THE MEASUREMENT OF OCTADECA-9CIS,11TRANS-DIENOIC ACID IN HUMAN NEOPLASIA: METHODS AND APPLICATIONS.

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ABSTRACT

Diene conjugation is an established marker of free radical attack on lipids. The major diene conjugated compound in human body fluids is octadeca-9cis,11trans-dienoic acid, 18:2(9c,11t). The serum concentration of 18:2(9c,11t) is significantly raised in some pathological conditions.

The method for assaying diene conjugated and non-diene conjugated fatty acids in serum was improved and modified to enable tissue, small biopsies and cells suspensions to be analysed. The assay was automated so that up to 75 specimens could be analysed daily and a computer program written to perform the post-run calculations. The concept of the molar ratio 18:2(9c,11t)/18:2(9c,12c), and its usefulness in a clinical context, was further developed.

The molar ratio, 18:2(9c,11t)/18:2(9c,12c), in neoplastic tissue from the cervix uteri (but not colon) was significantly higher than corresponding normal tissue. However, the assay was not sufficiently discriminating for the diagnosis of cervical cancer. A major reason for this was the enzymatic generation of 18:2(9c,11t) in the vagina by commensal bacteria.

It was concluded that changes in the concentrations of 18:2(9c,11t) in man should not be regarded as reflecting free-radical activity alone. By inference total diene conjugation in humans cannot be regarded as a simple function of free-radical damage to lipids.
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LIST OF ABBREVIATIONS

%MR  Percentage molar ratio, i.e. \([18:2(9c,11t)] \times 100/[18:2(9c,12c)]\)

18:2(9c,11t)  Octadeca-9cis,11trans-dienoic acid

18:2(9c,12c)  Octadeca-9cis,12cis-dienoic acid (linoleic)

18.1  Octadeca-9cis-enoic acid (oleic)

20:3  Eicosatrienoic acid

20:4  Eicosatetraenoic acid (arachidonic)

20:5  Eicosapentaenoic acid

22:6  Docosahexaenoic acid

amu  atomic mass units

aufs  Absorbance units full scale

CHP  Cumene hydroperoxide

CIN  Cervical intraepithelial neoplasia

CV  Coefficient of variation

DHSS  Department of Health and Social Security

EDTA  Ethyldiamine tetraacetic acid

Em  Emission wavelength

ESR  Electron Spin Resonance

Ex  Excitation wavelength

FAME  Fatty Acid Methyl Esters

GCMS  Gas Chromatography - Mass Spectrometry

HPLC  High Performance Liquid Chromatography

HPV  Human Papilloma Virus

HVS  High Vaginal Swab

IARC  International Agency for Cancer Research

IU  International Units

IUCD  Intra-Uterine Contraceptive Device

MDA  Malondialdehyde

MI  Myocardial Infarct
List of abbreviations continued:

OC Pill: Oral Contraceptive Pill

PBS: Phosphate Buffered Saline

PM: Post mortem

PUFA: Polyunsaturated fatty acids

DNA: Deoxyribonucleic acid

RBC: Red blood cells

RP18: HPLC column packing consisting of silica particles with attached eighteen carbon chain used for reverse phase chromatography.

RT: Retention Time

SD: Standard deviation

TBA: Thiobarbituric acid

TBARS: Thiobarbituric acid reactive substances

tBHP: Tert-butylhydroperoxide

TCA: Trichloroacetic acid

TCIN: All grades of cervical intraepithelial neoplasia

TDC: Total diene conjugation

TMP: Tetramethoxypropane

UV: Ultraviolet

v/v: volume/volume proportion

w/v: weight/volume proportion (g/100ml)
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SECTION ONE: INTRODUCTION.

The structure of lipids at the molecular level has been studied for more than two centuries. Before the end of the eighteenth century Scheele had obtained glycerol by saponifying olive oil (Scheele c1769-1785). However, he did not associate this significant finding with lipid structure. It was left to Chevreul to establish in the nineteenth century that lipids were compounds of alcohols, glycerol, and fatty acids (Chevreul 1814; Chevreul 1815)). From these early days the term lipid came to be used to describe the heterogenous group of compounds which were insoluble in water but soluble in organic solvents such as alcohols, hydrocarbons, and chloroform. The lipids, therefore, include fats, oils, and waxes, and related compounds. More detailed study of lipids was made difficult by their immiscibility with water. However, once the biological importance of lipids was appreciated techniques were developed to separate, analyse, and identify them. Thus it is only relatively recently that the vital roles of lipids have been understood and related to their molecular structure.

Lipids are a valuable source of energy either for immediate use or stored until required in adipose tissue. Furthermore, as components of membranes lipids are essential for the structure and function of all animals. The insulating properties of lipids are important: deposited subcutaneously they can provide thermal insulation; and, surrounding nerve fibres, a myelin sheath of high lipid content provides electrical insulation.
1.1. LIPID TYPES.

1.1.1. Fatty acids.
Common to all fatty acid molecules is the carboxyl group attached to a chain of partially or fully hydrogenated carbon atoms. The hydrocarbon chain is responsible for the hydrophobic nature of all fatty acids. Structurally fatty acids differ from each other in several respects; and these differences are reflected in their varied chemical and physical properties.

The longer the fatty acid chain, the higher the melting and boiling points. Unsaturation and branching prevents tight ordered packing of the fatty acid molecules and this is reflected in their melting and boiling points. For similar reasons cis fatty acids have less thermal stability than trans forms, and a fatty acid with a conjugated double bond system has more thermal stability than the corresponding unconjugated fatty acid.

At this point it is appropriate to consider the nomenclature of fatty acids, which has developed rather haphazardly. Many fatty acids were originally given trivial names which referred to their first discovered natural sources. Some were first isolated from seed fats: lauric acid for the Lauraceae, myristic acid from the Myristiceae, and palmitic acid from the Palmae. Although IUPAC has devised a systematic classification based on chain length, the number, position and configuration of any double bonds IUPAC names are typically long and cumbersome. Moreover, the trivial names are so widely accepted that the use of trivial names in this thesis has been unavoidable. However, the IUPAC name is given whenever a fatty acid is introduced. Use is also made of the popular shorthand detailing the number of carbon atoms, and the number, position and configuration of any double bonds. The fatty acids most abundant in humans are given below.
<table>
<thead>
<tr>
<th>IUPAC name</th>
<th>Common name</th>
<th>Shorthand name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecanoic</td>
<td>Palmitic</td>
<td>16:0</td>
</tr>
<tr>
<td>Octadecanoic</td>
<td>Stearic</td>
<td>18:0</td>
</tr>
<tr>
<td>All cis octadeca-9, 12-dienoic</td>
<td>Linoleic</td>
<td>18:2(9c,12c)</td>
</tr>
<tr>
<td>All cis eicosa-5, 8, 11, 14-tetraenoic</td>
<td>Arachidonic</td>
<td>20:4(5c,8c,11c,14c)</td>
</tr>
<tr>
<td>All cis docasa-4, 7, 10, 13, 16, 19-hexaenoic</td>
<td>-----------</td>
<td>22:6(4c,7c,10c,13c,16c,19c)</td>
</tr>
</tbody>
</table>

1.1.1.1. The synthesis of fatty acids in humans.

All saturated fatty acids required by humans can be synthesised *in vivo* from acetyl CoA. The required unsaturated fatty acids, however, are derived from four precursor unsaturated fatty acids by elongation or desaturation. These precursors, which are characterised by the distance of the methyl group from the nearest double bond, are oleic, linoleic, palmitoleic, and linolenic acids. The structure of each is given below:

- Linoleic: \( \text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH} \)
- Linolenic: \( \text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH} \)
- Oleic: \( \text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH} \)
- Palmitoleic: \( \text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH} \)
Only fatty acids without double bonds between the seven carbon atoms of the methyl terminal can be synthesised by humans. Thus, oleic acid and palmitoleic can be synthesised; but linoleic and linolenic acids must be obtained through the diet. The latter are, therefore, termed 'essential' fatty acids. In man, the double bonds of unsaturated fatty acids are almost exclusively of the cis configuration; trans bonded fatty acids being very uncommon. Fatty acids present in humans are Figure 1 shows the biosynthesis of the major fatty acids in humans.

The effects of essential fatty acid deficiency have been extensively studied in rats. Much of the early work was carried out by Evans, Burr and Burr, who produced acute deficiency states in rats through fat-free diets (Evans and Burr 1926). The effects of this deficiency included general weight loss, and a wide range of specific systemic abnormalities (Burr and Burr 1929). Burr and Burr showed that supplementing the fat-free diet with linoleic acid, linolenic acid, and arachidonic acids eliminated these deficiency states (Burr and Burr 1930). Docosahexaenoic acid was also found to have similar biological activity. However, supplementation of the diet with isomers of linoleic acid, which were trans bonded (octadeca-9trans,12trans-dienoic acid) or had conjugated double bonds (octadeca-9cis,11cis-dienoic acid), were shown to be biologically inactive in respect of the fat deficiency syndrome (Burr 1942). Thomasson (1953) later confirmed these findings using a bioassay and also found octadeca-10cis,12cis-dienoic acid was biologically inactive. The biological inactivity of fatty acids which contain a conjugated double bond system or double bonds in the trans configuration explains their rarity in animals. The effects of essential fatty acid deficiency in humans are not as well documented. However, Hansen (1937) found that the serum lipids of eczematous children have a lower degree of saturation than normal infants of the same ages. When linoleic acid was added to the
diet the lesions were found to improve and sometimes disappear completely. It is now accepted that an adult requires 2-10g linoleic acid daily. This is almost universally fulfilled as sufficient linoleic acid is available in most diets.
Figure 1. Synthetic pathways of fatty acids in humans. Fatty acids without a double bond within the first seven carbon atoms of the methyl terminal can be synthesised de novo, others must be derived from the diet.

**De novo synthesis**

```
Acetate + Malonate -> Acetate /Malonate / 16:0 / 18:0  ->  CH₃(CH₂)₇CH=CH(CH₃)₂COOH
                        / Oleic
                        / lipids
                        / lipids
     CH₃(CH₂)₇CH=CH.CH₂CH=CH(CH₂)₄COOH -> lipids
     18:2(6c,9c)  -> lipids
     CH₃(CH₂)₇CH=CH.CH₂CH=CH.CH₂CH=CH(CH₂)₃COOH
     20:3(6c,9c,12c)  -> lipids
```

**From diet only:**

```
CH₃(CH₂)₄CH=CHCH₂CH=CHCH₂CH=CH(CH₂)₄COOH  -> g-linolenic
                                              -> lipids
CH₃(CH₂)₄CH=CHCH₂CH=CHCH₂CH=CH(CH₂)₄COOH  -> Prostaglandins
                                            -> lipids
CH₃(CH₂)₄CH=CHCH₂CH=CHCH₂CH=CH(CH₂)₃COOH
D-6 series (essential fatty acids)
```

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1.1.2. Complex lipids.
Fatty acids do not exist in biological systems as free acids as they have a high affinity for many proteins and consequently an inhibitory effect on most enzymes. Almost all fatty acids in humans have an even number of carbon atoms and most are esterified as glycerides, phospholipids, and cholesterol esters. Where significant free fatty acid concentrations are found they are usually artifacts of cell damage. Cell damage allows lipases to hydrolyse tissue glycerides. The structure and properties of the major complex lipids will be briefly considered.

1.1.2.1. Phospholipids.
Much of the pioneering study of phospholipids was carried out by Thudichum (1884) more than 100 years ago. Thudichum isolated and analysed lipids from several animals which contained a fatty acid chain, a phosphate group, and an alcohol. Further studies were hindered, in part, by technical problems in obtaining pure phospholipids. In addition the mistaken belief that phospholipids were not metabolically active was widespread; and so the study of phospholipids was largely neglected for half a century. This myth was finally laid to rest when Hahn and Hevesy (1937) demonstrated that inorganic phosphate labelled with the newly isolated radioactive P$^{32}$ could be incorporated into tissue phospholipids. It is the amphipathic character of phospholipids (due to the strong ionic phosphate group and highly hydrophobic fatty acid chains) that is essential to their metabolic activity.

In humans most phospholipids are derived from glycerol and of these the nitrogen containing phosphatides are the most abundant. In human blood
phosphatides account for more than 90% of the phosphoglycerides. Phosphoglycerides, are not only vital components of the membrane lipid bilayer, but also play special roles in secretory processes, ion transport and the selective permeability of membranes.

1.1.2.2. Triglycerides.
Each triglyceride molecule consists of one molecule of glycerol esterified with three fatty acid molecules. Triglycerides form the bulk of the tissue lipids and account for approximately 25% of plasma lipids. Triglycerides are distributed in all organs and in particular adipose tissue in which triglycerides account for almost all the total lipid. The composition of triglycerides is strongly dependent on the diet. Rats fed a diet high in unsaturated fats showed a subsequent increase in the proportion of unsaturated fats esterified as triglycerides.

1.1.2.3. Cholesterol esters.
Berthelot was the first to show in 1859, that fatty acids could be esterified with cholesterol. Subsequently it was shown that the fatty acid esterified with the hydroxyl group on the third carbon of the cholesterol molecule. The general structure of cholesterol esters is shown in Figure 2.

The fatty acid is typically palmitic, stearic, oleic or linoleic. The bulk of cholesterol esters are present in plasma lipoproteins; significant concentrations are also present in the adrenal glands where they are precursors of the hormones released from those sites. As components of lipoproteins cholesterol esters play an important role in the transport of fatty acids and cholesterol throughout the body.
Figure 2. The general structure of a cholesterol ester. Carbon atoms are numbered according to convention.

is usually 16:0, 18:0, 18:1, or 18:2
1.2. Peroxidation of lipids.

1.2.1. The process of lipid peroxidation.

The problems associated with the storage of fats and oils exposed to air have long been recognised. Peroxidation of lipids results in rancidity and is accompanied by the development of foul odours. Early last century De Saussure (1820) described the oxidation of a layer of walnut oil on water when exposed to air. The oil absorbed three times its own volume of air in the first eight months, but during the course of the next ten months absorbed sixty times its own volume of air. Berzelius subsequently proposed that reaction of fats and oils with oxygen might also be responsible for other observations such as the spontaneous combustion of wool lubricated with linseed oil (c1825). At the end of the nineteenth century Engler and Wild proposed a mechanism for the peroxidation unsaturated fatty acids (Engler and Wild 1897). This involved the addition of oxygen at the double bond to form a cyclic peroxide.

Several workers reported that peroxidation is accompanied by the development of a characteristic absorption peak. Gillam et al. 1931). Vitamin A was not responsible for this absorption as it could be measured in fats with a high vitamin A content (eg. cod liver oil) and those in which the vitamin A content is minimal (eg. whale oil and butter). Dann and Moore found that the longer that methyl esters of fish oils were subjected to saponification conditions (boiling alcoholic potassium hydroxide), the greater the quantity of 234nm absorbing fatty acids produced. Edisbury et al. (1933 and 1935) postulated that the development of absorption at 230nm in fish oils during prolonged saponification was due to isomerisation, cyclisation or some other chemical change. It is now known that conjugated double bond systems (-C=C-C=C-) are responsible for this characteristic
absorption peak, an idea rejected in the 1930's in favour of the cyclisation hypothesis.

Once industry became interested in the spontaneous oxidative deterioration of oil-based products the sequence of peroxidation was intensively studied. Much of this work was carried out by Farmer and Bolland and their associates at the British Rubber Producers Association. These workers, by contrast with the Engler mechanism, showed there was no correlation between the decrease in double bonds and the addition of oxygen; the addition of oxygen occurred at a far faster rate than the reduction in double bonds. Also, the initial products of peroxidation were found to be fatty acid hydroperoxides with molecular oxygen incorporated at the methylenic group adjacent to the double bond. In a series of papers in the 1940's Farmer proposed that peroxidative events were initiated by the abstraction of a hydrogen atom from such a methylene group because its C-H bonds are weakened by the double bond. In a polyunsaturated fatty acids the methylene group between two double bonds would be very susceptible to hydrogen abstraction. Farmer et al. (1943) suggested this was followed by the displacement of double bonds to give an intermediate hybrid radical. On reaction with molecular oxygen, three possible hydroperoxides would be formed. If all were equally favoured two thirds of the hydroperoxides would be diene conjugated and therefore absorb at 230-235nm. Bolland and Koch (1945) found the product of the peroxidation of ethyl linoleate was a monohydroperoxide. which was at least 70% diene conjugated. However, they were unable to separate and identify these products further. Bergstrom (1945) meticulously analysed the products of the peroxidation of methyl linoleate by chromatography on alumina. Only two isomers were identified and both were diene conjugated. He concluded that initial peroxidative attack is at the methylene group (carbon eleven) between the two double
bonds. Following molecular rearrangement, subsequent reaction with oxygen yielded a 9-hydroperoxide and 13-hydroperoxide. It was subsequently confirmed that the 11-hydroperoxide was not produced (Sephton and Sutton 1956). Frankel commented that it was not surprising the hybrid radical reacts preferentially et al. (1961) with oxygen at the 9 and 13 positions as these products and the transition states leading to them would be resonance stabilised by the conjugated diene system. On the basis of studies with methyl linoleate, it was generally believed that the diene conjugation produced during autooxidation of PUFA was due solely to hydroperoxides.

Privett and others at the Hormel Institute in the United States, showed that the temperature at which the autooxidation of methyl linoleate occurred had a bearing on the stereochemistry of the hydroperoxides (Privett et al. 1953). Near 0°C 90% of the hydroperoxides formed were diene conjugated, and of those 90% were in the cis, trans configuration. At room temperature (24°C), however, the proportion of conjugated hydroperoxides in the cis, trans configuration was smaller and a significant proportion was present as the trans, trans isomer. It was postulated that the higher temperatures allowed a proportion of the cis, trans isomer to be converted to the thermodynamically stable trans, trans isomer.

Figure 3 shows the current understanding of the peroxidation sequence in the case of arachidonic acid.
Figure 3. The peroxidation of arachidonic acid.

Arachidonic acid

\[ \text{Hydrogen abstraction} \]

Arachidonic hydroperoxide

\[ \text{Molecular rearrangement} \]

Cyclic peroxide

\[ \text{Hydrolysis or heat} \]

Cyclic endoperoxide

\[ + \text{other products} \]

MDA
1.2.2. The reactions of lipid peroxidation: Initiation and propagation.
The peroxidation of polyunsaturated fatty acids is initiated by the abstraction of a hydrogen atom from the methyl group adjacent to a double bond, to leave a carbon centred radical. This reaction is effected by any radical species with sufficient energy. For example a peroxy, alkoxy or hydroxyl radical, but not hydrogen peroxide or superoxide. The acyl radical undergoes a spontaneous molecular rearrangement to give a carbon centred radical with a conjugated double bond system, which has specific absorbance at 234nm.

When oxygen is present the acyl radical combines with one molecule of oxygen to form a peroxyl radical which has two principal fates.

First, the peroxyl radical can be stabilised with a hydrogen atom donated by an antioxidant to form a peroxide as shown in the reaction scheme given below:
\[
\text{RH} \quad \rightarrow \quad \text{R}^* + \text{H}^* \\
\text{R}^* + \text{O}_2 \quad \rightarrow \quad \text{ROO}^* \\
\text{ROO}^* + \text{AH} \quad \rightarrow \quad \text{ROOH} + \text{A}^* \quad \text{(where AH is an antioxidant)}
\]

This stabilising reaction is the feature of the lag phase of lipid peroxidation and explains why De Saussure (1820) observed that the uptake of oxygen during the initial stages of the peroxidation of walnut oil is markedly slower than the later stages.

Second, when the supply of reduced antioxidant is exhausted the peroxyl radical is free to react with another fatty acid molecule to form a peroxide and a new acyl radical. This is termed the propagation stage of lipid peroxidation as the peroxyl radical formed when the newly formed acyl
radical reacts with oxygen can itself attack yet another fatty acid molecule. The reaction chain continues until the substrate fatty acid is exhausted. These reactions are shown below:

\[ \text{ROO}^* + \text{RH} \rightarrow \text{ROOH} + \text{R}^* \]

\[ \text{R}^* + \text{O}_2 \rightarrow \text{ROO}^* \]

Fatty acid peroxides are much less stable than fatty acids and can react further with oxygen to form cyclic endoperoxides, short chain alkanes (eg. pentane and ethane), and short chain aldehydes. These breakdown products of peroxides can also react with other molecules such as proteins.

1.2.3. Measurement of lipid peroxidation.

1.2.3.1. Oxygen Uptake.

The uptake of oxygen has long been used to monitor the reaction of oxygen with peroxidising lipid. De Saussure (1820) used a simple mercury manometer for his pioneering studies with walnut oil. One hundred and seventy years later oxygen uptake is typically measured with an oxygen electrode. Once calibrated, modern oxygen electrodes can measure minute changes in dissolved oxygen concentrations.

1.2.3.2. Peroxides.

Various methods have been developed to assay peroxides.

a) Iodine Liberation

Lipid peroxides can oxidise iodide ions to iodine, which can be titrated against thiosulphate ions by the reaction shown overleaf.
ROOH + 2I^- + 2H^+ \rightarrow I_2 + ROH + H_2O

I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-}

Though this simple method can be used for pure lipid systems it is of little use in biological systems because hydrogen peroxide and other oxidising agents present can also oxidise iodide to iodine. This measure of peroxides provides a 'snapshot' of the amount of peroxide present during the assay. It does not, therefore, yield any information concerning the rate of initiation of peroxidation or the rate at which peroxides are decomposing.

b) Glutathione Peroxidase

Glutathione peroxides catalyse the reduction of hydrogen peroxide and fatty acid hydroperoxides by glutathione. The reaction yields water, fatty acid alcohols, and dimerised glutathione. When glutathione, glutathione peroxidase and glutathione reductase are in excess and in the presence of NADPH GSSG can be converted back to GSH. The disappearance of NADPH can, therefore, be related to the amount of peroxide present. The reactions are shown below:-

2GSH + ROOH \rightarrow GSSG + ROH + H_2O

GSSG + NADPH \rightarrow 2GSH + NADP^+

The method is reportedly sensitive to 3nmol peroxide per ml, which makes it too insensitive for use on biological material.

c) Cyclo-oxygenase

Micromolar amounts of lipid peroxides have been shown to increase the rate at which cyclo-oxygenase reacts with arachidonic acid. By measuring enzyme activity Pendleton and Lands (1987) have been able to assay peroxides in biological specimens. However, since different lipid peroxides
stimulate cyclo-oxygenase activity by different extents the estimation will be affected by the nature of the peroxides being measured.

d) Haem Degradation of Peroxides.

Haem, and the haem moiety of proteins, can decompose lipid peroxides to form reactive intermediates. A haem peptide, prepared by the pepsin digest of cytochrome c and termed microperoxidase, MP-11, (Feder 1970), is particularly reactive in the decomposition of peroxides. Radicals produced by this reaction can react with isoluminol to produce light which can be quantitated (Frei et al. 1988). This method is particularly sensitive and can be used to detect picomolar concentrations of peroxides in biological systems.

Haem stimulated peroxides decomposition have also been reacted with a redox dye. The assay can be used to detect nanomole concentrations of peroxides.

1.2.3.3. Diene conjugation.

The oxidation of polyunsaturated fatty acids is accompanied by the production of diene conjugated fatty acids which have a characteristic absorption at 230-235nm. The measurement of diene conjugation has become one of the most widely used assays of lipid peroxidation in pure lipid systems because the measurement yields useful information on an early stage of lipid peroxidation and is simple to perform. Though it can be used to study lipid peroxidation in biological systems it must be borne in mind that several substances present in these specimens also absorb at 234nm. In addition since PUFA absorb at only slightly lower wavelengths the high concentrations of PUFA could give rise to significant 234nm absorption.
The method has been improved to give greater sensitivity for conjugated dienes by the application of second derivative spectroscopy (Corongiu et al, 1983). A simple spectrum plots absorbance against wavelength whilst the first derivative plots rate of change of absorbance against wavelength. The second derivative, therefore, is a plot of the rate of change of the first derivative against wavelength. The effect of these transformations is to convert what can sometimes be a broad based hump into a sharp peak. Furthermore, the increased resolution can enable the separation of different diene conjugates.

1.2.3.4. Thiobarbituric acid reactivity.

The assay of TBA reactivity is probably the most frequently used of all the measures of peroxidation. The peroxidation of PUFA results in the production of carbonyl compounds, such as malondialdehyde. The reaction of MDA with TBA produces a fluorescent compound. Calibration of the method with MDA allows quantitation of the carbonyl compounds in specimens. However, it must be remembered that almost all of the carbonyl compounds are formed during the assay from lipid peroxides present in the specimen. The assay is, therefore, a measure of the lipid peroxides in the specimens and not a measure of free malondialdehyde present in the specimen. The assay is extremely useful for the study of pure lipid systems but can be applied to biological systems if steps are taken to remove compounds such as proteins, carbohydrates, and nucleic acids which can react with TBA to give chromogens which have absorption maxima similar to the TBA-MDA complex. Yagi (1982) has developed a method suitable for the analysis of serum.
1.3. DIENE CONJUGATION IN MAN.

1.3.1. The nature of diene conjugation in man.

Bolland and others established that the diene conjugated lipid species produced during the peroxidation of PUFA in pure lipid systems are monohydroperoxides (Bolland and Koch 1945)). On the basis of this evidence it became widely accepted that hydroperoxides were also the compounds absorbing at 234nm in biological material although this had not in fact been conclusively proven.

Cawood et al. (1983) used HPLC to investigate the nature of the diene conjugation measurable in human body fluids. Hydrolytic enzymes were used to release free fatty acids from triglycerides, phospholipids and cholesterol esters. Good correlation was shown between total diene conjugation measured in chloroform extracts of human plasma and the sum of the esterified diene conjugated fatty acids measured by HPLC.

Reverse phase HPLC revealed several peaks with short retention times which were identified as lipid hydroperoxides. The most abundant diene conjugated fatty acid, in all lipid fractions of all human specimens analysed, eluted retention time between that of linoleic acid (18:2(9c,12c)) and a synthetic diene conjugated isomer of linoleic acid, octadeca-9\textit{trans}, 11\textit{trans}-dienoic acid (18:2(9t,11t)). Chemical analysis showed the lipid was not a hydroperoxide, epoxide or ketone. GCMS established it was in fact an eighteen carbon fatty acid with two double bonds positioned between carbon atoms 9 & 10, and 11 & 12. Although the configuration of the double bonds could not be determined the mass spectrum closely resembled the partial spectrum published for octadeca-9\textit{cis}, 11\textit{trans} dienoic acid (Andersson, Christie, and Holman 1975). The melting point of the 18:2(9,11) present in
biological specimens determined as 26°C, which is similar to the melting point reported for 18:2(9c,11t) (Nichols, Herb, and Reimenschneider 1951).

Cawood and his associates (1984) concluded that the serum diene conjugated fatty acid was most likely 18:2(9c,11t) and, that the measurement of 18:2(9c,11t) might be used as a specific marker for free radical activity in biological systems. Mass spectrometry studies of Diels-Alder adducts of the 18:2(9,11) isomer found in humans supports the isomer being 18:2(9c,11t) (Tay et al. 1985), and the findings of second derivative spectroscopic studies of diene conjugated lipids in plasma are also consistent with this assertion (Situnayake et al. 1990).

1.3.2. The method for the measurement of 18:2(9c,11t) in biological specimens.

The measurement of total diene conjugation is associated with significant methodological problems including the inability to standardise the method, the expression of the results in arbitrary units and non-specificity of the assay. Cawood et al. recognised the potential of 18:2(9c,11t) as a convenient marker of diene conjugation in biological systems (Iversen, Cawood and Dormandy 1985). Although GC is conventionally used for the analysis of fatty acids HPLC was the obvious choice for the assay of 18:2(9c,11t) for two main reasons. First, since the abundance of linoleic acid in human serum is typically twenty times more abundant than 18:2(9c,11t) it is impossible to optimise the GC detector sensitivity for both fatty acids. However, HPLC permits the use of two UV detectors set at 200nm and 234nm to measure simultaneously diene conjugated and non-diene conjugated fatty acids. Second, whilst GC analysis requires the preparation of methyl esters under conditions which can alter or damage the 18:2(9c,11t), HPLC can assay free
fatty acids directly. The destructive effect of chemical hydrolysis on 18:2(9c,11t) was also the reason for using enzymes to hydrolyse the esterified lipids.

The assay was directed towards fatty acids esterified as serum phospholipids due to the availability of highly specific and efficient hydrolytic enzymes, and towards phosphatidyl choline in particular as this is the predominant serum phospholipid.

As there can be considerable variation between the PUFA concentrations from different individuals, the relative concentration of 18:2(9c,11t) compared with the concentration of PUFA present is calculated. This is conveniently expressed as a 'molar ratio' of 18:2(9c,11t) to linoleic acid. Normal ranges have been established for the serum concentration of phospholipid esterified 18:2(9c,11t) and linoleic acid, and the molar ratio. In a group of normal male and females the range for the concentration of 18:2(9c,11t) was found to be 7.9-36.0 μmol/l (mean +/- 2SD) and the range for linoleic acid 550 - 1310 μmol/l (mean +/- 2SD). The range for the molar ratio was established as 1.0-3.8% (mean +/-2SD). The %MR has been shown to increase with age; and women have been found to have a higher %MR than men (Cawood PhD thesis 1988).

Daily variation in these three measures within an individual have been shown to be insignificant and no significant difference between fasting and post-prandial blood specimens has been demonstrated. Serum concentrations of linoleic acid and 18:2(9c,11t) and the molar ratio have been shown to be unaffected by acute alcohol, carbohydrate or lipids loads (Cawood PhD thesis 1988). However, initial results from dietary studies running parallel with this project indicate that the serum concentration of
18:2(9c,11t) and the molar ratio can be affected by longterm alterations in diet. Increased intake of dairy produce and meat of ruminant animals has been shown to lead to an increased serum concentration of 18:2(9c,11t) and molar ratio (Britton et al. 1992).
1.3.3. 18:2(9c,11t) and the %MR in human pathology.

The serum concentration of 18:2(9c,11t) and the %MR have been evaluated in normal individuals and in several clinical conditions.

Chronic alcoholics were found to have a significantly raised serum phospholipid esterified 18:2(9c,11t) and %MR concentration than control subjects. On withdrawal from alcohol these measures fell rapidly over the first two days and a steady fall for several weeks thereafter (Fink et al. 1985). In a non-alcoholic, however, the serum phospholipid esterified 18:2(9c,11t) concentration and %MR are not significantly affected by and acute alcohol load. These findings have been supported by studies by Szebeni et al. (1986) in Arizona, USA which confirmed that chronic alcoholics have a significantly raised serum phospholipid esterified 18:2(9c,11t) concentration and %MR compared with volunteers from teetotal Mormon church.

Crump et al. (1985) reported elevated concentrations of phospholipid esterified 18:2(9c,11t) in serum of four cases of paraquat poisoning.

The assay of 18:2(9c,11t) and 18:2(9c,12c) in serum is unable to distinguish non-esterified from phospholipid esterified fatty acids. Since free fatty acids normally account for only a negligible proportion of serum lipid the assay is essentially a measure of phospholipid esterified lipids. However, the influence of free fatty acids must be considered in procedures such as cardiopulmonary bypass surgery (Hind et al. 1986) or renal haemodialysis (Maher et al. 1986). During these conditions increased concentrations of non-esterified fatty acids (particularly 18:2(9c,11t), 18:2(9c,12c) and 18:1) have been recorded although the concentrations of phospholipid esterified fatty acids are essentially unchanged. These findings are almost certainly due to the systemic heparinisation which activates lipoprotein lipases thereby
releasing free fatty acids from triglycerides into the blood (Wickens et al. 1987).

The falling %MR observed in patients in diabetic coma during their treatment has been shown to reflect decreasing concentrations of non-esterified 18:2(9c,11t) and not a change in the phospholipids (Cawood Harrison and Dormandy 1985).

The serum concentrations of 18:2(9c,11t) and 18:2(9,12) have been investigated during the pregnancy of prima gravida women. Three separate prospective studies have established that 50% of women whose %MR increased during the pregnancy developed pre-eclamptic toxaemia or some other clinical complication (Erskine, Iversen and Davies. 1985; Tay SK personal communication; Fong CF personal communication). Why an increasing %MR should be associated with the subsequent development of a complicated pregnancy remains unclear but it may reflect a deterioration in antioxidant concentrations (Erskine, Iversen and Davies 1985).

Other studies have found that a significant proportion of patients with non-alcohol related liver disease, especially those with gall stone disease and primary biliary cirrhosis, had a higher %MR than that established for normal controls (Cawood PhD thesis 1988).

No alteration in the concentration of 18:2(9c,11t) or %MR has been found in patients with ARDS (Hind et al. 1987) or hypertension (Griffin and Yeoman unpublished data).
1.3.4. The origins of the $18:2(9c,11t)$ in human specimens.

1.3.4.1. The free radical origins of $18:2(9c,11t)$ in man.

Experiments demonstrating that $18:2(9c,11t)$ can be produced \textit{in vitro} by subjecting linoleic acid to a free radical generating system have lent support to a free radical origin for the $18:2(9c,11t)$ present in humans. Production of $18:2(9c,11t)$ \textit{in vitro} requires the presence of a thiol containing protein such as albumin (Cawood, Iversen and Dormandy 1984). These studies gave rise to the working hypothesis that $18:2(9c,11t)$ radicals generated in this \textit{in vitro} system must be stabilised by abstracting the hydrogen atom from a protein thiol group. The finding that the ratio of protein thiols to disulphide bonds decreases as the concentration of $18:2(9c,11t)$ increases is consistent with this theory (Cawood \textit{et al.} 1983). However, the UV irradiation of linoleic acid in the presence of a protein results in the generation of two other diene conjugated fatty acids in addition to $18:2(9c,11t)$; and one of these, $18:2(9t,11t)$, in greater abundance than the $18:2(9c,11t)$. The finding that the relative yield of $18:2(9c,11t)$ to $18:2(9t,11t)$ is greater at low partial pressures of oxygen suggests that $18:2(9c,11t)$ might be produced preferentially \textit{in vivo} in poorly oxygenated tissues such as neoplastic tumours.

It was, therefore, proposed that the generation of $18:2(9c,11t)$ \textit{in vivo} arises from a specific interaction between phospholipid esterified linoleic acid and membrane proteins constrained in the membrane such that free radical attack on the linoleic acid residue results in the production of a single diene conjugated product.
1.3.4.2. Dietary sources of 18:2(9c,11t).

Although no enzyme capable of producing 18:2(9c,11t) has been described in humans enzymatic conversion of 18:2(9c,12c) to 18:2(9c,11t) has been described in certain anaerobic commensal bacteria of the rumen. Studies carried out at North Carolina State University indicate that one such bacterium, *Butyrivibrio fibrisolvens* produces 18:2(9c,11t) as an intermediate during the conversion of linoleic acid to stearic acid (Kepler and Tove 1967). Figure 4 shows the biosynthetic mechanism for the production of 18:2(9c,11t) by *Butyrivibrio fibrisolvens*.. The first step of this hydrogenation pathway involves the isomerisation of 18:2(9c,12c) to 18:2(9c,11t) by the enzyme, linoleate 12cis, 11trans isomerase (EC 5.2.1.5.) which has been partially purified (Kepler Tucker and Tove 1971). The enzyme is specific for fatty acids with two cis double bonds at the 9 and 12 positions, and a free carboxylate group for the enzyme to be active (Kepler at al. 1971).

The reductase enzyme is specific for octadecadienoate having a cis double bond at the 9 position and a trans double bond at the 11 position and so is called 9cis, 11trans octadecadienoate reductase (Baker and Tove 1974). It has been partially purified and has a molecular weight of 60kD (Hughes, Hunter and Tove et al. 1982). Studies of the hydrogenation mechanism indicate that the hydrogen atoms are derived from water molecules (Hughes and Tove, 1980).

Other commensal rumen bacteria also hydrogenate linoleic acid. The spirochete, *Treponema (Borrelia)* strain B 2,5, uses a similar mechanism to *Butyrivibrio fibrisolvens* (Yokohama and Davis 1971). Precisely why bacteria should perform this elaborate conversion remains a mystery, although it has been suggested that unsaturated fatty acids are toxic to these bacteria and that hydrogenation serves to remove them (Nieman 1957). Removal of
linoleic acid by this pathway may prevent the fatal consequences of PUFA accumulation in the absence of adequate vitamin E (Blaxter 1957).

The high concentrations of 18:2(9c,11t) found in ruminant animals almost certainly result from the hydrogenation of linoleic acid by Butyribrio fibrisolvens and other commensal rumen bacteria and so it must be accepted that some of the 18:2(9c,11t) found in humans has a dietary origin.
Figure 4. The mechanism by which 18:2(9c, 12c) is converted into 18:0 by the ruminant bacteria *Butyriovibrio fibrisolvens*.
1.4. LIPIDS, FREE RADICALS AND CANCER.

As the number of aetiological agents (chemical, biological, and physical) known as carcinogens has grown, two hypotheses have emerged: cancer is not one but many diseases; or, more likely, these so called carcinogens act as potentiators of the real causes of cancer. Currently, it is felt that cancer is a single process but that carcinogenesis comprises two distinct aspects: initiation and promotion. In this section the influence of dietary fat and free radical reactions on the initiation or promotion of carcinogenesis will be considered.

1.4.1. Dietary lipids and cancer.

Considerable research effort continues to be directed towards investigating links between the diet and human diseases. Environmental factors have long been associated with the aetiology of certain types of cancer and in the last decade environmental factors are involved in the aetiology of as many as 90% of cancer types (Doll and Peto 1981). The studies of Doll and Peto indicate that the most important environmental factor is diet, accounting for an estimated 35% of cancer deaths in the USA. Epidemiological studies showing that the diet contains compounds which can either protect against or increase the risk of cancer by other agents have led scientific bodies to issue dietary guidelines. Amongst these it is recommended that fat intake is reduced as a diet high in fat is associated with increased incidence of certain cancers, particularly breast and colon cancer. In addition, the adequate intake of vitamins and minerals is advised as some of these appear to be protective. The finding that high fat intake is associated with increased risk of breast cancer is consistent with the conclusions of animal feeding experiments carried out more than 40 years ago. Experiments on mice
showed that calorie restriction leads to a decreased incidence of spontaneous mammary tumours (White et al. 1944), spontaneous lung tumours (Larsen and Heston 1945) and spontaneous leukaemia (Saxton, Boon, and Furth 1944). The decreased calorie intake also reduced the incidence of UV induced skin tumours (Rusch, Johnson and Kline 1945) and carcinogen induced papillomas (Boutwell, Busch, and Rusch 1949).

How dietary lipids and antioxidant influence the initiation and promotion of carcinogenesis is dependent on the nature and site of the tumour and the original carcinogen. Several mechanisms for the action of lipids and antioxidants have been proposed. Polyunsaturated fatty acids have been shown to influence the nutritional requirements of the tumour (Hopkins and West 1977). Based on experiments on mice Welsch and Aylsworth (1983) suggested that high fat diets may promote cancer via a hormone mechanism whilst Vitale and Briotman (1981) propose that the point of influence may well be the immune system. Another study suggests that lipids and antioxidants may change the microbiology of the intestine or the concentration of endogenous potentiators (Reddy 1981).

Accumulating evidence from animal experiments and epidemiological studies of human cancers shows that the composition of dietary fat has an important influence on the initiation and promotion of carcinogenesis. Several studies of various populations have reported an association of low serum cholesterol with increased cancer risk (Rose et al. 1974; Kark, Smith and Hames 1980; Garcia-Palmieri et al. 1981; Stemmermann et al. 1981), but others have not been able to confirm this (Salonen 1982; Kromhout et al. 1988). There have been suggestions that a low serum cholesterol concentration is merely a function of the disease and nothing at all to do with increased cancer risk (Rose and Shipley 1980; Sherwin et al. 1987). The
connection between cholesterol and increased cancer risk remains somewhat controversial. In one of the most recent studies Cowan et al. (1990) reported that total serum cholesterol and LDL cholesterol are significantly inversely related with overall cancer mortality in men but not women.

Over the last decade numerous studies have shown a link between a diet with a high saturated to unsaturated fat ratio and increased risk of cardiac disease. As a consequence Western populations have been encouraged to adopt a diet in which saturated fats are replaced by polyunsaturated fats, for instance by eating margarine derived from sunflower oil instead of butter. Whilst such a change in diet may lessen the risk of cardiac disease studies using experimental cancer models suggest it may increase the risk of cancer. In rats it has been shown that the incidence of chemically induced tumours is significantly increased when the intake of fat, particularly polyunsaturated fat, is high (Weisburger 1986; Reddy and Maruyama 1986). The finding in animal studies that the fatty acid composition of membranes is highly dependent on fatty acid composition of diet (Hammer and Wills 1978; Gower, Sayer and Wills 1986) may help to explain why this should be so. It has been shown that the activity of the mixed function oxidase and \( \text{P}_{450} \) systems, which are involved in the activation of carcinogens, are sensitive to changes in dietary fat. Mounie et al. 1986 showed that the levels of the enzyme systems fall when rats are fed a fat free diet. The levels of both are restored when fat is added to the diet but unsaturated fat was much more effective than saturated fat. In addition the studies of Vetter, Carey and Patton (1985) indicate that high dietary fat may make fat soluble carcinogens effective in reaching potential sites of carcinogenesis. A membrane which contains a high proportion of unsaturated lipids is more susceptible to free radical attack. The peroxidation of the lipids which can
follow such attack can play a part in the carcinogenesis process. The relation between free radicals, lipid peroxidation and cancer are discussed more fully in the section 1.4.2.

It can be concluded that the intake of high levels of fat and particularly dietary fat may increase the risk of cancer.

1.4.2. Free radicals, lipid peroxidation and cancer.

In recent years there has been much research interest in the role of free radicals in the aetiology and development of neoplastic cells. In considering the involvement of free radical species in cancer it is important to distinguish the initiation and promotion stages of tumour development.

There is no doubt that free radical species can initiate the mutagenic process. Pryor showed that free radicals in cigarette smoke can cause cancer (Pryor 1977; Pryor 1983) and Tso, Caspary and Lorentzen (1977) have demonstrated that free radicals can cause mutagenesis. Oxygen radicals generated by radiation, especially the highly reactive hydroxyl radicals, have been shown to damage DNA bases (Scholes 1983). The less reactive superoxide radicals can also damage chromosomes in cells (Iwata, et al. 1984). As well as direct mutagenic damage to DNA free radicals can promote DNA damage by activating latent carcinogens. The peroxidation of lipids have been shown to activate polycyclic aromatic hydrocarbons to carcinogens (Dix and Marnett 1983), and convert benzo(a)pyrene to active carcinogenic metabolites (Gower and Wills 1985).

Antioxidants have been shown to afford some protection against many types of free radical induced tumours in a variety of animals (Tso, Caspary
and Lorentzen 1977; Floyd and Soong 1977; Oberley and Buettner 1979; Slaga et al. 1981; Mason et al. 1982; Pryor 1982). Shamberger, Tytko and Willis (1976) discovered that high dietary intake of selenium correlates with a lower incidence of certain types of cancer. Schrauzer, White and Schneider (1977) suggested that this protection from cancer is due to the increased synthesis of selenium containing antioxidant enzymes. Free radicals, therefore, may be involved in the initiation of tumour development.

Once the carcinogenic process has been initiated there is long standing evidence that the rate of lipid peroxidation in tumour cells is lower than in corresponding normal cells (Donnan 1950). Regenerating rat liver was also found to have a low rate of lipid peroxidation. (Wolfson 1956). It was pointed out that as a consequence tumour cells and regenerating tissue are not exposed to the oxidative products which inhibit cell division. This led to the suggestion that oxidation products might have a role in the regulation of cell division (Wolfson 1956; Schuster 1958). Subsequent studies have confirmed the low rate of lipid peroxidation in growing intestine, testis and bone marrow (Barber and Wilbur 1959; Bernheim 1963; Mead 1976); and in regenerating tissues and tumours (Player 1982; Barber and Bernheim 1967; Ahmed and Slater 1981). In ESR studies of human cervix the strong peroxy radical signal detected in normal tissue is greatly attenuated or even absent in the tumour tissue (Slater and Cook 1970; Benedetto et al. 1981).

The low rate of lipid peroxidation in tumours and growing or regenerating tissue almost certainly reflects the degree of protection these tissues have against radical damage. Cheeseman (1984) showed that tumour tissues have a lower peroxidisability than corresponding normal tissues. Embryonic, foetal and neonatal tissues also have low susceptibility to lipid peroxidation.
(Cole 1956; Player, Mills and Horton 1977; Devasagayam 1986). The low peroxidisability of tumour tissues is mainly attributable to their high concentration of antioxidants (Lash 1966; Player 1982; Burton et al. 1982). In addition some tumour tissues have been shown to have a lower than normal content of PUFA (the fatty acids most susceptible to peroxidation) and radical generating systems (such as cytochrome P<sub>450</sub>) (Ahmed and Slater 1981). Tumour tissue has also been reported to have lower pO₂ than normal tissue.

There are, therefore, two aspects to free radical activity in cancer. Free radical species can play a role in the initiation of the carcinogenic process. In cancer promotion, however, free radical species are conspicuous by their absence as the tumour cells are well protected against them by high concentrations of antioxidants, low concentrations of unsaturated fatty acids and low concentrations of radical generating systems.

Dormandy (1983) has proposed that each cell has a self destruct mechanism, which is activated when the cell is damaged; but the failure of this mechanism allows damaged cells to survive and the tumour to grow. Clinical cancer might be conceived as an outcome of an imbalance between the carcinogenic process on the one hand and the protection mechanisms on the other. This multiple mechanism of carcinogenesis may account for the paradoxical effect of many causative therapeutic agents such as X-rays which can be both powerful carcinogens and effective anticarcinogens.
1.5. TYPES OF CANCER TO BE STUDIED

1.5.1. Colon cancer.

Colorectal cancer is a major health problem in Britain and throughout the developed world. The incidence of the disease is highest in the industrialised countries and lowest in equatorial Africa and South East Asia (Nigro and Bull 1987). However, environmental factors cannot be totally responsible for these variations. Migrants from a region in which the incidence of colorectal cancer is low to a region in which colorectal cancer is more common assume the increased risk of the new region.

Though the precise aetiology of the disease remains somewhat unclear three major risk factors have been identified.

First, it has long been noted that heredity is a significant risk factor in colorectal cancer (Fry, Fleshman and Kodner 1990). It has been calculated that first degree relatives of a subject with colorectal cancer have a 2 to 4 fold increased risk of developing colorectal cancer. It is known that some diseases such as familial polyposis and Gardener's syndrome are transmitted in an autosomal dominant pattern resulting in the development of numerous colorectal adenomatous polyps typically during adolescence. If the polyps remain untreated the patients almost certainly develop colorectal cancer. Cancer family syndrome is also transmitted in an autosomal dominant fashion and up to a third of individuals with this genetic composition develop colorectal cancer before the age of 50. The findings of recent genetic research implicates significant roles for oncogenes and cancer suppressing genes.
Second, studies have consistently shown that colorectal cancers often originate from or near colorectal polyps (Jarvinen, Ovaska and Mecklin-1988). Polyps are rare in young adults but become increasingly common with advancing age. When found polyps are, therefore, surgically removed as a preventative measure.

Third, a higher incidence of colorectal cancer has been identified in patients with inflammatory bowel disease and ulcerative colitis in particular (Fry, Fleshman and Kodner 1990). The degree of risk has been shown to be dependent on the extent of the colitis and the duration of the disease.

The proposed role of diet in the development of colorectal cancer has evoked considerable interest. It has been suggested that a diet high in fibre affords protection against colorectal cancer by diluting and binding carcinogens in food and by increasing the rate at which the bowels are cleared thereby limiting the exposure of the bowel to the carcinogens (Nigro and Bull 1987). It has also been proposed that high dietary fat intake increases the risk of colorectal cancer by increasing the concentration of faecal bile acid, which may produce carcinogenic metabolites.

Babbs (1990) has proposed a free radical mechanism for the development of colorectal cancer. This theory suggests iron derived from ingested meat can catalyse the production of highly reactive hydroxyl radicals from bacteria derived superoxide. A meat rich diet provides more catalytic iron and a diet high in fat more procarcinogens. The theory further suggests that patients with chronic ulcerative colitis are at increased risk of developing colorectal cancer because the condition leads to perimucosal superoxide from activated leucocytes and haem iron from the chronic bleeding.
Early detection and treatment of colorectal cancer stage improves the prognosis of the disease. It follows therefore that individuals in high risk groups should be regularly screened (Jarvinen, Ovaska and Mecklin 1988).

1.5.2. Cervical cancer.
This thesis is concerned with the premalignant and malignant stages of squamous cell carcinoma of the cervix and all references to cervical cancer refer to this type of carcinoma.

The incidence of cervical cancer varies considerably from one country to another and amongst racial and socio-economic groups within each country (Doll, Muir, and Waterhouse 1970; Waterhouse et al. 1976). After breast cancer cervical cancer is the most common cancer to affect women worldwide (Parkin, Stjernsward and Muir 1984). Any change in the incidence of cervical cancer will, therefore, have profound effects on the resources required for the screening for and the treatment of cervical cancer.

In the United Kingdom 4000 new cases of invasive cervical cancer are registered each year and of these about 2000 will eventually die from the disease (HMSO 1983). Although most deaths occur amongst older women the death rate amongst younger women is rising (Cook and Draper 1984). Walton (1976) reported that the age at which peak incidence of carcinoma in situ was occurring in British Columbia was decreasing. Comparable increases in the incidence of CIN amongst young women in the UK have been reported (Bamford, Barber and Beilby 1982; Wolfendale, King and Usherwood 1983). Cook and Draper (1984) suggested that these findings reflect the increased detection rate of CIN by screening programmes which have prevented a larger rise in the incidence of cervical cancer. The
introduction of a call and recall system in the UK will improve further the detection rate of CIN and treatment and follow up of these cases will place an increased burden on health service resources (Cuzick 1988).

1.5.2.1. The nature of cervical intraepithelial neoplasia.
The concept of premalignant cervical abnormalities capable of progressing to invasive cancer has become generally accepted over the last twenty-five years. Richart (1967) graded CIN into three stages but this is understood to represent a broad spectrum of changes rather than a series of distinct steps.

It had been thought that the milder conditions were more likely to regress, and the more severe conditions to progress to carcinoma in situ (Hall and Walton 1968; Fox 1967). In 1969 Richart and Barron reported that all stages of CIN had the potential to progress to carcinoma in situ. This study estimated the mean time for mild, moderate and severe dysplasia to progress to carcinoma in situ to be 58, 38, and 12 months respectively. More recently Campion et al. (1986) employed cytology, colposcopy and cervicography to study and follow up a group of women with CIN. This study concluded that the potential of CIN to progress to carcinoma in situ has been severely underestimated. Kottmeir (1961) showed that over a twelve year period 71% of a group of 31 women with untreated carcinoma in situ developed invasive cancer. However, Green and Donovan (1970) reported a much lower progression rate.

The interpretation of such studies is complicated by the possibility that the collection of biopsies might affect the natural progression of the disease. In addition it is ethically unacceptable to leave women with CIN untreated in a bid to devise a well controlled study.
Though the colposcopic examination may be suggestive of CIN, the presence of CIN can only be confirmed by histological examination of biopsies of cervical epithelium. CIN is typically associated with the presence of differentiated cells, nuclear abnormalities and abnormal mitotic activity.

A protocol for the treatment of CIN lesions by ablative techniques has been devised in order to effectively treat the CIN lesion with the minimum of surgical stress (Jordan and Mylotte 1982). Ablative laser treatment of CIN is of proven benefit both in terms of first treatment cure rates and prevention of later development of cervical cancer (Jordan et al. 1985).

1.5.2.2. The aetiology of cervical cancer.
Significant differences in the standardised mortality rates suggest some women have suffered greater exposure to risk factors than others. In this respect considerable evidence supports the involvement of sexual behaviour and sociological factors in the aetiology of cervical cancer. This is clearly illustrated by the increased risk of cervical cancer in women, who were sexually active during the World Wars, compared with those before, between and after the wars (Hill and Adelstein 1967). A study completed in the early sixties showed that married women in social class 5 were more likely to develop cervical cancer than those in social class 1 (Beral 1974). The same report suggested that women, whose husbands spend significant periods away from home on business are at increased risk.

The importance of sexual behaviour in the aetiology of cervical cancer is clearly indicated by the high incidence of cervical cancer in prostitutes (Rojel 1953) and low incidence in the Jewess (Steinitz and Costin 1970) and
strict religious orders (Gagnon 1950; Towns 1955). The most important sexual factor appears to be the age at first intercourse especially if this occurs before age 17 (Wynder et al. 1954; Rotkin and King 1962).

The risk of cervical cancer has also been shown to be related to the sexual activities of their partners. The regular use of prostitutes by Columbian men is thought to be responsible for cervical cancer in Columbia being more common amongst women in the higher social classes (Skegg et al. 1982). Wives of men with penile cancer are more likely to develop cervical cancer (Martinez 1969), as are wives of men whose first wife developed cervical cancer (Kessler 1976). In women, who reported only one sexual partner in their sexual history, the relative risk of developing cervical cancer was found to increase with the number of sexual partners of the husband (Buckley et al. 1981).

These studies led to the idea that the aetiology involves a sexually transmissible male factor (Singer 1973), and several agents have been proposed. Coppleson and Reid (1967) proposed that spermatozoa could influence the development of cervical neoplasia. This theory was based on the observation that the DNA metaplastic cervical epithelial cells is altered following phagocytosis of spermatozoa. These studies have been extended and French et al. (1987) have demonstrated that sperm protamine can induce neoplastic transformation in cultured cervical cells.

Cervical cancer frequently occurs with sexually transmitted infections (Beral 1974) but it is difficult to prove a cause and effect relationship. This association may simply reflect sexual promiscuity and exposure to a sexually transmissible agent or agents.
Viruses proposed to be causative agents include herpes simplex virus type II (Naib et al. 1969), cytomegalovirus (Melnick et al. 1978), and most recently the human papilloma virus (Baird 1983). Human papilloma virus type 16 is associated with high risk of cervical cancer development (McCance et al 1985).

Several studies have reported an increased risk of cervical cancer amongst women using the oral contraceptive pill for long periods (Stern et al. 1977; Vessey et al. 1983).

The risk of CIN in smokers is well documented (Thomas 1973; Marshall et al. 1983; Trevathan et al. 1983). Trevathan et al. estimated the relative risk of carcinoma in situ in smokers to be 12.7. Barton et al. (1988) found the concentrations of nicotine and cotinine in cervical mucus were fifty times higher than in blood although nicotine concentrations up to 100 times those of serum have been reported (Sasson et al. 1985). In addition this group showed these compounds were associated with immunocytological changes in the cervix. Passive smoking is an established risk in the development of lung cancer (Kuller et al. 1986; Wald et al. 1986), and the possibility that passive smoking contributes increased risk in the development of cervical cancer, as suggested by Hellberg, Valentin and Nilsson (1986), cannot be ruled out. However, it is difficult to assess passive exposure to cigarette smoke quantitatively.

The local immunology may play an important role in the progression of cervical cancer. Tay et al. found a decreased concentration of Langerhans cells and altered natural killer cells in women with HPV infection and CIN (Tay et al. 1987; Tay, Jenkins and Singer 1987)
1.5.2.3. Cervical cancer screening.

The importance of a well organised and available cervical cancer screening programme lies in its widely reported ability to cut the incidence and mortality rates for invasive cancer as demonstrated in Iceland (Johannesson, Geirson and Day 1978), British Columbia (Anderson et al. 1988), and parts of Britain (MacGregor and Teper 1978). Despite the considerable and increasing cost of the national cervical cancer screening programme introduced by the DHSS in 1964 the incidence of cervical cancer in Britain as a whole has failed to show a significant reduction (Roberts 1982). Both the administration of the programme and the screening techniques have been blamed for this failure.

It has been estimated that possibly 90% of women, who have died from cervical cancer, have never had a cervical smear (MacGregor and Teper 1978) and this has prompted the issue of new directives to all District Health Authorities to improve the administration of the programme and it is hoped that all women will be examined at least every five years. However, the International Agency for Research on Cancer (IARC) reported that whilst a five year screening interval can be expected to reduce the incidence of cervical cancer by 83.4 %, an interval of three years could improve this to 91.2% (IARC Working Group 1986). These studies have led to a call for a reduction in the screening interval recommended by the DHSS (Intercollegiate Report on Cervical Cancer Screening 1987).

All current screening programmes rely on the cytological technique devised in 1943 by Papanicolaou and Traut. The modification to the technique by Ayre (1949) led to increased detection of cervical abnormalities (Miller and von Hamm 1961), and Rubio et al. (1980) has further refined the preparation of the smear. Smears, however, can be difficult to interpret due to the
interference of red blood cells, microbial infection, and spermatozoa. In addition difficulties can arise from the variation in the appearance of squamous, columnar and metaplastic cells during the different phases of the menstrual cycle. Although the Papanicolaou smear test rarely gives false positive results (Hudson 1985) the problem of false negatives is widely recognised (Bjerre and Johansson 1983). Poor sampling technique and laboratory errors are thought to be equally responsible for these false negatives (MacGregor 1982), which have been variously estimated to occur at a rate of 5-30% (Dunn and Schweitzer 1981; Patterson, Peel and Joslin 1984).

The high cost of improving the effectiveness of the screening programmes, the inability to automate the reading of cervical smears and the poor sensitivity of the technique as a diagnostic test have prompted the quest for alternative techniques. The original method of Feulgen hydrolysis (Feulgen and Rossenbeck 1924) has been modified by Millett et al. (1982). In a recent study the technique clearly distinguished normal from abnormal cases (Sincock et al. 1987). Also under trial is the technique of cervicography, first proposed by Stafl (1981), in which a photograph is taken of the cervix following application of acetic acid and the enlarged photographs are examined later. Initial reports indicate that although the method has a high degree of sensitivity it has poor specificity (Blythe 1985; Tawa et al. 1988).
1.6. AIMS OF THIS STUDY.

The measurement of lipid diene conjugation has been used as a marker of lipid peroxidation. The predominant diene conjugated lipid in human tissues and tissue fluids has been shown to be 18:2(9c,11t). The concentration of 18:2(9c,11t) has been found to be altered in the body fluids of patients with pathological conditions in which changes in free radical activity have been implicated.

This project had three principal objectives. First the modification and improvement of the method for the measurement of 18:2(9c,11t) and of linoleic acid for the analysis of tissue biopsies. Second the application of the method to the study of normal and neoplastic tissue. Third the development of a analytical technique for routine use.

Many of the modifications made to the technique involved increasing sensitivity to cope with the minute amount of material in small biopsies. Since it was anticipated that a large number of specimens would be studied improvements to the technique also had to include automation of the method. Manual calculation of results was extremely time consuming and subject to some error. To address these matters it was hoped that a computer program could be devised to identify chromatographic peaks and calculate results.

Alterations in free radical activity and lipid content may be important in several pathological conditions. In particular it is known that free radical reactions can be involved in the initiation of cancer and that several mechanisms may protect the established tumour against free radical attack. The development of neoplastic tissue can be associated with changes in free radical activity: the concentration of peroxy radicals and thiol groups has
been shown to be reduced in carcinoma of the cervix. The lipid content of some tumours is known to be altered; and in particular tumours appear to have a higher saturated to unsaturated lipid ratio than normal cells. It was hoped that using the methodological advances and technical improvements projected the study of a large number of specimens from patients with various forms of cancer and precancer would shed more light on this.

It was also hoped that the results of these studies would help to establish a clinically useful diagnostic tool which could be of assistance in the field of cancer.
SECTION 2: MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Chemicals.

The following chemicals of Analar grade (unless otherwise stated) were obtained from British Drug Houses Chemicals Limited, Poole, Dorset, UK:

- Hydrochloric acid
- Hydrogen peroxide (Aristar grade)
- Potassium dihydrogen phosphate
- Potassium hydroxide
- Trichloroacetic acid
- Sodium azide
- Sodium chloride
- Disodium hydrogen phosphate
- Sodium hydroxide
- Sulphuric acid

The pyrrolidine and N-methyl-N-nitroso-p-toluene-sulphonamide, used for GCMS analysis, were obtained from Aldrich Chemical Company.

SIGMA Chemical Company Limited, Poole, Dorset, UK supplied the following chemicals and biochemicals:

Chemicals:

- tert-butylhydroperoxide, Cumene hydroperoxide
- Potassium ethyldiamine tetraacetic acid, 2-Thiobarbituric acid
- Sodium ethyldiamine tetraacetic acid
Proteins and enzymes:
Lecithinase type A (EC 3.1.1.4) phospholipase A2 from *naja naja* venom
Triacylglycerol acylhydrolase (EC 3.1.1.3.) type VII lipase from *candida cylindracea*.
Human albumin (essentially fatty acid free, prepared from factor V)

Fatty acids:
- Arachidonic acid
- Docosahexaenoic acid
- Eicosapentaenoic acid
- Eicosatrienoic acid
- Linoleic acid
- Linolenic acid
- Oleic acid
- *Trans*-vaccenic acid

The β-eleostearic acid was obtained as the methyl ester from Alltech Associates, Carnforth, Lancashire, UK.

The methyl ester of octadeca-9*trans*, 11*trans*-dienoic acid, prepared from dehydrated castor oil was kindly donated by Mr A. Sherwood of the Paint Research Association, Teddington, UK. Mr Sherwood regrets that further supplies are not available.

A small quantity of a crude preparation of octadeca-9*cis*, 11*trans*-dienoic acid, which was estimated to be 70% pure, was kindly donated by Professor F.D. Gunstone, Department of Chemistry, University of St.Andrews, Fife, UK.
2.1.2. Solvents.

The following HPLC grade solvents were purchased from Romil Limited, Shepshed, Loughborough, Leicestershire, UK:

- Acetonitrile (Far UV grade)
- Chloroform
- Methanol
- Water
- Propan-2-ol

The following solvents were obtained through the Aldrich Chemical Company:

- Diethylene glycol monoethyl ether
- Dichloromethane
- Ether

Acetic acid (sequencer grade) for HPLC use was obtained from Rathburns Limited, Walkerburn, Peebleshire, UK.

The analytical grade solvents, acetic acid and butan-1-ol, were obtained from British Drug Houses Limited, Poole, Dorset, UK.

2.1.3. Chromatographic media.

The 'Bond Elut' extraction system and the solid phase extraction cartridges, manufactured by Analytichem International Incorporated, California, USA, were supplied through Jones Chromatography Limited, Henyoed, Mid Glamorgan, UK. Two sizes of 'Bond Elut' cartridge were used:

- No.617303 - a 3ml cartridge with 200mg octadecasilyl sorbent and;
- No.617301 - a 1ml cartridge with 100mg octadecasilyl sorbent.
Lichroprep RP18 (octadecasilyl particles 25-40μm) was obtained from E Merck, Darmstadt, Germany.

2.1.4. Gases.
Oxygen free nitrogen and air were obtained from the British Oxygen Company Limited (Industrial Gases Division), Wembley, Middlesex, UK.

Helium used for GCMS analysis was supplied by British Oxygen Company Limited (Special Gases Division), The Priestley Centre, The Surrey Research Park, Guildford, Surrey, UK.
2.2. ANALYTICAL METHODS.

2.2.1. The measurement of diene conjugated and non-diene conjugated fatty acids by HPLC.

2.2.1.1. HPLC analysis.
All HPLC analysis was performed on equipment supplied by LDC Analytical Limited, Stone, Staffordshire, UK. The equipment comprised a Constametric III dual reciprocating pump; two Spectromonitor 3000 variable wavelength UV detectors in series (one initially set at 205nm and the other at 234nm); and a CI 3000 computing integrator.

The HPLC equipment was set up in accordance with the method described by Iversen, Cawood and Dormandy (1985) for the measurement of diene conjugated and non-diene conjugated unsaturated fatty acids in serum.

2.2.1.2. Preparation of the internal standards.
Both the octadeca-9trans,11trans-dienoic acid and the β-eleostearic acid were supplied as methyl derivatives. It was, therefore, necessary to prepare the free acids by saponification so that the compounds could be used as internal standards.

To 50ml methanol 8.5g potassium hydroxide was added and completely dissolved. The solution was filtered, cooled to -20°C and transferred to a 100ml double necked glass round bottomed flask containing approximately one gram of the octadeca-9trans,11trans-dienoic acid methyl ester. A reflux condenser was fitted to one of the necks and nitrogen passed through the other such that the gas passed over and into the solution. When the gas had passed for at least 10 minutes and whilst continuing to pass nitrogen into
the flask the contents of the flask were heated to 50°C for 20 minutes. The flask was cooled in ice and 10ml 2mol/l HCl added. Drops of concentrated HCl were added until the solution just became acidic. The nitrogen was switched off. The free acid was extracted twice with chloroform. Chloroform (20ml) was added, shaken thoroughly and the contents of the flask centrifuged at 1500g for 10 minutes. The chloroform (lower) layer was removed to a clean 100ml flask. The upper layer was extracted with 20ml chloroform as before and the second chloroform fraction was pooled with first. The chloroform fractions were washed with an equal volume of methanol/water (1:1,v/v) and the chloroform phase dried under nitrogen. Vials of the free acids were stored in liquid nitrogen until required.

2.2.2. Gas Chromatography-Mass Spectrometry.

2.2.2.1. Preparation of the fatty acid methyl esters.
Fatty acid methyl esters (FAME) were prepared by treatment with diazomethane in ether (Fales, Jaouni and Babshak 1973). The diazomethane reagent was prepared by adding a solution of 2g KOH in 3.3ml water to 7g N-methyl-N-nitroso-p-toluene-sulphonamide dissolved in 43ml ether and 12ml diethyleneglycol monoethyl ether. The released diazomethane was condensed in 55ml ether maintained at 0°C. The resuspended fatty acids were reacted with 1.7ml diazomethane in ether and the solution dried under a stream of nitrogen.

2.2.2.2. Preparation of pyrrolidide derivatives.
The pyrrolidide derivatives were synthesised from the methyl esters by dissolving them in 200μl pyrrolidine and adding 20μl acetic acid. After heating at 100°C for 30 minutes the amide formed was extracted with
dichloromethane, washed with 2mol/l hydrochloric acid, evaporated to dryness under nitrogen and resuspended in 50µl chloroform (Anderson and Holman 1974).

2.2.2.3. Gas chromatography-Mass Spectrometry analysis.
GCMS was performed on a Varian (Finnigan, Walton-on-Thames, Surrey, UK) MAT 112 instrument fitted with a Varian 1400 gas chromatography housing a 20m x 0.3mm open tubular glass capillary column coated with Sil 88 (Chrompack, Middleburg, Netherlands). Samples were introduced via an all glass solid injection system (Van den Berg and Cox 1972). The carrier gas was helium with an inlet pressure of 50kPs, which gave a flow rate through the column of 1ml per minute. FAME were separated isothermically at an oven temperature of 210°C. Methylene values were obtained by co-injection of a suitable hydrocarbon mixture with the samples. GC effluent was passed directly to the ion source via a heated line maintained at 250°C. The ion source temperature was 250°C, the ionisation current was 1.5mA and the accelerating voltage 800V. Repetitive magnetic scans (70 scans per minute) over the mass range 50-500 amu were taken and the mass spectra acquired and processed by the Varian (Finnigan) MAT SS.200 data system. Selective detection of single ions was also used to give greater sensitivity in some cases.

2.2.3. The assay of lipoperoxides in cervical cell suspensions and tissue.

2.2.3.1. Sample preparation.
Lipoperoxides present in cervical cells and cervical tissue homogenates were assayed using a method based upon that of Stocks and Dormandy (1974) in which protein in the homogenate or cell suspension was removed by
precipitation by 1.71mol/l trichloroacetic acid. The full procedure for each experiment is detailed in sections 3.3.1.1., 3.3.2.1., 3.3.3.1., 3.3.4.1. and 3.3.5.1..

2.2.3.2. The fluorimetric measurement of the TBA-MDA complex.
All spectrofluorimetry was performed on a MPF-3L Fluorescence Spectrometer Perkin Elmer, Beaconsfield, Buckinghamshire, which was standardised with a Perkin Elmer block no. 5 to 100% relative fluorescence at Ex 400nm, Em 475nm on sensitivity 1 with slit widths 12nm and 14nm respectively. The TBA-MDA complex was detected and measured spectrofluorimetrically with excitation wavelength 532nm (slit width 12nm) and emission wavelength 553nm (slit width 14nm).

2.2.4. Collection of samples from the cervix uteri.
2.2.4.1. The Papanicolaou smear.
An Ayre’s spatula was used to scrape cells from a 360° circumference around the cervical os under direct vision. In cases where the entire transformation zone was not visible an endocervical brush was also used. The cell scrape was spread onto a glass slide (Menzel-Glaser, Germany) and fixed with a solution containing methanol:acetic acid (95:5,v/v) and carbowax (DHSS central supplies, London, UK). Once dry the slides were examined by cytology staff.

2.2.4.2. Cytological specimens for biochemical analysis.
Cells were collected in the same manner as described above but washed into 10ml 20mmol/l phosphate (Na$_2$HPO$_4$/KH$_2$PO$_4$) containing 154mmol/l sodium chloride and 5mmol/l dipotassium EDTA. The cervix was scraped again to obtain sufficient material for the biochemical analysis. When the transformation zone was not visualised, it was sampled using an
endocervical brush (MedScand, Sweden). The samples were maintained at 4°C and immediately transported to the laboratory for biochemical analysis.

2.2.4.3. Cervical biopsies.

When an abnormal lesion on the cervix was visualised during colposcopical examination punch biopsies were taken from the most abnormal area of each lesion using Eppendorfer biopsy forceps. The biopsies were snap frozen in liquid nitrogen until histological assessment or biochemical analysis. The histological assessment was based on the criteria for CIN of Buckley et al. Butler and Fox (1982) and of Dyson et al. (1984) for human papilloma virus infection without neoplasia.

2.2.5. Statistical analyses.

Experimental and study results were analysed using a variety of tests as appropriate. Most of the statistical analyses were carried out on the North East Thames Regional Health Authority scientific computer using a statistical software package from the Pennsylvania State University. When the data were normally distributed the t-test were used to compare the test and control groups; when not normally distributed the Mann-Whitney non-parametric test was used. In some cases data were analysed by Duncan's multiple comparison method and correlation sought by using Spearman's rank correlation coefficient. Any suspected trends with diseases severity were verified using Wilcoxon's test for trend.
2.3. STUDY PROTOCOLS.

2.3.1. Protocol for the study of *post mortem* tissues.
Specimens of major organs were obtained during the *post mortem* examination of five subjects who had died at the Whittington Hospital, Highgate, London, UK. A full *post mortem* report was available on three of the subjects.

**Subject 1** was a man aged 78 who was examined 17 hours *post mortem*. The causes of death were considered to be a disseminated tumour in the lymph nodes, liver, adrenals (bilateral), and spleen; and pulmonary congestion and oedema. Tissue specimens from the brain, heart, lung, liver, kidney, spleen, and adrenal lymphoma were studied.

**Subject 2** was a man aged 55 who was examined 10 hours *post mortem*. The cause of death was considered to be a ruptured abdominal atherosclerotic aneurysm. Tissue specimens of the brain, lung, liver, kidney and spleen were studied.

**Subject 3** was a man aged 81 who was examined 82 hours *post mortem*. The causes of death were considered to be acute heart failure and coronary artery thrombosis and atherosclerosis together with chronic renal failure and renal ischaemia. Tissue specimens studied were of brain, heart, lung, liver, kidney, and spleen.

**Subject 4** was an elderly man. No *post mortem* report was available on this subject. Tissue specimens studied were of brain, heart, lung, liver, kidney, and spleen.
Subject 5 was an elderly woman. The cause of death was considered to be a primary tumour in the rectum causing obstruction and rupture of the intestine, and hepatic metastases. Tissue specimens of the apparently normal heart, lung, kidney, and spleen were studied. Specimens of the rectal tumour and a nasal skin tumour were also obtained as well as three hepatic specimens:

a) apparently normal hepatic tissue;

b) hepatic metastatic tissue;

c) interface of normal and metastatic tissue.

All tissue specimens were prepared on the day of collection for HPLC analysis of free, phospholipid esterified, and triglyceride esterified fatty acids.

2.3.2. Protocol for the study of colorectal biopsies.

The blind study involved 102 patients with carcinoma of the colon or rectum undergoing surgery at St. Mark’s Hospital, Islington. For comparison tissue samples from both diseased and normal bowel regions were collected where possible. The 91 biopsy specimens, which had been frozen in liquid nitrogen for up to 30 months, were transported to the Whittington hospital in liquid nitrogen. The 11 specimens collected during surgery were transported to the Whittington Hospital in phosphate buffered saline (20mmol/l KH₂PO₄/Na₂HPO₄ in 154mmol/l NaCl, pH 8.0) containing 5mmol/l potassium EDTA on ice for analysis within 24 hours.
2.3.3. Protocol for the study of cervical biopsies.

Cervical biopsy material from a total of 65 women was studied. Forty-six of the women had been referred for valuation of abnormal cytology or a cervical lesion suspected of showing cancerous changes to the Colposcopy Clinic at the Royal Northern Hospital, Islington, London, UK. In 42 colposcopic examination revealed atypical epithelium, whilst in 4 early cervical cancer (Stage 1B) was recognised. The control group comprised 19 women who had undergone operation for non-malignant cervical conditions. Two adjacent large punch biopsies (of wet weight approximately 10mg) were taken from each of the patients and operation specimens: one was snap frozen in liquid nitrogen for HPLC analysis; the other fixed in formal sublimate for histological diagnosis. From 11 women a third biopsy was taken from the squamous epithelium approximately 2-10mm outside the lateral margin of the site of the precancerous transformation. Histological diagnosis was made on six sections taken at three levels based on the criteria of Buckley et al. (1982).

2.3.4. Protocol for the initial studies with cervical cells.

An unselected group of 175 women, attending the Colposcopy Clinic at the Royal Northern Hospital for evaluation of an abnormal Papanicolaou smear, were studied. These cytological abnormalities comprised borderline nuclear abnormality not amounting to mild dyskaryosis (n=15), mild dyskaryosis (n=56), moderate dyskaryosis (n=43), and severe dyskaryosis (n=61). The control group (n=40) was drawn from women, who were attending family planning clinic or a general gynaecology clinic for reasons unrelated to cervical disease. All women in the control group had at least one negative smear result in the previous six months; all gave permission for colposcopic examination and the collection of a further cervical smear.
All 215 women were examined colposcopically and clinical details, including previous medical and social history, current method of contraception, smoking habits and the presence of any cervical erosion, were noted. Exfoliated cervical cells were prepared for HPLC analysis on the day of collection. A punch biopsy was taken, under colposcopic vision, from any suspected precancerous lesion for histological assessment according to the criteria of Buckley et al. (1982).

2.3.5. Protocol for the multicentre studies with cervical cells.
The normal population was drawn from women attending 'Well Woman' clinics at the Elizabeth Garrett Anderson, the Royal Free, and the Middlesex Hospitals in London; and Queen Elizabeth Hospital in Birmingham. The patient population was drawn from women newly referred to the Colposcopy Clinic at the Royal Northern Hospital, Islington, London following an abnormal smear result.

All women were examined colposcopically. Clinical and social information including age and method of contraception was obtained. Two specimens of exfoliated cells were collected: the first for cytological evaluation and the second for HPLC analysis. A punch biopsy was taken from any region suspected of neoplastic change for histological assessment. When no cervical abnormalities were detected cytologically or colposcopically the cervix was considered normal. Histological confirmation of cervical normality could not be sought as it was ethically unacceptable to biopsy a normal cervix.
Exfoliated cells for HPLC analysis were washed into PBS (pH 7.4) containing 5mmol/l dipotassium EDTA which had been prepared centrally and distributed to the participating clinics. All Papanicolaou smears were examined in the Cytology Department at Northwick Park Hospital, Harrow, London. All of the HPLC analyses were carried out in the Biochemistry Department at the Whittington Hospital, Highgate, London. The results of biochemical, cytological, and histological assessments were compared.

2.3.6. Protocol for the concluding studies with cervical cells.

Previous studies had shown that the amount of material available in samples collected from postmenopausal women was frequently insufficient for reliable determination of the %MR. This study was, therefore, confined to premenopausal women.

Women attending the Finsbury Family Planning Clinic who had no cervical abnormalities were recruited to the study as the normal group. Women with cervical abnormalities were drawn from patients attending the Colposcopy Clinic at the Royal Northern Hospital for evaluation of an abnormal smear. The average age of the group women found to have a normal cervix was 31 (range 18-43) and this was identical to the CIN group. Recent cytology was reported as normal (n=313), or showing the presence of minor abnormalities (n=55) or the presence of dyskaryosis suggestive of CIN (n=84).

All women were examined colposcopically by one of two gynaecologists within ten weeks of the a Papanicolaou smear being taken. Cervical cells for HPLC analysis were collected under colposcopic vision. A punch biopsy for histological assessment was taken from any region suspected of neoplastic
changes. Samples were transported to the laboratory at 4°C for HPLC analysis on the day of collection.
3.1. THE DEVELOPMENT OF METHODS FOR THE MEASUREMENT OF DIENE CONJUGATED AND NON-DIENE CONJUGATED FATTY ACIDS IN HUMAN TISSUE AND CELLS.

3.1.1. Verification of the published method.
It was necessary to confirm that the reverse phase HPLC technique of Iversen, Cawood and Dormandy (1985) could separate unsaturated fatty acids with good resolution and sensitivity. Furthermore, it had to be established that diene conjugated and non-diene conjugated fatty acids with the same or similar retention times could be clearly distinguished by detecting the non-diene conjugated fatty acids at 205nm and the diene conjugated ones at 234nm. It was also important to determine how long it took to analyse each sample.

3.1.1.1. Experimental series 1: The experimental verification of the published method.
In order to verify the method it was decided that samples of serum should be prepared and analysed in the manner described by Iversen, Cawood and Dormandy (1985). Serum (0.5ml) was mixed with 0.5ml of a solution containing 0.1mol/l methanol and 5000IU/l phospholipase A2 from naja naja venom. The mixture was incubated for 15 minutes at 25°C. Protein was precipitated and internal standard added with 2ml of a solution of 83mmol/l acetic acid in methanol containing approximately 5-10mmol/l β-eleostearic acid. The mixture was thoroughly mixed by vortex and
centrifuged at 1000g for 10 minutes. The lipids were extracted using a Bond-Elut solid phase extraction system (Analytichem, Harbor City, California, USA). The supernatant was applied to a 3ml column containing 200mg C_{18}-sorbent (no. 617303) which had been washed with 2ml propan-2-ol/acetonitrile (2:1, v/v) and 5ml methanol/water/acetic acid (66:33:0.03, v/v/v). The extraction column was washed with a further 5ml of the latter solvent. The lipids were eluted in 1ml propan-2-ol/acetonitrile (2:1, v/v). The eluate was injected onto an HPLC system, set up as described in section 2.2.1.1. in which a mobile phase consisting of acetonitrile, water and acetic acid (84.9:15:0.1, v/v/v): was pumped at a flow rate of 1.5ml/min through an ODS2 analytical column (HiChrom Ltd., Reading, Berkshire, UK) filled with 5μm spherical capped particles of octadecasilane. Samples were injected manually onto the HPLC, using a 90° Hamilton syringe (Bonaduz, Switzerland) via a Rheodyne valve 7125 fitted with a 50μl sample loop. The UV detectors were set up in series: one set at 205nm to detect the unsaturated fatty acids and the other at 234nm to detect diene conjugated fatty acids.

The resulting chromatograms, shown in Figure 5, demonstrate that the chromatography described by Iversen, Cawood and Dormandy (1985) was successfully reproduced. The four major peaks detected at 205nm were well resolved and had retention times similar to those published for linolenic (18:3(9,12,15)), arachidonic (20:4(5,8,11,14)), linoleic (18:2(9c,12c)) and oleic (18:1(9)) acids. The two peaks detected at 234nm had retention times similar to those published for β-eleostearic acid and 18:2(9c,11t). All peaks eluted within 20 minutes of injection.
Figure 5. Chromatograms of human serum prepared and analysed as described by Iversen, Cawood and Dormandy (1985).

Detector 205nm; Attenuation 0.2aufs
Mobile phase:
84.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min

Peak A=22:6 and 18:3
Peak B=20:4
Peak C=18:2(9c,12c)
Peak D=18:1

Detector 234nm; Attenuation 0.02aufs
Mobile phase:
84.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min

Peak E=18:3(9,11,13)
Peak F=18:2(9c,11c)
3.1.1.2. Experimental series 2: Confirmation of the identity of the major HPLC peaks.

In order to establish that the HPLC system was reproducing the separation described by Iversen, Cawood and Dormandy (1985) the identity of each of the six major peaks had to be confirmed.

A serum sample was prepared for HPLC but without added β-eleostearic acid. The chromatogram of this sample was compared with that obtained from an aliquot mixed 1:1 with propan-2-ol/acetonitrile (2:1, v/v) containing a fatty acid thought to be present in serum phospholipids. The presence of a particular fatty acid was deduced when the added fatty acid co-chromatographed with a peak present in the sample.

Figures 6 and 7 show that the fatty acids which co-chromatograph with the six peaks are eicosapentaenoic (20:5), linolenic (18:3), arachidonic (20:4), linoleic (18:2), eicosatrienoic (20:3), and oleic (18:1) acids. It was confirmed that docosahexaenoic acid (22:6) and linolenic acid (18:3) co-eluted. Figure 8 shows the relative eluting positions of β-eleostearic (18:3(9,11,13)), octadec-9cis,11trans dienoic and octadeca-9trans,11trans-dienoic acids.

The findings are consistent with presence of these fatty acids in the serum sample. A small quantity of synthesised 18:2(9c,11t), was available to spike the serum sample to confirm its retention time. However, this synthesised 18:2(9c,11t) was too impure to use to calibrate the assay.
Figure 6. The identification of chromatographic peaks of non-diene conjugated fatty acids present in human serum.

Serum sample
Detection 205nm
Attenuation 0.2

Serum spiked with 20:5
Detection 205nm
Attenuation 0.2
RT 20:5 = 5.99 min

Serum spiked with 22:6
Detection 205nm
Attenuation 0.2
RT 22:6 = 7.46 min

Serum spiked with 18:3
Detection 205nm
Attenuation 0.2
RT 18:3 = 7.34 min

Serum spiked with 20:4
Detection 205nm
Attenuation 0.2
RT 20:4 = 8.57 min

Serum spiked with 18:2(9c,12c)
Detection 205nm
Attenuation 0.2
RT 18:2(9c,12c) = 9.87 min
Figure 7. The identification of chromatographic peaks of non-diene conjugated fatty acids present in human serum.

- Serum sample
  - Detection: 205nm
  - Attenuation: 0.2

- Serum spiked with 20:3
  - Detection: 205nm
  - Attenuation: 0.2
  - RT 20:3 = 11.66min

- Serum spiked with 18:1
  - Detection: 205nm
  - Attenuation: 0.2
  - RT 18:1 = 15.10min
Figure 8. The identification of chromatographic peaks of diene conjugated fatty acids present in human serum.

Serum sample
Detection 234nm
Attenuation 0.02

Serum spiked with β-eleostearic acid
Detection 234nm
Attenuation 0.02
RT β-eleostearic = 8.03min

Serum spiked with 18:2(9c,11t)
Detection 234nm
Attenuation 0.02
RT 18:2(9c,11t) = 9.70min

Serum spiked with 18:2(9t,11t)
Detection 234nm
Attenuation 0.02
RT 18:2(9t,11t) = 11.27min
3.1.1.3. Criticisms of the published method.

The method offer high resolution but the complete separation of the fatty acids in each sample was slow, requiring approximately 20 minutes. Therefore, only three samples per hour could be analysed (less than 25 per working day). As it was intended that a large number of samples were to be analysed ways of increasing the sample throughput would have to be investigated.

It was noted that the non-diene conjugated fatty acids, which have maximal absorbance at approximately 200nm were, in fact, detected at 205nm. This was presumably because acetonitrile and acetic acid absorb strongly at this wavelength. The use of higher quality acetonitrile may enable the non-diene conjugated fatty acids to be detected with greater sensitivity at 200nm.

Since the assay measured free fatty acids in the specimen after phospholipase A₂ hydrolysis any free fatty acids present in the specimen prior to the addition of phospholipase A₂ would be assumed to have been hydrolysed from phospholipids by the phospholipase A₂. As almost all fatty acids in fresh serum are esterified this should not normally cause any difficulties. However, there are cases in which the free fatty acid fraction is significant, such as patients in diabetic coma (Cawood, Harrison and Dormandy 1985), or following myocardial infarct or heparinisation (Wickens et al. 1987).

3.1.2. Optimisation of the HPLC analysis.

The initial experiments showed the analytical method to have high resolution and good sensitivity, but the need for more rapid analysis was indicated. Experiments were carried out to determine whether the separation could be hastened at the expense of some resolution. The high
sensitivity had to be maintained as far as possible as it would be essential for the analysis of fragments of tissues and cell suspensions.

3.1.2.1. Experimental series 3: The effect on the chromatography of changing the solvent eluting strength.

The greater the eluting strength of the mobile phase the faster the chromatography and the shorter the analysis time. It was, therefore, decided that the effect of changing the proportion of acetonitrile in the mobile phase on the chromatography should be investigated.

The HPLC system equilibrated with mobile phase containing 84.9%, 89.9%, and 94.9% acetonitrile (by volume) was used to analyse a sample prepared as described in section 3.1.1.1.

Figure 9 shows the chromatograms obtained from the analysis. It is clear that when the solvent contained 94.9% acetonitrile the peaks were not completely resolved. When the mobile phase contained 89.9% acetonitrile the separation was markedly faster with adequate resolution; in addition the system gave superior sensitivity particularly for the diene conjugated fatty acids and oleic acids.

It was, therefore, decided that all subsequent analyses should be carried out using a mobile phase containing 89.9% acetonitrile.
Figure 9. Chromatograms obtained from the analysis of human serum using mobile phases varying in water/acetonitrile composition.

Mobile phase:
84.9% Acetonitrile
15% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.2aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.2aufs

Mobile phase:
94.9% Acetonitrile
5% Water
0.1% Acetic Acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.2aufs

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3.1.2.2. Experimental series 4: The effect of changing the pH of the mobile phase.

The presence of acetic acid in the mobile phase improves the resolution of the fatty acids by making them more polar. It was decided that the effect of increasing the proportion of acetic acid in the mobile phase still further should be investigated.

The HPLC system equilibrated with 0.1%, 0.2%, 0.3% acetic acid was used to analyse a sample of serum prepared as described in section 3.1.1.1.

Figure 10 shows the resulting chromatograms. In the absence of acetic acid the resolution was very poor: the non-diene conjugated fatty acids elute in two broad peaks. It is clear that the chromatography is not improved by increasing the proportion of acetic acid above 0.1%. Indeed, the sensitivity of the system is adversely affected.

It was, therefore, decided that the proportion of acetic acid in the mobile phase should remain at 0.1%
Figure 10. Chromatograms obtained from the analysis of human serum using mobile phases varying in acetic acid content.
3.1.2.3. Experimental series 5: The effect of increasing the flow rate of the mobile phase on the chromatography.

Increasing the solvent flow rate through column causes the fatty acids to be more rapidly eluted from the column, thereby reducing the analysis time. It was, therefore, decided that the effect on the chromatography of increasing the flow rate should be investigated.

A lipid extract of serum was injected onto the HPLC system with the flow rate set at 1.5ml/min, 1.75ml/min, and 2ml/min.

Figure 11 shows that increasing the flow rate did indeed speed up the chromatography. However, the diene conjugated fatty acid peaks merged when the flow rate was either 1.75ml/min or 2ml/min. Furthermore, increasing the flow rate led to a loss in sensitivity.

In conclusion, it was felt that the loss of sensitivity at flow rates above 1.5ml/min was too high a price to pay for reducing the analysis time by 100 seconds. It was, therefore, decided that the flow rate should remain at 1.5ml/min.
Figure 11. Chromatograms obtained from the analysis of human serum with the mobile phase pumped through the column at varying flow rates.

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.2aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.75ml/min
Detector 205nm
Attenuation 0.2aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 2.00ml/min
Detector 205nm
Attenuation 0.2aufs
3.1.2.4. Experimental series 6: The effect of reducing the detector attenuation of the detector response.

Increasing the sensitivity would not only increase the fatty acid peak heights but also accentuate baseline noise. However, since no baseline fluctuation was evident at the attenuation settings in use, it was decided that the minimum attenuation settings which provided a stable baseline should be determined.

A sample of serum was prepared as described in section 3.1.1.1. The extract was injected with the detectors set at 205nm, 0.2 aufs; and 234nm, 0.02 aufs. The attenuation settings of the detectors were reduced. The extract, diluted by the same factor as the reduction in detector attenuation, was again injected. The resulting chromatograms are shown in Figure 12. It can be seen that when detector attenuation was set below 0.0005 aufs it became difficult to determine the true base of a peak amongst the baseline fluctuation.

It was concluded that a maximum fourfold increase in sensitivity was possible by reducing detector attenuation.
Figure 12. Chromatograms obtained from the analysis of human serum using varying attenuation settings of the detectors.

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.2aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.1aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.05aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 205nm
Attenuation 0.02aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm
Attenuation 0.01aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm
Attenuation 0.005aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm
Attenuation 0.002aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm
Attenuation 0.0005aufs

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm
Attenuation 0.0002aufs
3.1.2.5. Experimental Series 7: The effect of sample loop volume on the chromatographic resolution.

The published method details the use of a 50μl sample loop. It was felt that greater resolution might be obtained if a sample loop of smaller volume was used. Since sensitivity was not a limiting factor it was decided that the effect on resolution of fitting a 20μl sample loop should be investigated.

A sample of serum was prepared as described in section 3.1.1.1.. The lipid extract of the sample was injected onto the HPLC fitted with either a 50μl or a 20μl sample loop. The attenuation of the detectors' response was reduced accordingly to maintain the magnitude of the peaks.

Figure 13 shows the greater resolution obtained when a 20μl sample loop was used with an appropriate reduction in the attenuation of the detectors' response. All further chromatographic analyses were carried out with a 20μl sample loop fitted and the attenuation of the detectors reduced accordingly.
Figure 13. Chromatograms obtained from the analysis of human serum using 20μl and 50μl sample loops.
3.1.2.6. Experimental Series 8: Optimisation of the detection wavelength for non-diene conjugated fatty acids.

Although the non-diene conjugated fatty acids absorb strongly in the range 190-200nm, Iversen, Cawood and Dormandy (1985) chose to detect these at 205nm. It was felt that the reason for this was the low transparency of the mobile phase components acetic acid and in particular acetonitrile over the range 190-200nm. It was, therefore, decided to investigate whether the non-diene conjugated unsaturated fatty acids might be detected at 200nm if a higher grade of acetonitrile was used in the mobile phase.

Far UV grade acetonitrile was obtained with 80% transparency at 200nm. The HPLC system with a UV detector set at either 200nm or 205nm was used to analyse a lipid extract of serum which had been prepared as described in section 3.1.1.1..

Figure 14 shows the resulting chromatograms from the analyses. The detection of the non-diene unsaturated fatty acids at 200nm resulted in a 30% increase in sensitivity.

It was, therefore, decided that the Far UV grade acetonitrile should be used in the mobile phase so that the non-diene conjugated unsaturated fatty acids could be detected at 200nm for all analyses. There was also an economical benefit of using this high quality solvent. After passing through the HPLC system the mobile phase could be returned to the 2.5 litre reservoir and recycled for up to four weeks since it was possible to 'back off' the rising background absorbance to zero the baseline throughout this period.
Figure 14. The detection non-diene conjugated fatty acids at 200 and 205nm.

Detector 205nm; Attenuation 0.2aufs
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min

Detector 200nm; Attenuation 0.2aufs
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
3.1.2.7. Automation of the assay.

The limiting factor which prevented the analysis of large numbers of samples was the restriction of the assay to attended operation. An autoinjector would enable injections to be made 24 hours a day.

It was, therefore, decided that a PROMIS autoinjector (Spark Holland, Emmen, Netherlands) should be attached to the HPLC system. It was anticipated that up to 75 specimens might be analysed in a 24 hour period and that whilst automatic injections were proceeding the analyst would be able to calculate results or prepare fresh ones.

Obviously, it had to be established that the autoinjector would make precise injections. This was confirmed in experiments detailed later (section 3.1.4.) in which the intrabatch and interbatch coefficients of variation for the method were calculated.

3.1.3. Calibration and standardisation of the HPLC method.

3.1.3.1. Experimental Series 9: Linearity of the detector response.

In order to assay fatty acids in a specimen it was necessary to determine the range over which the detector response was linear. It was decided to determine the detector response to concentrations of docosahexaenoic, eicosapentaenoic, arachidonic, eicosatrienoic, linolenic, linoleic, oleic, β-eleostearic and octadeca-9trans,11trans dienoic acids over the range 10μmol/l to 1000μmol/l.

Each of the fatty acids was dissolved in propan-2-ol/acetonitrile (2:1,v/v) to the following concentrations: 10μmol/l, 25μmol/l, 50μmol/l, 100μmol/l, 250μmol/l, 500μmol/l and 1000μmol/l.
Figures 15-23 show the detector response (peak height) plotted against fatty acid concentration. Each point plotted is the mean peak height from five injections. The standard deviation in each case was less than 4%. The detector response was linear for all fatty acids up to 250μmol/l; for oleic and linolenic up to 500μmol/l; and for eicosatrienoic, linoleic, β-eleostearic, and octadeca-9trans,11trans dienoic acids up to 1000μmol/l. Any lipid extracts containing concentrations of a fatty acid above the linear range would have to be diluted.
Figure 15. The eicosapentaenoic acid detector response curve.

Figure 16. The docosahexaenoic acid detector response curve.
Figure 17. The linolenic acid detector response curve.

![Linolenic acid detector response curve graph](image)

Figure 18. The arachidonic acid detector response curve.

![Arachidonic acid detector response curve graph](image)
Figure 19. Linoleic acid detector response curve

Figure 20. Eicosatrienoic acid detector response curve.
Figure 21. Oleic acid detector response curve.

Figure 22. The \( \beta \)-eleostearic acid detector response curve.
Figure 23. The 18:2(9t,11t) detector response curve.
3.1.3.2. Experimental Series 10: External standardisation.

In order to calculate the concentrations of fatty acids of interest in samples the detector response required calibration by use of external standards. The original method only assayed linoleic acid and octadeca-9cis, 11trans-dienoic acids but it was considered that the need may arise to measure other fatty acids as well. It was not possible to measure linolenic or docosahexaenoic acids in serum separately since it was shown in section 3.1.1.2. that these fatty acids co-chromatograph. It was not considered necessary to assay the eicosapentaenoic and eicosatrienoic acids.

It was, therefore, decided that the external standard should contain arachidonic, linoleic, oleic, and 18:2(9c,11t) acids at concentrations within the linear range and comparable with those found in the lipid extracts of serum. Unfortunately, because there was no source of pure 18:2(9c,11t) this fatty acid could not be used in the external standard. It was, therefore, decided to use the closest available fatty acid isomer, 18:2(9t,11t), donated by the Paint Research Association. The external standard, therefore, contained 4.17µmol/l 18:2(9t,11t), 166.7µmol/l linoleic acid, 41µmol/l arachidonic acid, and 500 µmol/l oleic acid in propan-2-ol/acetonitrile (2:1, v/v). Aliquots of the external standard were stored in liquid nitrogen for use throughout the project. An aliquot of the external standard was degassed on each day of analysis and run after every ten samples. Figure 24 shows chromatograms of the external standard at the start and end of the project.

It is clear that the fatty acids in the external standard were stable for the duration of the project: the peaks are in the same proportion. In addition hydroperoxide peaks, which would elute close to the solvent front and be most evident on the 234nm trace, are absent.
Figure 24. Chromatograms of the external standard at the start and end of the project.

External standard at start of project
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Detector 200nm; Attenuation 0.1aufs
Sample loop volume 20μl

External standard at start of project
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm; Attenuation 0.02aufs
Sample loop volume 20μl

External standard at end of project
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Detector 200nm; Attenuation 0.1aufs
Sample loop volume 20μl

External standard at end of project
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm; Attenuation 0.02aufs
Sample loop volume 20μl
3.1.3.3. Experimental Series 11: Internal standardisation.

For precise quantitation of the fatty acids in specimens it was necessary to assess the efficiency of the lipid extraction so that the lipid concentrations could be adjusted accordingly. Iversen, Cawood and Dormandy (1985) added β-eleostearic acid, a fatty acid not present in humans, to each sample prior to the lipid extraction as an internal standard. However, as this fatty acid was no longer commercially available an alternative had to be sought. The fatty acid to be used as internal standard have a retention time similar to the fatty acids of interest and could not be present in the samples under study. It was decided that the 18:2(9t,11t) donated by the Paint Research Association, which had a retention time close to that of linoleic acid and 18:2(9c,11t) might be a suitable internal standard. However, it had to be established that it was not present in any of the specimens to be studied.

Lipid extracts of different serum specimens were prepared as described in section 3.1.1.1. and injected onto the HPLC. Each extract was divided into two aliquots. One was injected directly onto the HPLC whilst the other was mixed with an equal volume of a 10μmol/l 18:2(9t,11t) in propan-2-ol/acetonitrile (2:1, v/v) before injection onto the HPLC.

Figure 25 shows the two chromatograms from one of the serum specimens. It can be seen that the 18:2(9t,11t) is only present in the lipid extract to which the fatty acid was added.

It was, therefore, clear that 18:2(9t,11t) was not a constituent of human serum and could be used as the internal standard. The extraction efficiency was assessed by comparing the peak height of the internal standard in the extracted sample with the peak height of a solution of internal standard injected onto the HPLC without prior extraction.
Figure 25. Chromatograms of human serum with and without added 18:2(9t,11t)

Serum without added 18:2(9t,11t)
Mobile phase:
89.9% Acetonitrile/ 10% Water/ 0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm; Attenuation 0.02aufs
Sample loop volume 20µl

Serum with added 18:2(9t,11t)
Mobile phase:
89.9% Acetonitrile/ 10% Water/ 0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm; Attenuation 0.02aufs
Sample loop volume 20µl
3.1.3.4. The calculation of results of HPLC analysis.

3.1.3.4.1. Calculation of fatty acid concentrations.
All calculations were based on the peak height. The concentration of a fatty acid could be calculated when its concentration in the lipid extract was within the established linear range of the detector response. Experiments reported in section 3.1.3.1. showed the linear range for linoleic acid and 18:2(9c,11t) up to 1000μmol/l, for oleic acid up to 500μmol/l, and for arachidonic acid up to 250μmol/l. Within these ranges fatty acid concentrations were calculated as follows:-

\[
\text{Fatty acid (μmol/l)} = \frac{\text{PH FA (sample)} \times \text{Conc. Std}}{\text{PH FA (Std)}}
\]

where:

- \(\text{PH FA (sample)}\) is the peak height of the fatty acid in the sample.
- \(\text{PH FA (Std)}\) is the peak height of the fatty acid in the external standard.
- \(\text{Conc. (Std)}\) is the concentration of the fatty acid in the external standard.

However, it was necessary to consider two other factors. 
First, the fatty acid concentrations in the lipid extract had to be related to the original volume of the sample. The fatty acid concentrations in the extract, therefore, were multiplied by the factor:-

\[
\frac{\text{Extract volume}}{\text{Sample volume}}
\]

where:

- \(\text{Extract volume}\) is the final volume (ml) of solvent in which the sample was dissolved prior to injection.
- \(\text{Sample volume}\) is the original volume of serum from which the lipid extract was prepared.
Second, the efficiency of the lipid extraction step.

The fatty acid concentrations were multiplied by the reciprocal of the efficiency of the extraction.

\[
\frac{1}{\text{Efficiency}} = \frac{\text{PH IS (U)} \times \text{Vol.IS}}{\text{PH IS (samp.)} \times \text{Extract volume}}
\]

where:-

Efficiency is the efficiency of the extraction.

PH IS (U) is the peak height of the internal standard injected without extraction.

Vol IS (samp.) is the volume of internal standard added to the sample prior to extraction.

Therefore, the full calculation for the concentration of a fatty acid in a serum specimen is:-

\[
([\text{Fatty acid}] = \frac{\text{PH FA (samp.)} \times \text{PH IS (U)} \times \text{Vol IS} \times \text{Conc.of Standard}}{\text{PH standard} \times \text{PH IS(sample)} \times \text{Sample Vol}})
\]

where:-

PH IS (sample) is the peak height of the internal standard in the sample.

It can be seen that the Extract volume cancelled out.

3.1.3.4.2. Calculation of the molar ratio of 18:2(9c,11t) to 18:2(9c,12c)

The relative concentration of 18:2(9c,11t) to 18:2(9c,12c) was expressed as a molar ratio and was calculated as follows:-
Molar Ratio (%MR) = \textbf{Concentration 18:2(9c,11t) x 100} \\
\textbf{Concentration 18:2(9c,12c)}

The molar ratio could also be calculated without first determining the concentrations of 18:2(9c,11t) and 18:2(9c,12c) as follows:

\[
\text{Molar Ratio (%MR)} = \frac{\text{PH}_X \text{ (sample)} \times \text{PH}_Y \text{ (S)} \times \text{Conc}_X}{\text{PH}_Y \text{ (sample)} \times \text{PH}_X \text{ (S)} \times \text{Conc}_Y} \times 100
\]

where:

\text{PH}_X \text{ (sample)} is the peak height of 18:2(9c,11t) in the sample.
\text{PH}_X \text{ (S)} is the peak height of 18:2(9c,11t) in the external standard.
\text{Conc}_X is the concentration of 18:2(9c,11t) in the external standard.
\text{PH}_Y \text{ (sample)} is the peak height of 18:2(9c,12c) in the sample.
\text{PH}_Y \text{ (S)} is the peak height of 18:2(9c,12c) in the external standard.
\text{Conc}_Y is the concentration of 18:2(9c,12c) in the external standard.
3.1.4. The improved method for the measurement of diene conjugated and non-diene conjugated fatty acids in serum

3.1.4.1. Summary of the major modifications to the method.
Several of the modifications had now been made to the original method and the principal ones are given below.

1. The mobile phase consisted of acetonitrile, water, acetic acid; 89.9:10:0.1 (v/v/v).
2. The sample volume required was reduced to 20μl.
3. The non-diene conjugated fatty acids were detected at 200nm.
4. An autoinjector was able to inject samples 24 hours a day if required.
5. Arachidonic acid and oleic acid could also be quantitated.
6. The internal standard used was now the diene conjugated linoleic acid, isomer, 18:2(9t,11t) donated by the Paint Research Association.

3.1.4.2. Experimental series 12: The calculation of the coefficients variation for the analysis of diene conjugated and non-diene conjugated fatty acids in serum.

In order to ensure the assay was precise coefficients of variation had to be determined. The intrabatch and interbatch CV's were, therefore, determined for the phospholipase A2 hydrolysis, the lipid extraction and subsequent automated HPLC analysis of serum. Approximately 35ml of sheep serum, which has a high proportion of 18:2(9c,11t), was divided into 0.5ml aliquots. To obtain the intrabatch CV twenty were prepared and analysed with appropriate standards according to the procedure described in section 3.1.1.1. and modified as described in section 3.1.4.1. The interbatch CV was obtained by preparing and analysing ten aliquots on 10 different days.
The results of the analyses and the intrabatch CV are given in Table 1. The intrabatch CV was 4.9% for the concentration of 18:2(9c,11t), 8.1% for the concentration of 18:2(9c,12c), 5.4% for the concentration of 20:4, 6.2% for the concentration of 18:1, 3.5% for the %MR and 18.0% for the recovery. The results of the analyses and the interbatch CV are given in Table 2. The interbatch CV was 6.3% for the concentration of 18:2(9c,11t), 5.7% for the concentration of 18:2(9c,12c), 7.6% for the concentration of 20:4, 5.4% for the concentration of 18:1, 5.8% for the %MR and 24% for the recovery.

Despite the considerable variation in recovery the CV for the concentrations of the fatty acids and the molar ratio were satisfactory, indicating that the method was precise and reliable. Measures to adapt the assay for the analysis of tissue samples could now be investigated.
Table 1. The intrabatch coefficient of variation calculated from the analytical results of 20 specimens of pooled serum prepared on the same day.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>18:2(9c,11t) μmol/l</th>
<th>18:2(9c,12c) μmol/l</th>
<th>20:4 μmol/l</th>
<th>18:1 μmol/l</th>
<th>%MR</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
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<td>29.0</td>
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<td>276</td>
<td>518</td>
<td>3.98</td>
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<td>277</td>
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<td>582</td>
<td>236</td>
<td>432</td>
<td>4.60</td>
<td>61</td>
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<td>410</td>
<td>4.66</td>
<td>62</td>
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<td>63</td>
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<tr>
<td>7</td>
<td>26.7</td>
<td>575</td>
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<td>446</td>
<td>4.64</td>
<td>65</td>
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<tr>
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<td>436</td>
<td>4.53</td>
<td>71</td>
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<tr>
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<td>237</td>
<td>440</td>
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<td>622</td>
<td>236</td>
<td>449</td>
<td>4.46</td>
<td>81</td>
</tr>
</tbody>
</table>

Mean: 28.0 μmol/l, 623 μmol/l, 243 μmol/l, 446 μmol/l, 4.51 %MR, 66.0 Recovery %

SD: 1.38, 50, 13, 27, 0.16, 11.9

CV %: 4.9, 8.0, 5.3, 6.1, 3.5, 18.0
Table 2. Interbatch CV for lipid extraction and HPLC analysis of diene conjugated and non-
diene conjugated fatty acids.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>18:2(9c,11t) μmol/l</th>
<th>18:2(9c,12c) μmol/l</th>
<th>20:4 μmol/l</th>
<th>18:1 μmol/l</th>
<th>%MR</th>
<th>Recovery %</th>
</tr>
</thead>
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<td>213</td>
<td>416</td>
<td>4.45</td>
<td>38</td>
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<tr>
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<td>433</td>
<td>4.21</td>
<td>42</td>
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<tr>
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<td>605</td>
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<td>416</td>
<td>4.18</td>
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</tr>
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<td>23.7</td>
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<td>208</td>
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<td>24.7</td>
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<td>377</td>
<td>4.41</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>25.1</td>
<td>592</td>
<td>246</td>
<td>428</td>
<td>4.24</td>
<td>74</td>
</tr>
<tr>
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<td>23.3</td>
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<td>240</td>
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<td>4.08</td>
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<td>4.96</td>
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<td>4.41</td>
<td>84</td>
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<tr>
<td>10</td>
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<td>573</td>
<td>216</td>
<td>441</td>
<td>4.66</td>
<td>85</td>
</tr>
</tbody>
</table>

Mean | 25.6 | 582 | 232 | 416 | 4.40 | 68.2 |
SD   | 1.6  | 33  | 18  | 22  | 0.26 | 16.8 |
CV % | 6.3  | 5.7 | 7.8 | 5.4 | 5.8  | 24.6 |
3.1.5. Experiments to determine the effect of enzyme inhibitors on the assay of phospholipid esterified diene conjugated and non-diene conjugated fatty acids in serum.

3.1.5.1. Experiments with potassium EDTA.

3.1.5.1.1. Experimental series 13: The effect of potassium EDTA on lipid hydrolysis by phospholipase A2.

EDTA is widely used as an anticoagulant for the collection of plasma. It was anticipated that some specimens might contain EDTA. It was known that the phospholipase A2 enzyme (from *naja naja* venom) used in the assay required calcium as a co-factor for activity. It was, therefore, important to determine whether the concentrations of EDTA normally present in such specimens would affect the assay.

Five hundred microlitre aliquots of pooled serum were mixed with an equal volume of 20mmol/l phosphate buffered saline (PBS) containing various concentrations of EDTA. The final concentrations of the potassium EDTA in the diluted serum ranged from 0-5mmol/l. The samples were prepared and analysed with appropriate standards according to the procedure described in section 3.1.1.1. and modified as described in section 3.1.4.1.. There were five replicates for each EDTA concentration.

Figure 26 shows the striking effect of the potassium EDTA on the assay of diene conjugated and non-diene conjugated fatty acids in the samples. The activity of the phospholipase A2 was significantly impaired at very low concentrations of EDTA. In the presence of 0.25mmol/l EDTA phospholipid hydrolysis was only approximately 80% complete. It was concluded that modifications to the assay would be required if specimens containing EDTA were to be analysed.
Figure 26. The effect of potassium EDTA on the release of phospholipid esterified fatty acids by phospholipase A₂.

a) Graph showing release of 18:2(9c,11t) from serum phospholipids by phospholipase A₂ with increasing potassium EDTA concentration. Each point plotted represents the mean of five replicate samples.

b) Graph showing release of 18:2(9c,12c), 18:1, and 20:4 from serum phospholipids by phospholipase A₂ with increasing potassium EDTA concentration. Each point plotted represents the mean of five replicate samples.
3.1.5.1.2. Experimental series 14: Studies to determine whether the effect of EDTA can be overcome by addition of calcium chloride to specimens prior to phospholipid hydrolysis.

Experiments described in section 3.1.5.1. showed that the method for assaying diene conjugated and non-diene conjugated fatty acids could not be applied to the assay of specimens containing EDTA. Since it was anticipated that EDTA might be present in some specimens for analysis it was decided to investigate how this difficulty might be circumvented.

Five hundred microlitre aliquots of pooled serum containing 5mmol/l potassium EDTA were mixed with an equal volume of a 20mmol/l PBS containing varying concentrations of calcium chloride. The final concentration of calcium chloride in the diluted serum ranged from 0.5-25mmol/l. There were five replicates for each calcium chloride concentration. The samples were prepared and analysed with appropriate standards according to the procedure described in section 3.1.1.1. and modified as described in section 3.1.4.1..

Figure 27 shows the experimental results. It can be seen that 25mmol/l calcium chloride is sufficient to allow full activity of the phospholipase A\textsubscript{2}. It was, therefore, concluded that specimens containing EDTA could be analysed if calcium chloride (final concentration 25mmol/l) was added to the specimen before addition of phospholipase A\textsubscript{2}.
Figure 27. The effect of calcium chloride in restoring full activity to phospholipase A2 in the presence of 5mmol/l potassium EDTA

**a**) Graph showing release of 18:2(9c,11t) from serum phospholipids by phospholipase A2 in the presence of 5mmol/l potassium EDTA and increasing calcium chloride concentration. Each point plotted represents the mean of five replicate samples.

**b**) Graph showing release of 18:2(9c,12c), 18:1, and 20:4 from serum phospholipids by phospholipase A2 in the presence of 5mmol/l potassium EDTA and increasing calcium chloride concentration. Each point plotted represents the mean of five replicate samples.
3.1.5.2. Experimental series 15: The effect of azide on the activity of phospholipase A₂.

Azide is used as an antioxidant enzyme (e.g., catalase) inhibitor in *in vitro* experiments. It might, therefore, be desirable to assay samples containing azide, and so the effect of sodium azide on the assay and specifically the activity of the phospholipase A₂ enzyme was investigated.

Pooled serum was divided into 250µl aliquots. Each aliquot was mixed with an equal volume of PBS (pH 7.4) which contained concentrations of sodium azide in the range 0-25mmol/l. The samples were prepared and assayed by the method described in section 3.1.1.1. with modifications detailed in section 3.1.4.3..

Figure 28 shows the concentrations of 18:2(9c,11t), linoleic, arachidonic, and oleic acids as determined by the assay. It can be seen that the presence of sodium azide does not inhibit phospholipase A₂ until its concentration exceeded 10mmol/l.

Since the azide concentrations required to inhibit antioxidant enzymes is less than 5mmol/l it was concluded that the analysis of samples from such *in vitro* experiments could be performed without any modifications to the method.
Figure 28. The effect of sodium azide on the release of phospholipid esterified fatty acids from serum phospholipids by phospholipase A2.

a) Graph showing release of 18:2(9c,11t) from serum phospholipids by phospholipase A2 with increasing sodium azide concentration. Each point plotted represents the mean of five replicate samples.

b) Graph showing release of 18:2(9c,12c), 18:1 and 20:4 from serum phospholipids by phospholipase A2 with increasing sodium azide concentration. Each point plotted represents the mean of five replicate samples.
3.1.6. Experiments to determine whether solvent lipid extraction could be used as an alternative to solid phase.

3.1.6.1. Experimental series 16: Comparison of lipid extraction by Solid phase (Bond-Elut) and solvent (chloroform/methanol)

During the course of the project it became necessary for economic reasons to change the method by which lipid extracts were prepared. The increasing cost of the Bond-Elut cartridges forced a move to chloroform/methanol extraction as described by Folch, Lees and Stanley (1957). It was, therefore, necessary to ensure that the two methods of lipid extraction gave comparable results.

Ten 0.5ml aliquots of the pooled serum were prepared and analysed (using the Bond-Elut extraction system) as described in section 3.1.1.1. modified as detailed in section 3.1.4.1.

Ten 100μl aliquots of the remaining pooled serum were prepared for analysis as follows. The 100μl serum was mixed with 100μl 5000IU/l phospholipase A₂ in a 5ml glass tube and incubated at 25°C for 15 minutes. To this mixture was added 200μl 5-10μmol/l 18:2(9c,11t) in methanol, 50μl 1mol/l HCl and 1200μl chloroform/methanol (2:1, v/v). Each preparation was mixed vigorously and centrifuged at 1000g for 10 minutes, the chloroform (lower) layer was removed to a clean glass tube and evaporated to dryness under a stream of oxygen free nitrogen. The lipids in each sample were resuspended in 200μl propan-2-ol/acetonitrile (2:1, v/v) and this extract injected onto the HPLC system described in sections 3.1.1.1. and 3.1.4.1.

The analytical results of the lipid extracts prepared using the 'Bond Elut' cartridges are listed in Table 3, and those of the extracts prepared using
chloroform/methanol extraction are listed in Table 4. It can be seen from the means and standard deviation values given that comparable results were obtained by the two methods. The results obtained when the solvent extraction was used were slightly lower than those obtained by the 'Bond Elut' extraction. However, the results were sufficiently close for the two methods of extraction to be considered equivalent. Use of solvent extraction effected substantial financial savings.
Table 3. Results of the 10 lipid extracts of pooled serum prepared using the solid phase (‘Bond Elut’) extraction system.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>20:4</th>
<th>18:1</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/l</td>
<td>µmol/l</td>
<td>µmol/l</td>
<td>µmol/l</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>42.8</td>
<td>781</td>
<td>320</td>
<td>453</td>
<td>67</td>
</tr>
<tr>
<td>2</td>
<td>46.2</td>
<td>862</td>
<td>353</td>
<td>468</td>
<td>53</td>
</tr>
<tr>
<td>3</td>
<td>45.3</td>
<td>838</td>
<td>344</td>
<td>478</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>42.5</td>
<td>775</td>
<td>315</td>
<td>442</td>
<td>65</td>
</tr>
<tr>
<td>5</td>
<td>40.8</td>
<td>751</td>
<td>305</td>
<td>421</td>
<td>61</td>
</tr>
<tr>
<td>6</td>
<td>44.3</td>
<td>792</td>
<td>323</td>
<td>454</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>43.0</td>
<td>776</td>
<td>316</td>
<td>437</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>40.6</td>
<td>751</td>
<td>310</td>
<td>411</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>42.2</td>
<td>775</td>
<td>320</td>
<td>437</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>45.4</td>
<td>838</td>
<td>347</td>
<td>469</td>
<td>59</td>
</tr>
</tbody>
</table>

Mean 43.3 789 325 447 61

SD 1.9 44 17 22 5

CV % 4.4 5.6 5.2 4.9 8.3
Table 4. Results of the analysis of 10 lipid extracts of pooled serum prepared using the solvent (chloroform/methanol) extraction method.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>20:4</th>
<th>18:1</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/l</td>
<td>pmol/l</td>
<td>pmol/l</td>
<td>pmol/l</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>43.7</td>
<td>797</td>
<td>326</td>
<td>462</td>
<td>65</td>
</tr>
<tr>
<td>2</td>
<td>41.6</td>
<td>766</td>
<td>311</td>
<td>429</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>43.9</td>
<td>792</td>
<td>322</td>
<td>446</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>44.3</td>
<td>845</td>
<td>339</td>
<td>459</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>41.7</td>
<td>760</td>
<td>309</td>
<td>413</td>
<td>59</td>
</tr>
<tr>
<td>6</td>
<td>43.4</td>
<td>776</td>
<td>316</td>
<td>445</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>44.5</td>
<td>821</td>
<td>340</td>
<td>460</td>
<td>63</td>
</tr>
<tr>
<td>8</td>
<td>41.0</td>
<td>736</td>
<td>304</td>
<td>403</td>
<td>51</td>
</tr>
<tr>
<td>9</td>
<td>40.2</td>
<td>745</td>
<td>299</td>
<td>414</td>
<td>63</td>
</tr>
<tr>
<td>10</td>
<td>39.8</td>
<td>732</td>
<td>286</td>
<td>401</td>
<td>56</td>
</tr>
</tbody>
</table>

Mean 42.4 777 315 433 59
SD 1.8 37 17 24 5
CV % 4.2 4.8 5.3 5.5 8.3
3.1.6.2. Experimental series 17: Investigation of the relative extraction efficiency of the chloroform methanol extraction

It was anticipated that specimens containing high and low fatty acid concentrations would be analysed. It was, therefore, important to establish that all specimens, regardless of fatty acid concentration, would be extracted with comparable efficiency.

Aliquots (100μl) of serum were spiked with 0-200μl of a stock fatty acid mixture which contained the following fatty acids at the concentrations given:-

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic</td>
<td>430μmol/l</td>
</tr>
<tr>
<td>Linoleic</td>
<td>1500μmol/l</td>
</tr>
<tr>
<td>18:2(9t,11t)</td>
<td>200μmol/l</td>
</tr>
<tr>
<td>Oleic</td>
<td>1800μmol/l</td>
</tr>
</tbody>
</table>

Volumes of the stock fatty acid mixture The samples were extracted and prepared for analysis using the chloroform/methanol lipid extraction method detailed in section 3.1.6.1. and analysed by HPLC as described for the chloroform/methanol extraction detailed in sections 3.1.1.1. and 3.1.4.1.

Figure 29 shows the concentration of each fatty acid in dilutions of the fatty acid mixture which are equivalent to their concentrations in the extract derived from serum spiked with various amounts of the fatty acid mixture. Figure 30 shows the concentration of each fatty acid on HPLC analysis plotted against volume of the stock fatty acid mixture added to the serum prior to extraction. It can be seen that the data produce a straight line for all fatty acids indicating that specimens with high and low fatty acid concentrations would be extracted with comparable efficiency.
Figure 29. The analysis of dilutions of the fatty acid mixture containing 18:2(9c,11t), 18:2(9t,11t), 18:2(9c,12c), 18:1, and 20:4.

Graph showing the relative extraction efficiency of 18:2(9c,11t) and 18:2(9t,11t) from dilutions of the fatty acid mixture.

Graph showing the relative extraction efficiency of 18:2(9c,12c), 18:1, 20:4 from dilutions of the fatty acid mixture.
Figure 30. The analysis of serum spiked with a fatty acid mixture containing $18:2(9c,11t)$, $18:2(9t,11t)$, $18:2(9c,12c)$, $18:1$, and $20:4$.

a) Graph showing the relative extraction efficiency of $18:2(9c,11t)$, $18:2(9t,11t)$ from serum spiked with increasing amounts of the fatty acid mixture.

b) Graph showing the relative extraction efficiency of $18:2(9c,12c)$, $18:1$, $20:4$ from serum spiked with increasing amounts of the fatty acid mixture.
3.1.7. Development of the method for the investigation of tissue.

It was clear that the method would have to be modified for the investigation of tissue specimens. The preparation of the tissue had to include a homogenisation step to allow the phospholipase A$_2$ access to the phospholipids in the tissue. The analytical results also had to be expressed differently: in terms of amount of fatty acid per gram wet weight.

3.1.7.1. Experimental Series 18: Preparation of tissue specimens.

The preparation of tissue specimens for analysis differs to that of serum specimens in that a homogenisation step must precede the phospholipase A$_2$ digestion to allow the enzyme to access as much of the tissue as possible. It was, therefore, decided that the initial developments of the method for tissue analysis should be carried out on sheep liver as this was readily available from the local butcher. The first task was to determine how much tissue was required for the assay.

A portion of fresh sheep liver (0.5g wet weight) was homogenised in 5ml 20mmol/l phosphate buffered saline (pH 7.4) by a Silverson homogeniser (Silverson Machines Ltd. Waterside, Chesham, Buckinghamshire, UK) to give a 10% (w/v) homogenate. An aliquot (0.5ml) of the homogenate was mixed with 0.5ml of a solution containing 100mmol/l Tris-HCl buffer (pH 8.9), 1mol/l methanol, and 5000IU/l phospholipase A$_2$. After incubation at 25°C for 15 minutes the protein was precipitated with 2ml of a solution of containing 83mmol/l acetic acid in methanol. The mixture was thoroughly mixed and centrifuged at 1000g for 10 minutes. The lipids were extracted using a ‘Bond Elut’ solid phase extraction system described in section 3.1.1.1. The eluate was loaded onto the HPLC described in section 3.1.1.1. with the modifications detailed in section 3.1.4.1.
Figure 31 shows the chromatograms resulting from the analysis of sheep liver. It can be seen from the height of the peaks that the concentration of the fatty acids in the tissue homogenate are comparable to those in the serum specimen previously analysed (shown in Figure 5). It was concluded that a tissue specimens of 0.5g wet weight was sufficient for the analysis.
Figure 31. Chromatograms of a lipid extract of sheep liver prepared from a homogenate of 0.5g sheep liver.

Detector 200nm; Attenuation 0.2aufs
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Sample loop 20μl

Detector 234nm; Attenuation 0.01aufs
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Sample loop 20μl
3.1.7.2. The analysis of small tissue specimens.

3.1.7.2.1 Experimental Series 19: The use of microhomogenisers.

Although the procedure detailed in section 3.1.7.1. could analyse tissue specimens as small as 0.5g, it could not be applied to the study of tissue biopsies, which are typically smaller than 100mg. It was, however, considered possible to increase the sensitivity of the method by an order of magnitude because although 0.5g tissue was homogenised, only 10% of the homogenate was actually subjected to enzyme hydrolysis, lipid extraction and HPLC analysis. Therefore, if the entire specimen was processed tissue specimens as small as 50mg might be analysed. However, the Silverson homogeniser required a minimum of 5ml in which to homogenise the tissue. The processing of this volume was considered too cumbersome, impractical, and expensive because it required ten times more reagents. It was, therefore, decided to investigate whether miniature hand homogenisers (nominal volume 0.1ml) could be used.

A fragment (50mg) of the same sheep liver specimen used in section 3.1.6.1. was finely chopped on a glass slide, transferred to a microhomogeniser and macerated in 0.1ml 20mmol/l PBS (pH 7.4) by 30 up and down strokes of the plunger. The homogenate was washed into a glass tube with 0.9ml of the same buffer and sonicated for 30 seconds at 23kHz with an amplitude of 14μm. To the homogenate was added 0.1ml 100mmol/l Tris-HCl buffer containing 1mol/l methanol and 50000 IU/l phospholipase A₂ (from naja naja venom). After incubation at 25°C for 15 minutes 0.1ml of the solution containing 5-10μmol/l internal standard, 18:2(9t,11t), in methanol was added. Protein was precipitated by mixing with 2ml of 83mmol/l acetic acid in methanol and centrifuging at 1000g for 10 minutes. The lipids were extracted using the ‘Bond Elut’ system. The supernatant was applied to a 3ml
'Bond Elut' cartridge that had been prewashed with 2ml propan-2-ol/acetoniitrile (2:1, v/v); and conditioned with 5ml of a solution containing methanol: water: acetic acid (67:33:0.03, v/v/v). The cartridge was washed with a further 5ml of the latter solvent. The lipids were eluted in 1ml propan-2-ol/acetonitrile (2:1,v/v) and injected onto the HPLC. The chromatogram and results obtained were compared with those from the 0.5g portion analysed in section 3.1.6.1.

It can be seen that the chromatograms from the analysis, of sheep liver using microhomogenisers shown in Figure 32, are comparable to those obtained by the method detailed in section 3.1.6.1. The calculated concentrations of the lipids in specimens analysed by the two methods were very close (coefficient of variation <8%; n=10). It was, therefore, decided that this micromethod could be used for tissue specimens as small as 50mg.
Figure 32. Chromatograms of a lipid extract prepared from 50mg sheep liver.

Detector 200nm; Attenuation 0.2aufs
Mobile phase:
89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Sample loop 20µl

Detector 234nm; Attenuation 0.01aufs
Mobile phase:
89.9% Acetonitrile/10% Water/0.1%Acetic acid
Flow rate 1.5ml/min
Sample loop 20µl
3.1.7.2.2. Experimental Series 20: The analysis of cell suspensions.

On ethical grounds it is only possible to remove a biopsy from a region in which an abnormality is suspected. It was, therefore, necessary to determine cell suspensions could be analysed for phospholipid esterified diene conjugated and non-diene conjugated fatty acids. It was decided that initial experiments to analyse cell suspensions should use buccal cells.

Collections of buccal cells from ten individuals were pooled and centrifuged at 1500g for 10 minutes at room temperature. The resulting supernatant was discarded. The cells were resuspended in 1ml 154mmol/l NaCl containing 40mmol/l CaCl$_2$ and the cell suspension was sonicated for 15 seconds at 23kHz and an amplitude of 14µm. One millilitre of a solution of 100mmol/l Tris-HCl buffer (pH 8.9), containing 1mol/l methanol and 5000IU/l phospholipase A$_2$ (from *naja naja* venom) was added. After incubation at 25°C for 15 minutes 0.1ml of a solution of 5-10µmol/l 18:2(9t,11t) was added as internal standard. The protein was precipitated with 2ml of a solution of 83mmol/l acetic acid in methanol and centrifuging at 1500g for 10 minutes. The lipids were extracted using the ‘Bond Elut’ system by applying the resulting supernatant to a 3ml ‘Bond Elut’ cartridge which had been prewashed with 2ml propan-2-ol/acetonitrile (2:1,v/v), and conditioned with 5ml of a solution containing methanol:water:acetic acid (67:33:0.03,v/v/v). The cartridge was washed with 5ml of the methanol:water:acetic acid. The lipids were eluted in 1ml propan-2-ol/acetonitrile (2:1,v/v) and injected onto the HPLC system described in section 3.1.1.1. and modified as detailed in section 3.1.4.1.

The chromatograms from the analysis of the lipid extract prepared from ten pooled cell collections is given in Figure 33. It can be seen that the fatty acid peaks are comparable in height to those for the serum specimen in Figure 5.
It was, therefore, possible to study buccal cells if ten collection were pooled. However, this method was not sensitive enough for the study of single cell collections.
Figure 33. Chromatograms of a lipid extract prepared from ten pooled buccal cell specimens.

Detector 200nm; Attenuation 0.2 aufs
Mobile phase: 89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Sample loop 20μl

Detector 234nm; Attenuation 0.02 aufs
Mobile phase: 89.9% Acetonitrile/10% Water/0.1% Acetic acid
Flow rate 1.5ml/min
Sample loop 20μl
3.1.7.2.3. Experimental Series 21: Concentrating the lipid extract.

The method described in section 3.1.6.2.2. could be used to study pooled cells but it was unsuitable for the study of individual cell collections. Although, it was necessary to elute the lipids from the ‘Bond Elut’ cartridge with 1ml of solvent only 20µl of this lipid extract was actually applied to the HPLC column. It was considered that biopsies smaller than 50mg and individual cell collections might be possible if the lipid extract was concentrated.

It was, therefore, decided to prepare 5mg specimens of the sheep liver used in section 3.1.7.1. by the method described in section 3.1.7.2.1. and an individual cell collection as described in section 3.1.7.2.2. The lipid extracts were dried under oxygen free nitrogen, and the lipids resuspended in 100µl propan-2-ol/acetonitrile (2:1,v/v). The concentrated extract was then injected onto the HPLC.

Chromatograms of the analysis of one of the 5mg sheep liver specimens and an individual buccal cell collection are shown in Figure 34. In each case the heights of the fatty acid peaks in the concentrated lipid extracts are comparable to those obtained from unconcentrated lipid extracts prepared from 50mg sheep liver and ten buccal cell collections.

It was, therefore, decided that the technique of drying down the lipid extract and resuspending the lipids in a smaller volume could be used to investigate biopsies smaller than 50mg and individual cell collections.
Figure 34. Chromatograms of a lipid extract prepared from 5mg of sheep liver and a single specimen of buccal cells.

a) Chromatograms of the concentrated lipid extract prepared from 5mg sheep liver.

b) Chromatograms of the concentrated lipid extract prepared from a single specimen of buccal cells.
3.1.7.3. Calculation of the results from analysis of tissue and cells.

3.1.7.3.1. Calculation of the fatty acid concentrations.

The calculation of the fatty acid concentrations in tissue and cells followed the same format and was subject to the same limitations as those for serum (given in section 3.1.3.4.). However, the fatty acid concentrations in the lipid extract had to be related to the original 'size' of the sample (e.g., mass, protein content, number of cells). Therefore, the calculation for the concentration of a fatty acid in a tissue specimen or cell suspension is:

\[
\text{[Fatty acid]} = \frac{\text{PH FA(sample)} \times \text{PH IS (U)} \times \text{Volume IS} \times \text{Conc.Std}}{\text{PH FA (Std)} \times \text{PH IS (sample)} \times \text{Sample mass}}
\]

(nmol/g)

where:

- **PH FA (sample)** is the peak height of the fatty acid in the sample.
- **PH FA (Std)** is the peak height of the fatty acid in the external standard.
- **PH IS (U)** is the peak height of the internal standard injected without extraction.
- **PH IS (sample)** is the peak height of the internal standard in the sample.
- **Volume IS** is the volume (ml) of the internal standard solution added to the sample.
- **Conc. Std** is the concentration (μmol/l) of the fatty acid in the external standard.
- **Sample mass** is the mass (g) of the tissue sample.

Clearly, **Sample mass** could be replaced by the mass of protein or number of cells (10^6). In these cases, the fatty acid concentrations would be expressed in nmol/g of protein and nmol/10^6 cells respectively.
3.1.7.3.2. Calculation of the molar ratio of 18:2(9c,11t) to 18:2(9c,12c).

The molar ratio of 18:2(9c,11t) to a 18:2(9,12) was calculated as described in section 3.

When the sample 'size' was unknown the molar ratio could be calculated:

\[
\% MR = \frac{PH_X (sample) \times PH_Y (Std) \times Conc. X}{PH_Y (sample) \times PH_X (Std) \times Conc. Y} \times 100
\]

where:

- \(PH_X (sample)\) is the peak height of the 18:2(9c,11t) in the sample.
- \(PH_X (Std)\) is the peak height of the 18:2(9c,11t) in the external standard.
- \(Conc. X\) is the concentration of 18:2(9c,11t) in the external standard.
- \(PH_Y (sample)\) is the peak height of 18:2(9c,12c) in the sample.
- \(PH_Y (Std)\) is the peak height of 18:2(9c,12c) in the external standard.
- \(Conc. Y\) is the concentration of the 18:2(9c,12c) in the external standard.
3.1.8. Computerised data handling.

3.1.8.1. The need for computerised calculations.

The manual calculation of results was a lengthy process as a separate calculation was required for the concentration of each of the four fatty acids, the extraction efficiency (recovery) and the percentage molar ratio (%MR) of 18:2(9c,11t) to 18:2(9c,12c). The 300 separate calculations required for an overnight run of 50 samples were also prone to error as each involved seven variables. Although double checking served to minimise the errors this was even more time consuming.

It was felt that if the calculations were performed by computer not only would the results be more reliable but this would also 'free' the analyst to prepare further samples or interpret results. However, none of the software available for use on the CI3000 integrator was able to perform all the calculations required or cope with the fluctuations of the fatty acid retentions times with temperature.

It was, therefore, decided to devise a dedicated program for the CI3000 to recognise all peaks of interest, perform all necessary calculations and print out the results after the analysis of each sample. The program had to be 'user friendly' to enable its use by analysts not familiar with the assay, and be versatile enough to allow the results to be calculated in different manners according to the nature of the specimens.

3.1.8.2. Characteristics of the calculation program.

The programming language was a version of BASIC. Figure 35 shows the principal steps of the calculation program in the form of a flow chart. A full listing of the program may be found in Appendix 1. Subroutines were used...
to prompt injections, extract peak height data from data files, perform calculations and print results.

It was apparent from previous experiments that the speed of the chromatography was temperature dependent: fatty acid retention times shortened with increasing ambient temperature. To overcome this problem a mechanism was included in the program for updating the time windows when the actual retention times differed from the preset retention times. The program also allowed retention times to be entered manually if rapid changes in temperature ever caused fatty acids to elute outside the preset time windows.

Prior to the run the program prompted input of all the constants required for the calculations such as sample preparation details and the concentration of fatty acids in the external standard.

Calibration, which required the injection of the external standard followed by the internal standard, was prompted once the constants had been entered and after a preset number of sample injections. It was also possible to request recalibration to be brought forward if desired. When chromatography of the external standard was complete the program prompted the input of the retention times of fatty acids of interest and a percentage tolerance value to set the time window for each peak of interest. Peaks corresponding with each fatty acid of interest were then recognised and the response factors calculated. When chromatography of the internal standard was complete the response factors were updated.

Following calibration the injection of samples was prompted. If the injections were made manually the sample number or name could be
entered so each chromatogram could be labelled. On completion of the chromatography the fatty acid concentrations in the samples, molar ratio of 18:2(9c,11t) to 18:2(9c,12c) and the extraction efficiency were printed out and the injection of the next sample prompted. When injections were being made manually certain special functions were available including the recalculation of results for a previous sample.
Figure 35. Flow chart showing the principal steps of the computer program for calculation of the results of HPLC lipid analysis.
3.2. THE APPLICATION OF THE METHOD FOR THE MEASUREMENT OF DIENE CONJUGATED AND NON-DIENE CONJUGATED FATTY ACIDS TO HUMAN TISSUE AND CELLS.

3.2.1. The study of post mortem tissue.

It was decided that the initial application of the method modified for the assay of tissues and cells should be with post mortem (PM) tissue specimens as these were available in sufficient amount for replicate assays.

Specimens (approx. 0.5g) of heart, lung, liver, kidney, and spleen were obtained at post mortem examination of five subjects who had died in hospital. Specimens of cerebellum, liver metastases, cancerous adrenal gland, rectal tumour and skin tumour were also available from some subjects.

Approximately 0.5g of each specimen was weighed and assayed for linoleic acid and 18:2(9c,11t) as described in section 3.1.7.1. The remaining homogenate was used to assay free and triglyceride esterified fatty acids. Free fatty acids were assayed as for the phospholipid esterified fraction except that phospholipase A₂ was omitted from the 100mmol/l Tris-HCl buffer containing 1mol/l methanol. With the exception of the enzyme hydrolysis the assay of triglyceride fraction was similar to the assay of the phospholipid fraction. To 0.5ml homogenate was added 0.5ml 20mmol/l PBS (pH 7.4) containing 1000 IU/l lipase (triacylglycerol acylhydrolase EC 3.1.1.3. type VII lipase from candida cylindracea ). Following incubation at 37°C for one hour 2ml of a solution containing 83mmol/l acetic acid and internal standard (5-10μmol/l 18:2(9t,11t)) was added. The mixture was vortex mixed and centrifuged at 1500g for 10 minutes. The resulting supernatant was applied
to a 3ml ‘Bond Elut’ cartridge which had been prewashed with 2ml propan-
2-ol/acetonitrile (2:1, v/v) and conditioned with 5ml methanol/water/acetic
acid (67:33:0.03, v/v/v). The cartridge was washed with a further 5ml of the
methanol/water/acetic acid. The lipids were eluted in 1ml propan-2-ol/acetonitrile (2:1, v/v) and injected onto the HPLC.

The concentrations of the non-esterified 18:2(9c,11t) and 18:2(9c,12c) in the
tissues were calculated. Corrected concentrations of 18:2(9c,11t) and
18:2(9c,12c) esterified as triglycerides or phospholipids were obtained by
deducting from each fraction the concentrations of non-esterified fatty acids.
It was not possible to assay the cholesterol fraction.

The findings are summarised below; the detailed results are given in
Appendix 2.

3.2.1.1. Analysis of post mortem tissue: Case reports.

Subject 1

Male; Aged 78; Examined 17 hours post mortem.

With the exception of the brain tissue a significant proportion of the
18:2(9c,11t) and 18:2(9c,12c) in each tissue examined were present as free fatty
acids indicating that a considerable degree of hydrolysis of lipid esters had
occurred particularly in the case of the spleen. In general more 18:2(9c,11t)
and 18:2(9c,12c) were present in the triglyceride fraction than the
phospholipid fraction: the main exception being the brain where
triglycerides are not stored. The %MR in the phospholipids and triglycerides
ranged from 0.37 to 3.05. The highest %MR values were found in the liver
and adrenals; both of these were sites of neoplasia and were contributory
causes of death.
Subject 2
Male; Aged 55; Examined 10 hours post mortem.
With the exception of the brain significant lipid hydrolysis had occurred in the tissue specimens prior to analysis though not as much as in the specimens from the first subject possibly because the second subject was examined sooner. Where markedly different the %MR and esterified 18:2(9c,11t) and 18:2(9c,12c) concentrations were higher in the triglycerides than the phospholipids. The %MR in the triglycerides and phospholipids ranged from 1.19 to 2.68.

Subject 3
Male; Aged 81; Examined 82 hours post mortem.
With the exception of the brain a significant amount of lipid hydrolysis had occurred prior to specimen analysis. The concentrations of 18:2(9c,11t) and 18:2(9c,12c) and %MR in the brain, lung, and spleen were higher in the phospholipids (%MR range 1.08 to 6.46) than the triglycerides. This was in contrast to the findings from heart and liver, where the triglyceride %MR's were 32.18 and 2.84 respectively. The PM report stated that considerable coronary atherosclerosis was evident. Heart and liver triglyceride concentrations of linoleic acid and 18:2(9c,11t) were much higher in this subject than any of the others studied. However, the increase in the lipid concentrations were not accompanied by high %MR's in either the triglyceride or phospholipid fractions.

Subject 4
Elderly male; post mortem report not available.
Again prior to analysis significant lipid hydrolysis had occurred in all tissues examined except in the brain. No triglyceride 18:2(9c,11t) or 18:2(9,12)
detected in the brain specimen. The concentrations of 18:2(9c,11t) and 18:2(9c,12c) released from the homogenate of the brain specimen were negligible. Concentrations of 18:2(9c,11t) and 18:2(9c,12c), and the %MR in the lung were greater in the triglycerides than the phospholipids. In the heart, liver, kidney, and spleen the concentrations of 18:2(9c,11t) and 18:2(9c,12c) were higher in the phospholipids than the triglycerides although the triglyceride %MR always exceeded the phospholipid %MR.

Subject 5
Elderly female; Examined 18 hours post mortem.

As noted in other subjects significant lipid hydrolysis had occurred in the tissues prior to specimens analysis. The triglycerides in the heart and kidney contained the highest concentrations of 18:2(9c,11t) and 18:2(9c,12c) and extremely high %MR (8.83 and 14.69 respectively). The spleen and lung phospholipids contained more 18:2(9c,11t) and 18:2(9c,12c) and higher had %MR's (5.92 and 4.89 respectively) than the triglyceride fractions from the same tissues.

Three specimens from the liver were obtained: normal, metastatic tissue, and a portion taken from the interface of the normal and metastatic tissue. Results from these specimens (shown in Table 5) show a clear trend of increasing %MR from normal to tumour tissue.

Compared with normal liver the concentrations of 18:2(9c,11t) and 18:2(9c,12c) in the triglyceride fraction of the tumour were considerably reduced: in fact no 18:2(9c,11t) and little 18:2(9c,12c) was measurable in the metastatic tumour. However, the concentrations of phospholipid esterified 18:2(9c,11t) and 18:2(9c,12c) were markedly increased in the tumour compared with the normal tissue.
The rectal tumour and the skin tumour from the nose both had higher %MR in the triglyceride fraction of both the rectal and skin tumours (4.61 and 6.75 respectively) were higher than the corresponding phospholipid fractions. Indeed phospholipid 18:2(9c,11t) was undetectable in the rectal tumour.
Table 5. Results from the HPLC analyses of fatty acids esterified phospholipids and triglycerides in normal and neoplastic hepatic tissue from a subject with liver metastases.

a) The concentration of 18:2(9c,11t) and 18:2(9c,12c) and the molar ratio in phospholipids of normal and metastatic liver, and a portion taken from the interface of the normal and metastatic regions.

<table>
<thead>
<tr>
<th>Nature of hepatic specimen</th>
<th>18:2(9c,11t) nmol/g</th>
<th>18:2(9c,12c) nmol/g</th>
<th>% Molar</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>11.7</td>
<td>1370</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Normal/tumour interface</td>
<td>131.0</td>
<td>3143</td>
<td>4.17</td>
<td></td>
</tr>
<tr>
<td>Tumour</td>
<td>26.2</td>
<td>517</td>
<td>5.07</td>
<td></td>
</tr>
</tbody>
</table>

b) The concentration of 18:2(9c,11t) and 18:2(9c,12c) and the molar ratio in triglycerides of normal and metastatic liver and a portion taken from the interface of the normal and metastatic regions.

<table>
<thead>
<tr>
<th>Nature of hepatic specimen</th>
<th>18:2(9c,11t) nmol/g</th>
<th>18:2(9c,12c) nmol/g</th>
<th>% Molar</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>30.0</td>
<td>1127</td>
<td>2.66</td>
<td></td>
</tr>
<tr>
<td>Normal/tumour interface</td>
<td>64.7</td>
<td>1849</td>
<td>3.50</td>
<td></td>
</tr>
<tr>
<td>Tumour</td>
<td>Not detected</td>
<td>30</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
3.2.1.2. Summary of results from the analysis of post mortem specimens.

The analysis of post mortem tissue showed that the fatty acids in tissues could be measured reliably. The triplicate and in the case of the non-esterified fatty acids quadruplicate samples from each homogenate gave results within 4%. The diene conjugated lipid present in serum, identified as 18:2(9c,11t), co-chromatographed with the predominant diene conjugated lipid in all tissue specimens.

The results of the analyses varied considerably form one subject to another. There was a delay between death and post mortem examination of up to 82 hours. For most of this period the bodies were kept at 4°C which explains the high degree of hydrolysis of the esterified lipids. The degree of hydrolysis, however, was not commensurate with the period of storage prior to examination. No non-esterified fatty acids were detected in brain tissue. As expected most fatty acids in the brain were esterified as phospholipids.

Tissues affected by tumours had relatively high %MR's. The three liver specimens from the fifth subject suggested that compared to normal hepatic tissue tumour tissue may contain less triglyceride esterified 18:2(9c,11t) and 18:2(9c,12c) but more as phospholipids with an increased %MR.

The heart and liver specimens from the subject with coronary atherosclerosis had high %MR's, possibly arising from the fatty deposits.

Although these findings suggested links between 18:2(9c,11t) and disease the considerable degree of hydrolysis which had occurred prior to post mortem examination made interpretation of the post mortem specimens very
difficult and almost impossible. For these reasons it was decided that fresh tissue biopsies and cell suspensions should be investigated.

3.2.2. The analysis of colorectal tissue.

The study of PM tissue suggested that the concentration of 18:2(9c,11t) may be raised in tumour tissue. However, clear interpretation of these findings was made difficult by the considerable degree of hydrolysis which had occurred prior to the analysis of the specimens. It was, therefore, decided that fresh tissue should be examined in order to overcome this problem. The technique was, therefore, applied to the study of normal and cancerous colorectal biopsies.

3.2.2.1. Experimental procedure.

In collaboration with the Gastroenterology Unit at St. Marks Hospital, Islington, London specimens from 102 patients undergoing surgery were studied. The full protocol for this study is given in section 2.3.2..

The phospholipid esterified fatty acid analysis was carried out using a modified version of the micromethod for tissue detailed in section 3.1.7.2.3.. Following homogenisation in 200μl 154mmol/l sodium chloride in the microhomogeniser the volume of the homogenate was made up to 1ml with 100μl 20mmol/l calcium chloride and 700μl 154mmol/l sodium chloride. The mixture was sonicated for 10 seconds using an Ultrasonics disintegrator (Shipley, Yorkshire, UK) fitted with a 3mm probe, and incubated for 15 minutes at 25°C with 1ml of a solution containing 100mmol/l Tris-HCl, 1mol/l methanol, and 5000 IU/l phospholipase A₂ from naja naja venom. The protein was precipitated by addition of 8ml
83mmol/l acetic acid in methanol. The internal standard was added as 100μl of 83mmol/l acetic acid in methanol containing 5-10μmol/l 18:2(9t,11t). The mixture was vortex mixed and centrifuged at 1000g for 10 minutes. The lipids were extracted using the 'Bond Elut' extraction system. The supernatant was applied to a 1ml 'Bond Elut' cartridge, which had been washed with 2ml propan-2-ol/acetonitrile (2:1,v/v). and conditioned with 2.5ml of a solution containing methanol/water/acetic acid (67:33:0.03, v/v/v). The column was washed with a further 2.5ml of methanol/water/acetic acid (67:33:0.03, v/v/v) and the lipids eluted in 0.5ml propan-2-ol/acetonitrile (2:1,v/v). The eluate was dried under a stream of oxygen free nitrogen, the lipids resuspended in 50μl propan-2-ol/acetonitrile (2:1,v/v) and the extract injected onto the HPLC system described in section 3.1.1.1. with the modifications detailed in section 3.1.6.1.. The concentrations of the 18:2(9c,11t) and 18:2(9,12) in terms of nmol/g wet weight of tissue, and the %MR were calculated as shown in section 3.1.7.3.1..

3.2.2.2. Study results.

3.2.2.2.1. Results of the analysis of surgical specimens of colorectal mucosa from patients with carcinoma of colon or rectum.

Table 6 shows the mean concentrations of 18:2(9c,11t) and 18:2(9c,12c) and the mean %MR for specimens from patients with carcinoma of the colon or rectum.

It can be seen that in the group of stored specimens the mean %MR is significantly lower (p=0.02) in the cancerous tissue (3.58) than the histologically normal tissue from the same patients (4.86). In contrast, when fresh specimens were analysed the %MR in the cancerous tissue was not significantly different (p=0.2) from histologically normal tissue from the
same patients. It was, therefore, felt that the significant difference between cancerous and histologically normal tissue observed in the stored specimens might simply have been a storage artifact.

3.2.2.2. The effect of storage on the 18:2(9c,11t) and 18:2(9c,12c) content of surgical specimens of colorectal mucosa.

The discrepancy between the findings of the studies of stored and fresh specimens prompted investigation of the effect of storage on the assay. It was, therefore, decided to study further specimens from patients with carcinoma of the colon or rectum.

The remaining portion of each of the eleven specimens, which had been assayed fresh (ie. within 24 hours of collection), were re-assayed after six months storage in liquid nitrogen by the method described in section 3.2.2.1. The results of this investigation are shown in the third row of Table 6.

In the set of specimens analysed within 24 hours there was no significant difference (p=0.2) in %MR between normal and cancerous tissue. However, after six months storage in liquid nitrogen the histologically normal tissue had a significantly higher (p=0.04) %MR than cancerous tissue. The discrepancy between the %MR in fresh and stored tissue was principally due to a more pronounced reduction in the concentrations of 18:2(9c,12c) in normal compared to cancerous tissue.

It was, therefore, apparent that storage even in liquid nitrogen affected normal and cancerous tissue to different extents.
Table 6 Concentrations of 18:2(9c,11t) and 18:2(9c,12c) and percentage molar ratio in fresh and frozen specimens of normal and cancerous colorectal mucosa from patients with colon or rectal cancer.

<table>
<thead>
<tr>
<th>Storage data</th>
<th>Clinical condition</th>
<th>18:2(9c,11t) nmol/g</th>
<th>18:2(9c,12c) nmol/g</th>
<th>% Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>First batch of biopsies. Normal analysed after up to - 30 months. (n=91)</td>
<td>Carcinoma</td>
<td>57.5 (27.6)</td>
<td>1605 (759)</td>
<td>3.58</td>
</tr>
<tr>
<td>Second batch of Normal biopsies analysed within 24 hours of Carcinoma collection. (n=11)</td>
<td>Carcinoma</td>
<td>56.7 (27.5)</td>
<td>1574 (333)</td>
<td>3.60</td>
</tr>
<tr>
<td>Second batch of Normal biopsies analysed after six months storage in Carcinoma liquid nitrogen. (n=11)</td>
<td>Carcinoma</td>
<td>60.3 (16.5)</td>
<td>1702 (683)</td>
<td>3.34</td>
</tr>
</tbody>
</table>

Standard deviation values are given in parentheses.
3.2.2.2.3. Summary of the study of colorectal mucosa.

The study showed that the specimens of colorectal mucosa could be analysed using the technique described in section 3.1.7. Tissue phospholipid concentrations of 18:2(9c,11t) and 18:2(9c,12c) were of the same order as detected in PM tissue, described in section 3.2.1.

In contrast to the studies of PM tissue the analysis of specimens of colorectal mucosa from patients with carcinoma of the colon or rectum suggested that cancerous tissue had a lower %MR than histologically normal tissue from the same patients.

However, there was no difference in %MR between cancer and histologically normal tissue in similar specimens when the samples were analysed within 24 hours of excision; but when fragments of these same specimens were analysed after six months of storage the % MR in the normal tissue had increased.

Since there was no difference in %MR between normal and cancer tissue (except on storage) no further studies were undertaken.
3.2.3. The study of cancerous, precancerous and normal biopsies from the cervix uteri.

Cancer of the cervix, in common with colorectal cancer progresses through clearly defined stages. Though investigations of PM tissue suggested that the %MR might be raised in neoplastic tissue, studies with surgical specimens of colorectal mucosa did not reveal a difference in %MR between normal and cancer tissue. It was, therefore, decided to investigate cervical cancer.

3.2.3.1. Experimental procedure.

Cervical biopsy material was studied from a total of 65 patients of the Royal Northern Hospital, Islington, London. The full protocol for this study is given in section 2.3.3. The specimens were obtained using the procedure described in section 2.2.4.3. homogenised as described in 3.1.7.2.2. and analysed in the manner used for the study of colorectal mucosa detailed in section 3.2.2.1..

3.2.3.2. Results of the study of cervical biopsies.

The results of the analysis of cervical biopsies are summarised in Table 7. Compared with the histologically normal group all grades of precancer had a significantly higher 18:2(9c,11t) concentration (p<0.04) and %MR (p<0.01). As the cancer group comprised only four samples the Mann-Whitney non-parametric statistical test was used to compare it with the other groups. The concentration of 18:2(9c,11t) and the %MR in the cancer specimens were both significantly higher than the normal and precancer groups. There was no significant difference in the concentrations of linoleic acid or arachidonic acid between the groups.
The distribution of the %MR values within the normal, CIN (grades CIN 1, CIN 2, and CIN 3) and cancer groups is shown in Figure 36. Figure 37 shows the distribution of %MR values in cervical biopsies taken from CIN lesions and histologically normal cervical tissue from the same patients. There was a significant correlation ($r=0.636; 0.05<p<0.01$) in the %MR between the pairs of biopsies.

It was concluded from this study that the higher concentration of 18:2(9c,11t) in precancerous and cancerous cervical tissue compared to normal cervix might reflect changes in free radical activity. Moreover, it was felt that by measuring 18:2(9c,11t) and 18:2(9c,12c) neoplastic changes in the cervix might be detected at an early stage. Further study of cervical cancer was clearly indicated.
Table 7. Results from the analysis of normal, precancerous and cancerous biopsies of the cervix uteri.

a) The concentration of 18:2(9c,11t) in normal (n=18), CIN 1 (n=10), CIN 2 (n=10), CIN 3 (n=19) and cancerous (n=4) cervical biopsies.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>n</th>
<th>Mean [18:2(9c,11t)] nmol/g</th>
<th>SD</th>
<th>t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>41.1</td>
<td>19.0</td>
<td>-</td>
</tr>
<tr>
<td>CIN 1</td>
<td>10</td>
<td>64.2</td>
<td>27.6</td>
<td>p=0.035</td>
</tr>
<tr>
<td>CIN 2</td>
<td>10</td>
<td>59.0</td>
<td>20.0</td>
<td>p=0.034</td>
</tr>
<tr>
<td>CIN 3</td>
<td>19</td>
<td>71.7</td>
<td>29.7</td>
<td>p=0.0007</td>
</tr>
<tr>
<td>All CIN grades</td>
<td>39</td>
<td>66.5</td>
<td>26.4</td>
<td>p=0.002</td>
</tr>
<tr>
<td>Cancer</td>
<td>4</td>
<td>81.0</td>
<td></td>
<td>p=0.03*</td>
</tr>
</tbody>
</table>

* by Mann-Whitney non-parametric test.

b) The concentration of 18:2(9c,12c) in normal (n=18), CIN 1 (n=10), CIN 2 (n=10), CIN 3 (n=19) and cancerous (n=4) cervical biopsies.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>n</th>
<th>Mean [18:2(9c,12c)] nmol/g</th>
<th>SD</th>
<th>t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>2106</td>
<td>1001</td>
<td>-</td>
</tr>
<tr>
<td>CIN 1</td>
<td>10</td>
<td>2659</td>
<td>1319</td>
<td>p=0.27</td>
</tr>
<tr>
<td>CIN 2</td>
<td>10</td>
<td>2118</td>
<td>775</td>
<td>p=0.97</td>
</tr>
<tr>
<td>CIN 3</td>
<td>19</td>
<td>2414</td>
<td>1073</td>
<td>p=0.25</td>
</tr>
<tr>
<td>All CIN grades</td>
<td>39</td>
<td>2401</td>
<td>1067</td>
<td>p=0.37</td>
</tr>
<tr>
<td>Cancer</td>
<td>4</td>
<td>1644</td>
<td>811</td>
<td>p=0.42*</td>
</tr>
</tbody>
</table>

* by Mann-Whitney non-parametric test.
Table 7 (continued). Results from the analysis of normal, precancerous and cancerous biopsies of the cervix uteri.

c) The molar ratio in normal (n=19), CIN 1 (n=10), CIN 2 (n=10), CIN 3 (n=19) and cancerous (n=4) cervical biopsies.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>n</th>
<th>Mean %MR</th>
<th>SD</th>
<th>t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>19</td>
<td>1.99</td>
<td>0.52</td>
<td>-</td>
</tr>
<tr>
<td>CIN 1</td>
<td>10</td>
<td>2.55</td>
<td>0.42</td>
<td>p=0.0087</td>
</tr>
<tr>
<td>CIN 2</td>
<td>10</td>
<td>2.85</td>
<td>0.59</td>
<td>p=0.0013</td>
</tr>
<tr>
<td>CIN 3</td>
<td>19</td>
<td>3.16</td>
<td>1.05</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>All CIN grades</td>
<td>39</td>
<td>2.93</td>
<td>0.87</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Cancer</td>
<td>4</td>
<td>5.57</td>
<td>-</td>
<td>p=0.0024*</td>
</tr>
</tbody>
</table>

* by Mann-Whitney non-parametric test.
Figure 36. The distribution of % molar ratios of cervical biopsies from normal women (n=19) and women with cervical intraepithelial neoplasia (grades CIN 1 (n=10), CIN 2 (n=10), and CIN 3 (n=19) and cancer (n=4).
Figure 37. Comparison of % molar ratio values of eleven sets cervical biopsies taken from the centre of a neoplastic region and from normal tissue outside the margin of neoplastic change in the same organ.
3.2.4. The measurement of 18:2(9c,11t) and 18:2(9c,12c) in exfoliated cervical cells.

3.2.4.1. Initial studies with cervical epithelial cells from women with and without cervical abnormalities.

The significant differences in the concentration of 18:2(9c,11t) and the %MR found in cervical biopsies from women with and without cervical neoplasia raised the possibility that this assay might form the basis of a discriminatory test for precancerous changes in the cervix. It was, therefore, decided to initiate a study comparing the sensitivity of the %MR measurement with the Papanicolaou smear test.

3.2.4.1.1. Experimental procedure.

In collaboration with the Department of Obstetrics and Gynaecology at the Royal Northern Hospital a group of 175 women, attending the Colposcopy Clinic for evaluation of an abnormal Papanicolaou smear, were studied. The control group comprised 40 women who were attending a Family Planning Clinic or an Out Patient Clinic for reasons unrelated to cervical disease. The detailed protocol of the study is given in section 2.3.4..

In order to be compared with the Papanicolaou smear test exfoliated cells, scraped from the cervix as described in section 2.2.4.2., were assayed for 18:2(9c,11t) and 18:2(9c,12c) The procedure used to prepare and analyse the specimens was based on that described for buccal cells in sections 3.1.7.2.2. and 3.1.7.2.3..

The cell suspensions were centrifuged at 1500g for 10 min at room temperature. The supernatant was discarded and the cells resuspended in
1ml 154mmol/l NaCl containing 40mmol/l CaCl₂. The cell suspension was sonicated for 15 seconds at a frequency of 23kHz and an amplitude of 14µm by a Soniprep 150 (MSE, Crawley, Sussex, UK) fitted with a 3mm probe. One millilitre of 100mmol/l Tris-HCl buffer (pH8.9) containing 1mol/l methanol and 5000IU/l phospholipase A₂ was added and the mixture incubated at 25°C for 15 minutes. Following incubation 50µl of methanol containing 5-10µmol/l internal standard was added. Lipids were extracted from the mixture either by the use of solvents (chloroform/methanol) or the 'Bond Elut' solid phase extraction system.

**Solvent extraction**

Following addition of the internal standard 1.5ml chloroform/methanol (2:1,v/v) and 100µl 42mmol/l H₂SO₄ was added. The mixture was vortexed and centrifuged at 1500g for 10 minutes. The chloroform (lower) layer was removed to a clean glass tube and evaporated to dryness under a stream of oxygen free nitrogen. The lipids were resuspended in 50µl of propan-2-ol/acetonitrile (2:1,v/v) and 20µl of this was injected onto the HPLC system.

**Solid phase extraction**

Following addition of the internal standard protein was precipitated by the addition of 4ml methanol containing 83mmol/l acetic acid. The mixture was vortexed and centrifuged at 1500g for 10 minutes. The supernatant was applied to the a 'Bond Elut' cartridge (nominal volume 1ml containing 100mg C18 sorbent) which had been washed with 1ml propan-2-ol/acetonitrile (2:1,v/v), and conditioned with 2ml methanol/water/acetic acid (67:33:0.03). The cartridge was washed with a further 2ml of the latter solvent and the lipids eluted with 0.5ml propan-2-ol/acetonitrile (2:1,v/v). The eluate was collected and evaporated
to dryness under a stream of oxygen free nitrogen. The lipids were resuspended in 50μl propan-2-ol/acetonitrile (2:1,v/v) and 20μl of this was injected onto the HPLC system.

As the amount of material present in cervical smears varies considerably only the %MR could be calculated. However, the findings of the study of fresh cervical biopsies had established that although the %MR was a function of both the 18:2(9c,11t) and not 18:2(9c,12c) the difference between normal and CIN tissues was due to an increased concentration of 18:2(9c,11t) rather than a reduction in the concentration of 18:2(9c,12c).

3.2.4.1.2. Study results.

The results of the study are summarised in Table 8 and the distribution of the %MR's within each histological group is shown in Figure 38.

The mean %MR in exfoliated cells from women with CIN was significantly higher than the %MR in cells from women in the control group (p<0.0001) by the Mann-Whitney test. The %MR was also raised significantly in cells from women with HPV infection without histologically evident neoplasia compared with the control group (p<0.01). The Wilcoxon test for trend showed that the increase in %MR from HPV infection to CIN was highly significant (p<<0.001).

The %MR could not be correlated with the age of the women (Table 9) or phase of menstrual cycle at time when the sample was collected (Table 10); nor with current method of contraception (Table 11), smoking habits (Table 12), nor the presence or absence of cervical erosions (Table 13).
By plotting the true positives and false positives for the %MR values within the range of observation onto a 'receiver-operator characteristic curve' (Detsky et al., 1984), a clinically useful 'cut-off' point was established which combined optimum sensitivity and specificity. With a 'cut-off' point for the %MR set at 2.10 the sensitivity of the test for all grades of CIN was 81.1%. In the CIN 3 group alone it significantly improved at 87.5%. The cell samples from the patients with invasive carcinoma all gave %MR's higher than the mean %MR in the CIN 3 group, although cytology reported two as dyskaryotic and the other as negative.

Eight women, whose smear gave a false negative results (< 2.10), were re-examined 2-3 months later. Figure 39 shows that in seven of these women the second test gave a %MR above 2.10.

It was concluded from this study that the %MR might prove a practical and useful screening method for cervical precancer. This possibility, however, had to be tested in a multicentre trial under the normal conditions of gynaecological screening.
Table 8. The molar ratios in cervical epithelial cells according to histological grade.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>n</th>
<th>Mean %MR</th>
<th>SD</th>
<th>Mann-Whitney Test (v normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>40</td>
<td>1.80</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>HPV infection</td>
<td>13</td>
<td>2.33</td>
<td>0.68</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>CIN 1</td>
<td>40</td>
<td>2.89</td>
<td>1.31</td>
<td>p&lt;&lt;0.0001</td>
</tr>
<tr>
<td>CIN 2</td>
<td>38</td>
<td>3.06</td>
<td>1.42</td>
<td>p&lt;&lt;0.0001</td>
</tr>
<tr>
<td>CIN 3</td>
<td>81</td>
<td>3.32</td>
<td>1.26</td>
<td>p&lt;&lt;0.0001</td>
</tr>
<tr>
<td>Cancer</td>
<td>3</td>
<td>5.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9. Variation of the %MR of cervical smears with age.

<table>
<thead>
<tr>
<th>Age</th>
<th>Normal</th>
<th>HPV</th>
<th>CIN 1</th>
<th>CIN 2</th>
<th>CIN 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean %MR</td>
<td>SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;19</td>
<td>1.70 (3)</td>
<td>2.02 (3)</td>
<td>2.68 (4)</td>
<td>3.08 (2)</td>
<td>3.20 (6)</td>
</tr>
<tr>
<td>20-24</td>
<td>1.83 (5)</td>
<td>2.60 (4)</td>
<td>2.94 (12)</td>
<td>3.00 (7)</td>
<td>3.15 (4)</td>
</tr>
<tr>
<td>25-29</td>
<td>1.74 (9)</td>
<td>2.11 (3)</td>
<td>2.94 (8)</td>
<td>3.04 (8)</td>
<td>3.36 (17)</td>
</tr>
<tr>
<td>30-34</td>
<td>1.83 (10)</td>
<td>2.53 (2)</td>
<td>2.93 (5)</td>
<td>3.23 (11)</td>
<td>3.53 (20)</td>
</tr>
<tr>
<td>35-39</td>
<td>1.89 (6)</td>
<td>2.47 (1)</td>
<td>2.80 (5)</td>
<td>2.93 (6)</td>
<td>3.11 (12)</td>
</tr>
<tr>
<td>40-49</td>
<td>1.67 (3)</td>
<td>-</td>
<td>2.89 (4)</td>
<td>2.99 (3)</td>
<td>3.36 (7)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>1.81 (4)</td>
<td>-</td>
<td>2.95 (2)</td>
<td>2.80 (1)</td>
<td>3.43 (5)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the number of patients in each group.
Table 10. Variation of the %MR of cervical smears with phase of the menstrual cycle.

<table>
<thead>
<tr>
<th>Time of Menses</th>
<th>Normal HPV infection</th>
<th>CIN 1</th>
<th>CIN 2</th>
<th>CIN 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>1.79 (12)</td>
<td>2.48 (3)</td>
<td>3.07 (18)</td>
<td>3.09 (18)</td>
</tr>
<tr>
<td>Mid-cycle</td>
<td>1.82 (9)</td>
<td>2.26 (3)</td>
<td>2.68 (11)</td>
<td>3.07 (6)</td>
</tr>
<tr>
<td>Luteal</td>
<td>1.94 (9)</td>
<td>2.20 (5)</td>
<td>3.05 (10)</td>
<td>3.03 (8)</td>
</tr>
<tr>
<td>Menstruating</td>
<td>1.70 (6)</td>
<td>2.53 (2)</td>
<td>2.73 (8)</td>
<td>3.01 (4)</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>1.64 (4)</td>
<td>-</td>
<td>3.07 (3)</td>
<td>3.01 (2)</td>
</tr>
</tbody>
</table>

The figures in parentheses indicate the number of patients in each group.

Table 11. Variation of the %MR of cervical smears with method of contraception

<table>
<thead>
<tr>
<th>Method of contraception</th>
<th>Normal HPV infection</th>
<th>CIN 1</th>
<th>CIN 2</th>
<th>CIN 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. C. Pill #</td>
<td>1.85 (10)</td>
<td>2.45 (6)</td>
<td>2.88 (12)</td>
<td>3.15 (15)</td>
</tr>
<tr>
<td>IUCD*</td>
<td>1.91 (4)</td>
<td>2.14 (2)</td>
<td>2.76 (7)</td>
<td>2.93 (6)</td>
</tr>
<tr>
<td>Barrier</td>
<td>1.90 (6)</td>
<td>2.36 (2)</td>
<td>2.92 (9)</td>
<td>3.03 (7)</td>
</tr>
<tr>
<td>Sterilised</td>
<td>1.73 (20)</td>
<td>2.21 (3)</td>
<td>2.95 (12)</td>
<td>3.03 (10)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the number of patients in each group.

#O.C. Pill - Oral contraceptive pill.
*IUCD - Intrauterine contraceptive device.
Table 12. Variation of the %MR of cervical smears with smoking habits

<table>
<thead>
<tr>
<th>Cigarette smoking (no./day)</th>
<th>Normal</th>
<th>HPV</th>
<th>CIN 1</th>
<th>CIN 2</th>
<th>CIN 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.78 (20)</td>
<td>2.02 (3)</td>
<td>2.80 (16)</td>
<td>3.10 (21)</td>
<td>3.31 (28)</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.88 (8)</td>
<td>1.81 (1)</td>
<td>2.84 (3)</td>
<td>2.93 (4)</td>
<td>3.10 (6)</td>
</tr>
<tr>
<td>11-20</td>
<td>1.69 (7)</td>
<td>2.47 (5)</td>
<td>3.00 (16)</td>
<td>3.03 (10)</td>
<td>3.36 (32)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>1.96 (5)</td>
<td>2.25 (4)</td>
<td>2.80 (5)</td>
<td>3.07 (3)</td>
<td>3.34 (15)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the number of patients in each group.

Table 13. Variation of the %MR of cervical smears with presence or absence of cervical erosions.

<table>
<thead>
<tr>
<th>Cervical erosions</th>
<th>Normal</th>
<th>HPV</th>
<th>CIN 1</th>
<th>CIN 2</th>
<th>CIN 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>1.70 (16)</td>
<td>2.42 (8)</td>
<td>2.89 (32)</td>
<td>3.11 (27)</td>
<td>3.32 (49)</td>
</tr>
<tr>
<td>Absent</td>
<td>1.87 (24)</td>
<td>2.37 (5)</td>
<td>2.90 (8)</td>
<td>2.93 (11)</td>
<td>3.26 (32)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the number of patients in each group.
Figure 38. Initial studies with cervical exfoliated cells: Distribution of %MR values within histological groups: Normal (n=40), HPV infection (n=13), CIN 1 (n=40), CIN 2 (n=38), CIN 3 (n=81) and Cancer (n=3).
Figure 39 Comparison of %MR value of a second specimen of cervical exfoliated cells with the first specimen in eight false negative cases.
3.2.4.2. Multicentre studies of cervical epithelial cells from women with and without cervical abnormalities.

3.2.4.2.1. Experimental procedure.
A multicentre trial was set up in which exfoliated cervical smears were collected from a total of 1103 women at five centres as described in section 2.3.5..

The normal population was drawn from women attending ‘Well Woman’ clinics at the Elizabeth Garrett Anderson Hospital, and General Practices associated with the Royal Free and Middlesex Hospitals in London; and the Queen Elizabeth Hospital in Birmingham. The samples were collected as described in section 2.2.4.2. and analysed by the same procedure used for the previous studies with cervical cells described in section 3.2.4.1.1..

3.2.4.2.2. Study results.
The biochemical and cytological and colposcopy results were compared with the histology. However, the study was insufficiently controlled and of the 1103 women studied, cytological assessment was not possible on 276 due to incorrect smear collection. The %MR could not be obtained on 398 women: most of these were insufficient but some were unsuitable for analysis having arrived in the laboratory up to 18 days after collection. Others were unlabelled or lost during transport to the laboratory. Documentation was incomplete on 403 women. These difficulties left only 354 on whom results were available from colposcopy, cytology, %MR measurement, and where appropriate histology.
3.2.4.2.2.1. Comparison of the cytology and histology.

The efficacy of the Papanicolaou smear in detecting cervical abnormalities is shown in Table 14. Cytology reported as normal six of the nine cases of HPV infection without neoplasia. Four cases of CIN 1 and one of CIN 2 were similarly reported as normal. However, cytological examination detected dyskaryosis in all women with CIN 3 indicating that although the technique lacked sensitivity for minor abnormalities, it had high specificity.

3.2.4.2.2.2. Comparison of the %MR and cytology.

Table 15 shows the %MR in each of the 5 cytological categories. Women with either HPV infection or dyskaryosis had a significantly higher %MR compared with the normal group. However, when the dyskaryotic group was divided into mild, moderate and severe dyskaryosis the significant difference was only confined to the mild and moderate dyskaryosis and the %MR in women with severe dyskaryosis was not significantly different from that of women with normal cervices.

3.2.4.2.2.3. Comparison of %MR with histology.

Table 16 shows the %MR of smears from colposcopically normal women compared with the %MR of smears from women with histological evidence of CIN.

There was a significant difference in %MR of smears from colposcopically normal women and women with CIN. However, this difference did not correlate with the severity of the disease. There was no difference in the %MR of smears from colposcopically normal women and women with HPV infection without neoplasia.
The distribution of %MR within the normal and CIN groups is shown in Figure 40. Figure 41 shows the distribution of %MR within the different grades of CIN. It can be seen that in all histological groups the values of %MR are scattered over a broad range with considerable overlap between the groups.
Table 14. The efficacy of the Papanicolaou smear in recognising cervical intraepithelial neoplasia (CIN).

<table>
<thead>
<tr>
<th>Cytology result</th>
<th>CIN 1 (n=25)</th>
<th>CIN 2 (n=7)</th>
<th>CIN 3 (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>HPV infection</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild dyskaryosis</td>
<td>14</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Moderate dyskaryosis</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Severe dyskaryosis</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 15. The molar ratio in exfoliated cervical cells against result of most recent Papanicolaou smear

<table>
<thead>
<tr>
<th>Result of most recent Papanicolaou smear test.</th>
<th>n</th>
<th>Mean %MR</th>
<th>Median %MR</th>
<th>SD</th>
<th>Mann-Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>296</td>
<td>2.28</td>
<td>2.36</td>
<td>3.05</td>
<td>-</td>
</tr>
<tr>
<td>HPV infection</td>
<td>2</td>
<td>3.22</td>
<td>3.22</td>
<td>0.16</td>
<td>p=0.19</td>
</tr>
<tr>
<td>Mild dyskaryosis</td>
<td>21</td>
<td>3.02</td>
<td>2.58</td>
<td>1.22</td>
<td>p=0.0497</td>
</tr>
<tr>
<td>Moderate dyskaryosis</td>
<td>14</td>
<td>4.04</td>
<td>3.54</td>
<td>1.88</td>
<td>p=0.0004</td>
</tr>
<tr>
<td>Severe dyskaryosis</td>
<td>21</td>
<td>2.80</td>
<td>2.51</td>
<td>1.51</td>
<td>p=0.4</td>
</tr>
<tr>
<td>All dyskaryosis</td>
<td>56</td>
<td>3.19</td>
<td>2.63</td>
<td>1.57</td>
<td>p=0.0011</td>
</tr>
</tbody>
</table>
Table 16. Multicentre studies with cervical smears: Distribution of %MR values within histological grades

<table>
<thead>
<tr>
<th>Cervical condition</th>
<th>n</th>
<th>Mean %MR</th>
<th>Median %MR</th>
<th>SD</th>
<th>Mann-Whitney test *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>285</td>
<td>2.72</td>
<td>2.35</td>
<td>2.84</td>
<td>--------------------</td>
</tr>
<tr>
<td>HPV infection</td>
<td>9</td>
<td>2.35</td>
<td>2.23</td>
<td>0.59</td>
<td>p=0.8</td>
</tr>
<tr>
<td>CIN 1</td>
<td>15</td>
<td>3.18</td>
<td>2.82</td>
<td>1.67</td>
<td>p=0.0237</td>
</tr>
<tr>
<td>CIN 2</td>
<td>17</td>
<td>6.02</td>
<td>3.09</td>
<td>8.04</td>
<td>p=0.12</td>
</tr>
<tr>
<td>CIN 3</td>
<td>28</td>
<td>3.18</td>
<td>2.59</td>
<td>1.53</td>
<td>p=0.0122</td>
</tr>
<tr>
<td>All CIN grades</td>
<td>60</td>
<td>3.51</td>
<td>2.69</td>
<td>3.10</td>
<td>p=0.0005</td>
</tr>
</tbody>
</table>

* Mann-Whitney test used as data was not normally distributed.
Figure 40 Multicentre studies with exfoliated cervical cells: Distribution of molar ratios within normal (n=285) and CIN (n=60) groups.
Figure 41. Multicentre studies with exfoliated cervical cells: Distribution of %molar ratios within each grade of CIN: CIN 1 (n=15), CIN 2 (n=17), CIN 3 (n=28).
3.2.4.2.3. Summary of the results from the multicentre trial.

Although cytological examination detected dyskaryosis in all cases of CIN 3 its failure to detect abnormalities in the five cases of CIN 1 and CIN 2 demonstrated that the Papanicolaou technique is less sensitive for the minor abnormalities.

The %MR is increased in smears from women with CIN although the measurement lacks specificity because the %MR does not increase with severity of disease. This was further demonstrated by the considerable overlap between the values of %MR in the normal and CIN groups.

The findings of the multicentre study, in contrast to the initial study, did not support propositions that determination of the %MR could contribute to the screening for cervical cancer. However, the study was clearly unsatisfactory in a number of respects. First, the %MR could only be calculated in 30% of cases; 25% of the cytology samples could not be assessed. Second, other factors were not properly controlled including insufficient cell collections, samples accompanied by incomplete documentation, and severely delayed arrival in laboratory of specimens kept at ambient temperatures. It was, therefore, unclear whether or not the results of the study were genuine.
3.2.4.3. Concluding studies with cervical epithelial cells from women with and without cervical abnormalities.
Although the findings of the multicentre trial suggested that the %MR was of no benefit in cervical cancer screening the study was conducted in such an unsatisfactory manner that it was unclear whether or not the study findings were reliable. It was, therefore, decided that a rigourously controlled study should be carried out to determine whether the study of cervical epithelial cells should be continued.

3.2.4.3.1. Experimental procedure.
A total of 488 premenopausal women attending a Colposcopy Clinic at the Royal Northern Hospital and the Bloomsbury Family Planning Clinic, were studied. The specimens were prepared and analysed on the same day of collection as for the previous studies (described in sections 3.2.4.1. and 3.2.4.2.). The protocol for these studies is given in section 2.3.6..

3.2.4.3.2. Study results.
The %MR could be calculated for 452 of the 488 specimens as insufficient material was collected from 36 women for reliable determination of %MR.

3.2.4.3.2.1. Comparison of the cytology and histology reports.
Histological examination of biopsies taken during colposcopical examination revealed a total of 86 women with CIN. Of these cases 57 were CIN 1, 13 CIN 2 and 16 CIN 3: but none had any evidence of microinvasive cancer. Table 17 shows the cytological findings in smears from women with each of the three grades of histologically proven CIN.
The false negative rates for the Papanicolaou smear test in this study were 42% for CIN 1 (24/57), 15% for CIN 2 (2/13), and 13% for CIN 3 (2/16). It is clear that the smear test has poor sensitivity for the milder condition. However, the test was highly specific because histologically proven CIN was found in all cases in which cytology reported moderate or severe dyskaryosis.

3.2.4.3.2.2. Comparison of the results of the molar ratio determination with the Papanicolaou smear test.

Table 18 relates the %MR values with the cytological reports of the Papanicolaou smear test. It can be seen that there is no difference in %MR between any of the cytological categories.

3.2.4.3.2.3. Comparison of the molar ratio with results of histology.

The results of statistical analysis of the %MR values in each histological group are given in Table 19.

A scattergram showing the distribution of %MR values in smears from women with normal cervices (n=366) and women with histologically proven CIN (n=86) is given in Figure 42. Marked on the scattergram is the 'cut-off' point (%MR=2.10) between normal and CIN groups, calculated from the initial study. It can be seen that there is considerable overlap between the CIN group and the normal group.

Figure 43 shows the distribution of %MR values within each of the three histological grades of CIN. It is clear that the %MR values did not reflect the severity of the precancer.
There was no significant difference in %MR between the normal and combined CIN groups (p=0.09). Although there was a significant difference in %MR between the normal and the CIN 1 groups, it is unlikely that this difference is related to the neoplastic changes because the %MR in the CIN 2 and CIN 3 groups are not significantly different from the normal group. It was felt that the difference in %MR between the normal and CIN 1 groups was not caused by a generally higher %MR's in the CIN 1 group but by a disproportionate number of specimens in the CIN 1 group with very high %MR's. Of the eight %MR values above 6.00 in the CIN group seven were from women with CIN 1. This suspicion was confirmed when the study results were re-analysed with all %MR values above 6.0 excluded. The significant difference between the CIN 1 and normal groups disappeared.

However, it was noted that the very high %MR's in both the CIN and normal groups were frequently found in women with anaerobic vaginosis. The possibility that vaginal bacteria might be a source of 18:2(9c,11t) was later investigated.

3.2.4.3.2.4. The effect of anaerobic vaginosis on the %MR.
Following indications that the %MR of cervical smear specimens might be influenced by the presence of anaerobic vaginosis, specimens from women with this condition were examined.

High vaginal swabs had been taken during colposcopical examination from any women with symptomatic or heavy discharge. Bacterial culture confirmed the presence of anaerobic vaginosis in nine otherwise colposcopically normal women. Table 20 shows the %MR values of cervical
cells from women whose high vaginal swab culture showed the presence of anaerobes.

Four of the nine women had %MR's well in excess of 6.00 and one had a %MR of 73. There was, however, no significant difference in molar ratio between these nine cases and the other colposcopically normal women.

3.2.4.3.2.5. The effect of menstruation on the %MR.
In order to assess any influence the menstrual cycle might have on the %MR the colposcopically normal women were divided into four categories:

Amenorrhoeic;
Postmenstrual/ovulatory;
Peri-ovulatory; and
Pre-menstrual /menstruating.

There was, however, no significant difference in molar ratio between any of these groups by Mann-Whitney non-parametric test (p>0.4).

3.2.4.3.2.6. The effect of smoking on the %MR.
The effect of smoking on the %MR was assessed both for colposcopically normal women and women with CIN. Absolute non-smokers were compared with women who smoked at least ten cigarettes per day. Since occasional smokers are notoriously vague about how much they smoke, 43 women claiming to smoke less than ten cigarettes per day were excluded from this comparison. There was no significant difference in %MR between non-smokers and smokers amongst women in either the normal or CIN groups.
Table 17. Final studies with cervical smears: Efficacy of the Papanicolaou smear test for the recognition of CIN.

<table>
<thead>
<tr>
<th>Result of most recent Papanicolaou smear test.</th>
<th>CIN 1</th>
<th>CIN 2</th>
<th>CIN 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>24</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Squamous atypia</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Borderline dyskaryosis</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mild dyskaryosis</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Moderate dyskaryosis</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Severe dyskaryosis</td>
<td>0</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 18. Final studies with cervical smears: The %MR of cervical smears against results of most recent Papanicolaou smear.

<table>
<thead>
<tr>
<th>Result of most recent Papanicolaou smear test</th>
<th>n</th>
<th>Mean</th>
<th>Mann-Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>307</td>
<td>2.84</td>
<td>-----------------</td>
</tr>
<tr>
<td>Inadequate specimen</td>
<td>6</td>
<td>2.45</td>
<td>p=0.2</td>
</tr>
<tr>
<td>Borderline dyskaryosis</td>
<td>55</td>
<td>2.88</td>
<td>p&gt;0.8</td>
</tr>
<tr>
<td>Squamous atypia</td>
<td>23</td>
<td>2.79</td>
<td>p&gt;0.4</td>
</tr>
<tr>
<td>Mild dyskaryosis</td>
<td>28</td>
<td>2.76</td>
<td>p&gt;0.7</td>
</tr>
<tr>
<td>Moderate dyskaryosis</td>
<td>25</td>
<td>2.96</td>
<td>p&gt;0.6</td>
</tr>
<tr>
<td>Severe dyskaryosis</td>
<td>8</td>
<td>2.52</td>
<td>p&gt;0.7</td>
</tr>
</tbody>
</table>
Table 19. Final studies with cervical smears: Distribution of %MR values within histological grades.

<table>
<thead>
<tr>
<th>Cervical condition</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Mann-Whitney test %MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>366</td>
<td>3.47</td>
<td>4.57</td>
<td></td>
</tr>
<tr>
<td>CIN 1</td>
<td>57</td>
<td>4.02</td>
<td>2.99</td>
<td>p=0.043</td>
</tr>
<tr>
<td>CIN 2</td>
<td>13</td>
<td>2.98</td>
<td>1.49</td>
<td>p&gt;0.35</td>
</tr>
<tr>
<td>CIN 3</td>
<td>16</td>
<td>2.87</td>
<td>1.26</td>
<td>p&gt;0.8</td>
</tr>
<tr>
<td>All CIN grades</td>
<td>86</td>
<td>3.83</td>
<td>2.81</td>
<td>p=0.09</td>
</tr>
</tbody>
</table>

Table 20. The %MR of smears from women with anaerobic vaginosis.

<table>
<thead>
<tr>
<th>Study number</th>
<th>%MR value</th>
<th>Bacteria cultured</th>
</tr>
</thead>
<tbody>
<tr>
<td>W59</td>
<td>2.6</td>
<td>Anaerobes</td>
</tr>
<tr>
<td>W64</td>
<td>0.4</td>
<td>Anaerobes</td>
</tr>
<tr>
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<tr>
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<td>Anaerobes &amp; Candida species</td>
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<tr>
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<td>Anaerobes</td>
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</table>
Figure 42. Final studies with exfoliated cervical cells: Distribution of %MR in specimens from normal women (n=366) and women with CIN (n=86).
Figure 43 Final studies with exfoliated cervical cells: The distribution of %MR values amongst the grades of CIN: CIN 1 (n=57), CIN 2 (n=13), and CIN 3 (n=16).
3.2.4.3.3. Summary of the results of the concluding studies with cervical cells.

Histologically proven CIN was found in all cases in which cytology reported moderate or severe dyskaryosis confirming the high specificity of the Papanicolaou smear test. A third of women with histologically proven CIN were reported as normal. However, these false negatives were predominantly cases of CIN 1 as dyskaryosis was reported in 87% of CIN 3 cases, 85% of CIN 2, and 58% of CIN 1. This clearly demonstrates the insensitivity of the technique for the minor abnormalities.

There was no significant difference in %MR between the normal group and either the CIN 2 or CIN 3 groups. The significant difference in %MR between the normal and CIN 1 groups was due to a few specimens in the CIN 1 group with very %MR's. The large number of false positives (%MR>2.10 but colposcopically and cytologically normal) indicates the measurement has poor specificity.

The %MR was not found to be influenced by the menstrual cycle, smoking habits or anaerobic vaginosis. However, because high vaginal swabs were only taken when symptomatic or heavy discharge was evident some cases of minor undetected infection may have been overlooked.

The findings of this study are in sharp contrast with those of the initial studies. The considerable overlap between the normal and CIN groups clearly preclude the use of the technique for discriminating normal from precancerous cervical conditions. Further studies to establish factors capable of influencing the %MR are clearly indicated; in particular the relationship between %MR and the presence of anaerobic vaginosis.
3.2.4.4. Summary and conclusions from the analysis of exfoliated cervical cells from women with and without CIN.

All three studies demonstrated very clearly the inadequacies of cytological screening. It had poor sensitivity for minor abnormalities and even for CIN 3 it had a false negative rate greater than 10% in the carefully controlled final study. However, the technique was shown to have high specificity as it did not report any false positive results.

Though the findings of the initial study suggested the %MR estimation may contribute to cervical cancer screening the findings of the larger later studies provided conclusive evidence that the technique as it stood could not discriminate between normal and precancerous cervical conditions. Much of the overlap between the normal and CIN groups in the latter two studies was caused by false positives as defined by the 'cut-off' point (%MR=2.10) suggested by the initial studies.

The final study considered factors potentially having a bearing on the %MR. It was found that the %MR was not significantly affected by the phase of the menstrual cycle, smoking, or the presence of anaerobic vaginosis. However, since high vaginal swabs were only taken for bacterial culture when symptomatic or heavy discharge was evident a number of cases of minor infections may well have been missed.

It was concluded that whatever the cause of the contrast in findings between the initial and later studies the %MR estimation could make no practical contribution to the screening for cervical cancer. However, further investigation of anaerobic vaginosis and other factors possibly influencing the %MR were indicated.
3.2.5. Factors possibly affecting molar ratio in specimens of exfoliated cervical cells.

3.2.5.1. The effect of endocervical mucus on the molar ratio.
It is known that the amount and viscosity of the mucus changes during the menstrual cycle and that progesterone oral contraceptives can cause the production of a reduced amount of highly viscous mucus. In these studies the mucus was most apparent in samples collected during the progestational phase of the menstrual cycle when it entraps endocervical and ectocervical cells and other debris from the uterus and vagina. It was, therefore, decided to investigate the effect of endocervical cells on the %MR.

3.2.5.1.1. Experimental procedure.
The %MR was determined in endocervical and ectocervical specimens from 22 women attending the Bloomsbury Family Planning Clinic. Specimens of ectocervical endocervical cells were collected as described in 2.2.4.2. The specimens were analysed as described in sections 3.2.4.1.1..

3.2.5.1.2. Study results.
The ectocervical specimens had a mean %MR of 3.01 (SD=0.98); and the endocervical specimens a mean %MR of 2.98 (SD=1.09). The paired t-test showed no significant difference (p>0.9) between the endocervical and ectocervical specimens despite the different populations of cells in each type of specimen. The correlation coefficient (r) was 0.67 which was highly statistically significant (p=0.001). It was, therefore, concluded that endocervical mucus has no effect on the %MR.
3.2.5.2. Investigation of bacterial generation of 18:2(9c,11t).

The observation that specimens from women with anaerobic vaginosis frequently had high %MR's prompted suggestions that vaginal bacteria could produce 18:2(9c,11t) possibly via a mechanism similar to *Butyrivibrio fibrisolvens*. Bacteria isolated from the vagina were, therefore, cultured and screened for 18:2(9c,11t) production.

3.2.5.2.1. Experimental procedure.

The study involved 150 women examined in the Colposcopy Clinic at the Royal Northern Hospital, London. High vaginal swabs (HVS) were collected from all the women who had evidence of anaerobic vaginosis. Bacteria present in the HVS were cultured on horse blood agar under anaerobic conditions for 48 hours.

Bacterial growth was removed from the surface of a culture plate with a sterile cotton tipped swab and washed into 2ml chloroform/methanol (2:1, v/v). The suspension was sonicated by a Soniprep 150 (MSE, Crawley, Surrey, UK) fitted with a 3mm exponential probe oscillating at 23kHz with an amplitude of 14µm.

The sonicated samples were mixed with 1.5ml deionised water and centrifuged at 1000g for 10 minutes. The chloroform (lower) layer was removed and dried under a stream of oxygen free nitrogen. The lipids were resuspended in 100µl propan-2-ol/acetonitrile (2:1, v/v) and 20µl was injected onto the HPLC system described in section 3.1.1. which incorporated the modifications detailed in section 3.1.4.1.. The residual lipid extracts of bacteria thought to produce 18:2(9c,11t) were stored at -20°C for further investigations.
Two control experiments were carried out to establish that the culture medium neither contained $18:2(9c,11t)$ nor interfered with the assay. Lipids were extracted from one gram of horse blood agar and a cotton tipped swab wiped across the same culture medium. These lipid extracts were injected onto the HPLC and analysed along with the bacterial extracts.

3.2.5.2.2. Results of the investigation of bacterial generation of $18:2(9c,11t)$.

Both control experiments showed the culture media did not contain $18:2(9c,11t)$. The 234nm trace of the lipid extract prepared from one gram of horse blood agar is shown in Figure 44. The short broad peak with retention time 8.72 minutes elutes approximately one minute later than $18:2(9c,11t)$. Since $18:2(9c,11t)$ has a retention time of approximately 7.70 minutes and the peak represents 1g of horse blood agar it was concluded that the concentration of the $18:2(9c,11t)$ in the culture media would not influence the assay of $18:2(9c,11t)$ in cultured bacteria. Chromatography of the sample prepared from the swab wiped across the culture plate showed no definite peaks. It was, therefore, concluded that neither the culture of the bacteria nor the sampling of cultured bacteria caused any artifactual results.

Lipid extracts of some of the bacterial cultures contained high concentrations of a fatty acid which co-eluted with $18:2(9c,11t)$. Figure 45 shows the chromatograms of one such sample in which the concentration of the putative $18:2(9c,11t)$ was so high that the sample had to be diluted 20 fold for analysis. It was possible to identify three strains of bacteria which produced the fatty acid as:

1. *Lactobacillus brevis*;
2. *Propinibacterium acnes*;
3. *Coryneform species*.

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It was, unfortunately, not possible to identify the Coryneform bacteria further despite additional biochemical tests and comparison with the computer databank at a reference laboratory (National Collection of Typed Cultures, Colindale, London, UK).

The lipid extract of one strain of *Lactobacillus brevis* also contained significant concentrations of a fatty acid which was detected at 200nm and eluted close to oleic acid. Figure 46 shows that this fatty acid co-chromatographed with added *trans* vaccenic acid, 18:1(11t).

### 3.2.5.2.3. Summary of the investigation of bacterial production of 18:2(9c,11t).

The findings of these studies strongly suggested that bacteria present in the vagina can produce 18:2(9c,11t). Moreover, *Lactobacillus brevis* appeared to produce 18:1(11t) suggesting that any 18:2(9c,11t) formed was an intermediate of the conversion of 18:2(9,12) to 18:1(11t).

In some cases although the initial mixed bacterial culture produced 18:2(9c,11t) none of the subsequent subcultures of individual bacteria did. This was considered due either to the loss of the bacteria during subculture or the requirement of more than one bacteria for 18:2(9c,11t) production. Neither of these possibilities were pursued.

It was concluded that vaginal bacteria could influence the %MR of cervical smears. However, since different fatty acids can co-elute (*cf.* 22:6 and 18:3) further investigation to identify the putative 18:2(9c,11t) in the bacterial extracts was indicated.
Figure 44. Chromatograms of a lipid extract prepared from 1g horse blood agar.

Mobile phase:
89.9% Acetonitrile/ 10% Water/ 0.1% Acetic acid
Flow rate 1.5ml/min
Detector 200nm; Attenuation 0.2aufs
Sample loop volume 20µl

Mobile phase:
89.9% Acetonitrile/ 10% Water/ 0.1% Acetic acid
Flow rate 1.5ml/min
Detector 234nm; Attenuation 0.02aufs
Sample loop volume 20µl
Figure 45. Chromatograms demonstrating the production of putative 18:2(9c,11t) by *Lactobacillus brevis*.

**a) Bacterial extract**

- **Mobile phase:**
  - 89.9% Acetonitrile
  - 10% Water
  - 0.1% Acetic acid
- **Flow rate:** 1.5ml/min
- **Detector:** 234nm
- **Attenuation:** 0.01aufs
- **Sample loop volume:** 20µl

**b) 18:2(9c,11t) Standard**

- **Mobile phase:**
  - 89.9% Acetonitrile
  - 10% Water
  - 0.1% Acetic acid
- **Flow rate:** 1.5ml/min
- **Detector:** 234nm
- **Attenuation:** 0.01aufs
- **Sample loop volume:** 20µl

**c) Bacterial extract spiked with 18:2(9c,11t)**

- **Mobile phase:**
  - 89.9% Acetonitrile
  - 10% Water
  - 0.1% Acetic acid
- **Flow rate:** 1.5ml/min
- **Detector:** 234nm
- **Attenuation:** 0.01aufs
- **Sample loop volume:** 20µl
Figure 46. Chromatograms of a lipid extract of a *trans*-vaccenic acid producing bacteria (*Lactobacillus brevis*) spiked with *trans*-vaccenic acid.

a) Bacterial extract

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 200nm;
Attenuation 0.2aufs
Sample loop volume 20µl

b) *trans*-vaccenic acid

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 200nm;
Attenuation 0.2aufs
Sample loop volume 20µl

c) Bacterial extract spiked with *trans*-vaccenic acid

Mobile phase:
89.9% Acetonitrile
10% Water
0.1% Acetic acid
Flow rate 1.5ml/min
Detector 200nm;
Attenuation 0.2aufs
Sample loop volume 20µl
3.2.5.3. Gas Chromatography-Mass Spectrometry analysis of bacterial lipid extracts.

The findings of HPLC studies were consistent with the production of 18:2(9c,11t) by some bacterial species present in the vagina. But, further supporting evidence was required. It was decided to study it using Gas Chromatography - Mass Spectrometry (GCMS).

3.2.5.3.1. Experimental procedure.

These investigations used the remainder of the lipid extracts of bacteria suspected of producing 18:2(9c,11t) from the experiment described in section 3.2.5.2..

Samples of sufficient purity for GCMS analysis were prepared by isolating the putative 18:2(9c,11t) from other fatty acids in each lipid extract. To achieve this each lipid extract was injected onto the HPLC several times and the eluate fractions containing the putative 18:2(9c,11t) pooled. The pooled eluate of each sample was mixed with an equal volume of chloroform and centrifuged at 1000g for 10 minutes. The chloroform (lower) layer was removed and dried under a stream of oxygen free nitrogen. The residue of each sample was resuspended in 0.3ml methanol. These samples together with 18:2(9c,11t) and 18:2(9t,11t) reference standards were submitted for GCMS analysis to the Department of Mass Spectrometry, Clinical Research Centre, Northwick Park Hospital, Harrow, London, UK. Full details of the GCMS analysis are given in section 2.2.2..

3.2.5.3.2. Results of the GCMS analysis.

GCMS analysis of the methyl ester and pyrrolidide derivatives of the reference standards 18:2(9c,11t) and 18:2(9t,11t) could not distinguish the two
stereoisomers. The amount of lipid from each of the bacterial extracts was unfortunately insufficient for exhaustive GCMS investigations. However, the strongest mass spectra were obtained from an extract of *Lactobacillus brevis*. The spectra of the pyrrolidide derivative of the methyl ester of the putative 18:2(9c,11t) from this source, shown in Figure 47, were consistent with the lipid being an isomer of 18:2(9,11) and closely resembled those of 18:2(9c,11t) given in Figure 48.
Figure 47. Spectra from GCMS analysis of the diene conjugated fatty acid produced by

*Lactobacillus brevis*

a) The GCMS spectrum of the pyrrolidine derivative of the methyl ester of the putative
18:2(9c,11t) prepared from a lipid extract of cultured *Lactobacillus brevis*.

b) An expanded portion (150-250 amu) of the GCMS spectrum shown in Figure 47(a)
Figure 48. Spectra from the GCMS analysis of reference 18:2(9c,11t).

a) The GCMS spectrum of the pyrrolidide derivative of the methyl ester of reference 18:2(9c,11t).

b) An expanded portion (150-250amu) of the GCMS spectrum shown in Figure 48(a).
3.2.5.4. Summary of the studies of potential influencers of the %MR of cervical smears.

The findings of studies comparing the %MR in endocervical and ectocervical cells showed a significant correlation between the two cell populations. It was, therefore, concluded that the variable proportion of endocervical cells in cervical smears was unlikely to affect the %MR.

In contrast the findings of the studies investigating the production of 18:2(9c,11t) by vaginal bacteria suggested that the %MR can indeed be influenced by bacterial production. Three strains of bacteria were found to produce a fatty acid which co-chromatographed with 18:2(9c,11t) on HPLC. Furthermore, mass spectra of derivatives of the fatty suggested that it was 18:2(9,11) and consistent with being 18:2(9c,11t).

One of the strains of Lactobacillus brevis also produced a fatty acid which co-chromatographed on HPLC with 18:1(11t). This finding suggested that the bacteria was converting linoleic acid to 18:1(11t) via 18:2(9c,11t) enzymatically possibly using a similar mechanism to the ruminant bacteria, Butyribibrio fibrisolvens.

It was concluded that bacterial production of 18:2(9c,11t) was almost certainly responsible for the high %MR's frequently found in cervical smears from women with anaerobic vaginosis.
3.3 THE PEROXIDISABILITY OF CERVICAL CELLS.

The experimental findings reviewed in section 1.4.2. reported that the rate of lipid peroxidation, and the concentration of peroxidation products and antioxidants can be altered in cervical cancer. It was, therefore, decided to investigate the effect on the concentrations of fatty acids and thiobarbituric acid reactive substances (TBARS) of added inducers of peroxidation with a view to devising a stress test suitable for cervical cells, which could be used to assess the peroxidisability of cells from women with and without CIN.

3.3.1. Peroxidation induction studies with cervical cells.

3.3.1.1. Experimental procedure.

The study involved 20 women with cytologically and colposcopically normal cervical epithelium, who were attending the Bloomsbury Family Planning Clinic. Specimens of cervical cells were collected from each women as described in section 2.2.4.2.

The specimens were pooled and centrifuged at 1500g for 10 minutes. The supernatant was discarded and the cells resuspended in 1ml 20mmol/l PBS containing 5mmol/l sodium azide (to inhibit catalase activity). The cell suspension was mixed for two minutes with a Silverson mixer emulsifier. After the suspension had been allowed to equilibrate at room temperature it was mixed thoroughly by vortex and 5ml of the suspension was placed into each of four glass tubes. One millilitre was removed from each tube for HPLC analysis detailed later in this section. Hydrogen peroxide was added to two of the tubes (B1 and B2) to give a final concentration of 10mmol/l. No hydrogen peroxide was added to the other two tubes (A1 and A2). All four tubes were stoppered and incubated at 37°C for 60 minutes. After incubation
1ml was taken from each tube for HPLC analysis and 2.5ml for measurement of TBARS.

**HPLC Analysis**

One millilitre of each incubated cell suspension was mixed with 0.2ml 40mmol/l calcium chloride and 0.1ml of a solution containing 1mol/l methanol and 50 000 IU/l phospholipase A2 in 100mmol/l Tris-HCl buffer. Following 15 minutes incubation at 25°C the mixture was vortex mixed with 2ml chloroform/methanol (2:1,v/v) and centrifuged at 1500g for 10 minutes. The chloroform (lower) layer was removed to a clean glass tube and evaporated to dryness under a stream of oxygen free nitrogen. The lipids were resuspended in 100μl of propan-2-ol/acetonitrile (2:1,v/v) and injected on to the HPLC system described in section 3.1.1.1. and incorporating the modifications detailed in section 3.1.4.1..

**The measurement of thiobarbituric acid reactivity.**

The 2.5ml of the incubated cell suspension was mixed with 1.5 ml 1.71mol/l trichloroacetic acid for the measurement of TBA reactive substances by a modified version of the stress test of Stocks and Dormandy (1974). The mixture was centrifuged at 1500g for 10 minutes and 1ml of the resulting supernatant was added to 1ml of a solution of 70mmol/l thiobarbituric acid in 50mmol/l sodium hydroxide. The tubes were incubated at 95°C for 15 minutes and allowed to cool in cold water. One millilitre butan-1-ol was added to each tube and the tubes shaken vigorously for two minutes. Following centrifugation at 1500g for 10 minutes the butan-1-ol (upper) layer was removed for fluorimetric measurement (Ex532nm, slit width 12nm; Em553nm, slit width 14nm) on the instrument described in section 2.2.3.2..
3.3.1.2. Results.

Figure 49 clearly shows the marked increase in TBA reactivity of the samples incubated with hydrogen peroxide compared with control samples incubated with H$_2$O$_2$.

Figure 50 shows the change in the fatty acid content of cervical cell suspensions incubated with and without H$_2$O$_2$. This experiment shows that the concentrations of the more unsaturated (and so more susceptible to peroxidation) phospholipid esterified fatty acids declined considerably on incubation with H$_2$O$_2$ compared to the controls in which no significant decrease was noted. The concentrations of docosahexaenoic and arachidonic acids fell by 43% and 36% respectively, whilst the concentrations of the more saturated linoleic acid and 18:2(9c,11t) were reduced by 21% and 31% respectively. However, the concentration of phospholipid esterified oleic acid was unchanged after incubation with H$_2$O$_2$. It was, therefore, apparent that the more unsaturated the fatty acid the more pronounced was its decrease on incubation with H$_2$O$_2$. 
Figure 49. Histogram showing the effect of incubation with H$_2$O$_2$ on TBA reactive material in exfoliated cervical cells.

![Histogram showing the effect of incubation with H$_2$O$_2$ on TBA reactive material in exfoliated cervical cells.]

Figure 50. The reduction in the fatty acid content of cervical cells on incubation with hydrogen peroxide.

![Bar graph showing the reduction in the fatty acid content of cervical cells on incubation with hydrogen peroxide.]

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3.3.2. Incubation of cervical cells with hydrogen peroxide, tert-butylhydroperoxide and cumene hydroperoxide.

The amount of material in each cervical smear sample was limited so it was important to devise a sensitive stress test to assess the peroxidisability of cervical cells. The effect on the production of TBARS of different peroxide compounds was investigated.

3.3.2.1. Experimental procedure.

The study involved a further 20 women with cytologically and colposcopically normal cervical epithelium from whom cervical cells were collected.

The specimens were pooled and centrifuged at 1500g for 10 minutes. The supernatant was discarded and the cells resuspended in 20ml 20mmol/l PBS (pH 7.4) containing 1mmol/l sodium azide. The suspension was homogenised with a Silverson mixer emulsifier for 30 seconds. The cell suspension was divided into 8 aliquots of 2.5ml. To each aliquot was added 100µl of one of the following:

- 20mmol/l PBS (pH 7.4);
- 260mmol/l hydrogen peroxide (final concentration 10mmol/l);
- 260mmol/l tert-butylhydroperoxide (final concentration 10mmol/l);
- 260mmol/l cumene hydroperoxide in PBS (final concentration 10mmol/l).

The aliquots were incubated at 37°C for 60 minutes. After incubation TBARS were measured fluorimetrically as described in sections 2.2.3.2. and 3.3.1.1.
3.3.2.2. Results.

Figure 51 shows percentage increase in concentration of TBARS in the cell suspension following incubation with the different peroxides.

Although incubation with any of the peroxide compounds gives rise to increased TBARS, it can be seen that tert-butylhydroperoxide produced the greatest rise.

It was concluded from this study that the magnitude of the increase in fluorescence (Ex 532nm, slit width 12nm; Em 553nm, slit width 14nm) was sufficient to allow the assay of peroxidisability in individual specimens of cervical cells. It was decided that since tBHP produced the greatest increase in TBARS it would offer the highest sensitivity for such an assay.
Figure 51. Comparison of the effect of incubation with hydrogen peroxide, tert-butylhydroperoxide and cumene hydroperoxide on the TBA reactive material in exfoliated cervical cell suspensions.
3.3.3. Intrabatch coefficient of variation for the cervical epithelial cell stress test.

The findings of the previous studies showed it was possible to measure the peroxidisability of individual cervical smears. First, however, it was important to determine the coefficient of variation for the assay.

3.3.3.1. Experimental procedure.

The assay involved 10 women with cytologically and colposcopically normal cervical epithelium from whom cervical cells were collected. The specimens were pooled prior to centrifugation at 1500g for 10 minutes. The supernatant was discarded and the cells resuspended in 20ml 20mmol/l PBS (pH 7.4) containing 1mmol/l sodium azide. The suspension was homogenised by a Silverson mixer emulsifier for thirty seconds. The suspension was divided into 20 aliquots of 2.2 ml. Each aliquot was further divided into two aliquots (A and B) and 30µl 0.4mol/l tert-butylhydroperoxide was added to each of the B aliquots. The samples were incubated at 37°C for 60 minutes.

Following incubation 1ml of each incubated cell suspension was vortex mixed with 0.6ml 1.71mol/l TCA and the mixture centrifuged at 1500g for 10 minutes to precipitate the protein. One millilitre of the resulting supernatant was added to 1ml of a solution of 70mmol/l TBA in 50mmol/l sodium hydroxide in a glass tube. The tubes along with freshly prepared blanks and TMP standards run in triplicate were incubated at 95°C for 15 minutes. The blanks consisted of 667µl deionised water, 333µl 1.71mol/l TCA, and 1ml TBA reagent.

The blanks, standards and samples were cooled with cold water and each shaken vigorously with 1ml butan-1-ol. Following centrifugation at 1500g
for 10 minutes and the fluorescence of the butan-1-ol (upper) layer measured against the blanks and standards. The conditions for the measurement of fluorescence were as described earlier in section 2.2.3.2..

3.3.3.2. Results.
The amount of material in each aliquot of cell suspension was clearly adequate for analysis as fluorescence was readily measurable in all the samples using low sensitivity settings whether or not they had been incubated with tert-butylhydroperoxide.

The concentrations of MDA in the 'A' samples and corresponding 'B' samples are given in Table 21 together with the calculated intrabatch coefficient of variation (CV).

The intrabatch CV of 7.2% shows the measurement of the lipoperoxides by this method is precise. Since the lipoperoxide concentration of a specimen increases with storage (even if stored in liquid nitrogen) it was not possible to assess the interbatch CV of the method. However, it was concluded that the method could be applied to cervical smears analysed on the day of collection.
Table 21. Intrabatch CV for the measurement of TBA reactive material in cervical smears.

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<th>Post-incubation with tBHP [TBARS] pmol/ml</th>
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3.3.4. The peroxidisability of cervical cells from women with and without CIN.

3.3.4.1. Experimental procedure.
A total of 33 women attending the Bloomsbury Family Planning Clinic or the Colposcopy Clinic at the Royal Northern Hospital were studied. The specimens were centrifuged at 1500g and the cells resuspended in 2.2ml 20mmol/l PBS containing sodium azide and homogenised in a mixer emulsifier (Silverson Machines Ltd.). Each cell suspension was divided into two aliquots of 1.1ml (A and B). These were processed as described in section 3.3.3.1.

3.3.4.2. Results.
Figure 52 shows the percentage increase in TBARS in specimens from colposcopically normal women and women with CIN. There was no significant difference (p=0.32) in the increase in TBARS between specimens from women with normal cervical epithelium and those with CIN.

Figure 53 shows the increase in TBARS on incubation with tBHP of the cervical specimens according to degree of red cell contamination. It was noted, however, that the increase in TBARS on incubation with tBHP was greater in specimens which were contaminated with red blood cells than those which were not.

The difference in the increase in TBARS between specimens, which were contaminated with red blood cells and those that were not, was highly significant (p<0.0005 on Mann-Whitney test).
It was concluded that although there may be a difference in peroxidisablity between cervical cells from women with and without CIN any such difference was obscured by the presence of red blood cells. Further investigation of the effect of red blood cells on the TBA reactivity of specimens were indicated.
Figure 52. Scattergram showing the increase in TBA reactive material in exfoliated cervical cell suspensions from normal women (n=20) and women with CIN (n=13) on incubation with tert-butylhydroperoxide.
Figure 53. Scattergram comparing the increase in TBA reactive material in cervical cell suspensions with high (n = 12) and low (n = 21) red blood cell contamination.
3.3.5. The effect of the presence of red blood cells on the assessment of peroxidisability.

3.3.5.1. Experimental procedure.
The investigation involved 20 women attending the Bloomsbury Family Planning Clinic who had normal cervical epithelium. Cervical smears collected from each woman were pooled and centrifuged at 1500g for 10 minutes. The supernatant was discarded. The cells, resuspended in 25ml 20mmol/l PBS containing 2mmol/l sodium azide, were homogenised in a mixer emulsifier (Silverson Machines Ltd.) and 1.1ml aliquots were placed into test tubes. Various concentrations (range 0.25-8.0 x 10⁵/ml) of red blood cells suspended in 154mmol/l sodium chloride were prepared. To each tube of epithelial cells was added 1.1ml of one of the red blood cell suspensions or 1.1ml 154mmol/l sodium chloride. The contents of the tubes were mixed and the divided into two aliquots (A and B). Thereafter the peroxidisability of each sample was assessed as described in section 3.3.3.1.

3.3.5.2. Results.
Figure 54 shows the increase in fluorescence intensity on incubation with tBHP plotted against degree of RBC contamination. It can be seen that the presence of RBC's even at low levels causes a significant increase in TBA reactivity.

It was concluded that the peroxidisability of cervical cells could not be assessed unless any contaminating blood is first removed. Investigation of methods of removing red blood cells from cervical cells were, therefore, indicated.
Figure 54. Graph showing the increase in TBA reactive substances in pooled cervical cell suspensions with increasing concentration of red blood cells.

Each point represents the mean of five specimens. Standard error bars are shown.
4.1.1. An overview of established techniques used in the study of the role of free radicals in human disease.

Progress in the investigation of free radical involvement in human disease has long been hampered by the lack of analytical methods which can be applied to the study of biological specimens. The highly reactive nature of free radical species makes their direct detection difficult.

Free radicals can be detected directly using ESR spectroscopy but the expense and complexity of the equipment has limited its widespread use. Furthermore the amount of tissue required for ESR studies has restricted its use to post mortem and operation specimens. However, the development of more sensitive and smaller ESR instruments is well advanced and will probably make an impact outside the specialist research laboratory within the next ten years.

More often free radicals are monitored indirectly by measuring reaction products. Of all the methods developed for the indirect study of free radical reactions involving damage to lipids the two most frequently used have been the measurement of total diene conjugation and malondialdehyde. However, both have limitations of specificity. Total diene conjugation measures both lipid hydroperoxides and non-peroxide lipids whilst the TBA reagent used in the measurement of MDA also reacts with breakdown products of other macromolecules.
4.1.2. The method for the measurement of conjugated and non-diene conjugated phospholipid esterified fatty acids.

Against the background of a limited repertoire of assays suitable for the study of free radical reactions, the development of an HPLC method which could measure the concentrations of individual diene conjugated lipids (Iversen, Cawood and Dormandy 1985) appeared to represent a distinct advance in the methods available for free radical research.

This HPLC technique offered significant advantages over established methods. First, diene conjugated lipid hydroperoxides could be separated from the non-peroxide diene conjugated lipids. Lipid hydroperoxides, by virtue of the highly polar hydroperoxide group pass rapidly through the reverse phase column and elute in a single peak. The non-peroxide diene conjugated lipid fraction is retained longer on the column and its component lipids are well resolved. Second by using two UV detectors in series, the HPLC system could simultaneously monitor the diene conjugated and non-diene conjugated unsaturated fatty acids, thus enabling detection of micromolar concentrations of 18:2(9c,11t) and 18:2(9c,12c). Third, the analysis gave results in absolute units of concentration instead of arbitrary ones (cf. total diene conjugation).

It was intended to use the technique to study large numbers of tissue specimens. It was, therefore, clear that the method of sample preparation had to be adapted and improvements made to the HPLC system.
4.1.3. Modifications and improvements to the method sample preparation to allow the analysis of tissue, small biopsies, and cell suspensions.

The principal modifications made to the method involved the homogenisation of the specimens, the extraction of the lipids and the concentration of the lipid extract.

Clearly it was necessary to homogenise the tissue and cells so that the phospholipase A\textsubscript{2} enzyme could access the phospholipids. This was reasonably straightforward when large amounts of tissue were available for analysis. For example a 10\% (w/v) homogenate was prepared from specimens obtained at \textit{post mortem} examination using a mixer emulsifier. The results produced from replicate samples were in close agreement (within 4\%) indicating that the analysis was reproducible and reliable. However, this homogenisation technique was unsuitable for small biopsies and other means had to be sought to finely homogenise these specimens. The use of microhomogenisers generally gave an uniform homogenate after 30 strokes, but some less friable specimens required in excess of 30 strokes to obtain a suitable homogenate.

The original HPLC method was too insensitive to detect the low concentration of fatty acids in the lipid extracts of small biopsies and cell suspensions. The incorporation of a drying and resuspension step was, therefore, crucial for the assay of tissue biopsies and individual specimens of cervical cells.

During the course of the project, increases in the cost of the 'Bond Elut' solid phase extraction columns prompted a switch to chloroform/methanol lipid extraction. Serum lipid extraction by chloroform/methanol was found to be
as effective as extraction by 'Bond Elut' cartridge, and good correlation was observed between assay results using the two methods.

4.1.4. Improvements to the HPLC analysis

The principal changes to the HPLC system involved measures to increase the throughput of samples but other modifications were made to allow the quantitative estimation of arachidonic and oleic acids from the same injection.

The HPLC method developed by Iversen Cawood and Dormandy (1985) was essentially a research technique for the analysis of a few samples at a time. As this project was concerned with the analysis of a large number of samples on an almost routine basis it was important to seek ways to increase the number of samples which could be analysed each day.

The most significant change to the running conditions of the HPLC analysis was the increase in the proportion of acetonitrile in the mobile phase from 85% to 90%. This considerable increase in the eluting strength of the solvent reduced the sample analysis time from just under twenty minutes to around fourteen minutes. Although chromatographic resolution was marginally compromised all the fatty acid peaks continued to be well separated.

Although up to 32 analyses (6 standards and 26 specimens) could be performed each working day. The use of an autoinjector, which allowed specimens to be injected 24 hours a day, increased this throughput to 96 analyses (18 standards and 78 specimens) in a 24 hour period. However, the fatty acid concentrations and %MR still had to be calculated manually. This
was an extremely time consuming task which was prone to error. Fully automated analysis only became possible with the introduction of the computing integrator programmed to detect the peaks of interest and calculate fatty acid concentrations and %MR values. Of all the features of the computer, probably the most notable was the automatic resetting of the time windows to accommodate the fluctuation in the speed of chromatography with ambient temperature.

It was felt that the absolute concentrations of arachidonic and oleic acid might be required in certain studies. Once the range of concentrations over which there was a linear detector response had been established, arachidonic and oleic acids were included in the external standard. These additional data were obtained from the same injection and so made efficient use of specimens and analysis time. The computer program was adjusted accordingly so that the concentrations of these fatty acids were also calculated and printed out.

Since β-eleostearic acid was no longer commercially available, it was necessary to use a different internal standard for this project than that detailed in the method described by Iversen, Cawood and Dormandy (1985). The β-eleostearic acid was in any case impure because on HPLC there was a shoulder on its peak. Having established that human serum and tissue did not contain endogenous 18:2(9t,11t) the fatty acid was used as the internal standard. The 18:2(9t,11t), prepared by the Paint Research Association, had high purity and was better resolved from the 18:2(9c,11t) than was β-eleostearic acid.
4.1.5. Limitations of the HPLC assay.
In the original method published by Iversen, Cawood and Dormandy (1985) it was stated that the assay is specific for fatty acids esterified as phospholipids. However, this is not always the case because the free fatty acids are assayed following phospholipase A$_2$ hydrolysis. The method cannot, therefore, distinguish fatty acids released by the action of phospholipase A$_2$ from those present in the specimen prior to hydrolysis. In blood from normal individuals this is not a problem as almost all fatty acids are esterified but in patients in diabetic coma and systemically heparinised subjects (Wickens et al. 1987) a significant proportion of fatty acids in the blood may not be esterified. When specimens were suspected of containing significant proportions of free fatty acids these were assayed using the same method of preparation except that phospholipase A$_2$ was omitted from the Tris-HCl buffer.
4.2. THE APPLICATION OF THE HPLC ASSAY TO TISSUE AND CELLS.

4.2.1. The measurement of diene conjugated and non-diene conjugated fatty acids in post mortem tissues.

The application of the method for measurement of diene conjugated and non-diene conjugated fatty acids in post mortem tissues showed the technique gave reliable and consistent results. The results from the analysis of aliquots of the same homogenate varied by less than 4%. The technique could, therefore, be applied to single specimens with confidence.

There was a wide variation in the analytical results from each subject so standard ranges could not be established. Initially it was felt the wide variation in the values of fatty acid concentrations and %MR may have been due to the different periods of time each subject was stored at 4°C prior to examination. These delays allowed considerable hydrolysis to occur as shown by the high concentrations of free fatty acids found in specimens. However, the degree of hydrolysis was not commensurate with the duration of storage. This variation would suggest that either the degree of hydrolysis was dependent on the tissue content of antioxidants or the availability of enzyme cofactors.

The low concentrations of free fatty acids in the brain homogenate indicated that little hydrolysis had occurred at this site. It could be that the brain is better protected by a higher concentration of antioxidants or that enzyme cofactors are unavailable after death.

The most interesting finding of these studies was that tumour tissue had a generally higher %MR than normal tissue. In one of the subjects there was a clear trend of rising %MR from normal hepatic tissue through the interface
of normal and tumour tissue to tissue from the centre of the metastatic lesion. This finding raised the possibility that the %MR could be a tumour marker.

The relatively high %MR in heart tissue from the subject with coronary atherosclerosis may have been due to free radical attack of the fatty deposits.

Although these studies suggested links between 18:2(9c,11t) and disease the considerable hydrolysis which had occurred prior to PM examination made interpretation impossible. It was, therefore, important to investigate the concentrations of 18:2(9c,11t) and 18:2(9c,12c), and %MR of fresh tissue.

4.2.2. The measurement of diene conjugated and non-diene conjugated fatty acids in colon biopsies.

Colorectal biopsies were chosen for the next studies: primarily because these tissue specimens were snap frozen in liquid nitrogen immediately after collection thus, avoiding the problems of gross lipid hydrolysis encountered with PM specimens. The study also allowed the comparison of normal and cancerous tissue from the same patient.

The %MR values of the cancerous biopsies were significantly lower than those of the adjacent histologically normal tissue from the same group of patients. This initial finding from the same group was in sharp contrast to the findings from the study of PM specimens and evoked concern that the discrepancy was due to the six months the colorectal specimens had been stored prior to analysis. Comparison of the %MR of specimens analysed on the day of collection with a duplicate set of specimens stored for six months in liquid nitrogen indicated that the %MR in normal tissue rises
significantly on storage. On closer examination this increase in %MR was found to be due to a faster decrease in 18:2(9c,12c) concentration in normal than cancerous tissue. Autooxidation is almost certainly responsible for the reduced 18:2(9c,12c) content of stored tissue and the different rates of the decrease in normal and cancerous tissue may reflect a higher antioxidant content in neoplastic tissue.

A lower rate of lipid peroxidation and the presence of high concentrations of antioxidants in cancer tissue is well established. In 1950 Donnan found that tumour tissue had a lower rate of peroxidation than normal tissue and this finding has since been confirmed (Ahmed and Slater 1981; Slater and Cook 1970; Benedetto et al. 1981). This lower rate of peroxidation in tumour tissue is related to the degree of protection tumours have against radical damage. Cheeseman (1984) discovered that tumours are less peroxidisable than normal tissue and this is principally due to the high concentrations of antioxidants in tumours (Lash 1966; Player 1982; Burton et al. 1982). Reduced pO₂ and lower than normal concentrations of readily peroxidisable PUFA and radical generating systems (eg. cytochrome P₄₅₀) also contribute to the low peroxidisability of tumour tissue (Ahmed and Slater 1981).

These studies concluded that the initial differences in %MR between normal and cancerous tissue were most likely artifacts of the longterm storage of the specimens and not related to malignant change.

4.2.3. The measurement of diene conjugated fatty acids in biopsies of the cervix uteri.

Cancer of the cervix, in common with cancer of the colon, progresses through clearly defined stages: so the assay of diene conjugated and nondiene conjugated fatty acids was next applied to cervical biopsies.
The cervical specimens had, like the colon biopsies, been frozen in liquid nitrogen immediately after collection. However, the cervical biopsies were assayed within three days thus minimising the opportunity for appreciable oxidation to occur.

Some specimens of both normal and CIN tissue were of sufficient size (i.e. greater than 20mg) to be split for assay within three days of collection and reassay a week later. In all cases the %MR values on reassay were similar to those previously obtained on the corresponding specimens. Moreover, no reduction in the 18:2(9c,12c) content was apparent in the specimens of normal cervix reassayed after one week stored in liquid nitrogen.

Some tissue specimens were less friable than others and so these were homogenised until a suitable homogenate was obtained. It was suggested that the higher concentration of 18:2(9c,11t) in cancer specimens was due to the extremely friable nature of neoplastic tissue. However, this could not be the case as the concentration of 18:2(9c,12c) in the CIN tissue did not differ significantly from that in normal tissue yet the %MR in CIN was higher.

The findings of this study were in direct contrast with those of the study of colon cancer and suggested a clear trend of increasing %MR through the grades of CIN to cancer stage 1A. This trend was similar to that observed in the post mortem specimens from a subject with liver metastases. In that case the phospholipid %MR of tissue from the histologically normal tissue had a %MR of 0.85, a specimen from the interface of the normal and metastatic tissue a %MR of 4.17, and metastatic tissue a %MR of 5.07.
The %MR of biopsy specimens taken 3-5mm outside the colposcopically visible margin of the neoplastic lesion correlated significantly with the %MR in biopsies from the centre of the lesion. This finding raised the possibility that cancerous changes were occurring in a field outside the lesion. If this was indeed the case the estimation of the %MR might allow the detection of neoplastic change before it is histologically evident.

There are various reasons for the considerable overlap between the %MR values of the CIN histologically normal groups. However, this overlap may reflect individual differences in the histological diagnosis of the grades of CIN. Alternatively it could be that neoplastic changes can be detected biochemically before they are apparent histologically.

4.2.4. Initial studies with cervical epithelial cells.

These studies confirmed that the analysis of single specimens of exfoliated cells was possible. Previous studies of cervical biopsies had established that the %MR was essentially a function of the 18:2(9c,11t). It was therefore unnecessary to quantitate the amount of material in the specimens as assay of the 18:2(9c,12c) provided an internal reference. The straightforward sample preparation and automated analysis made the technique convenient to perform, so enabling up to 75 specimens to be analysed daily.

The findings of earlier work on cervical biopsies were confirmed. A clear trend of %MR increasing with severity of CIN was established. However, this may only be of academic interest as it has been suggested that histological grading of CIN is of little practical importance and that cervical tissues should only be examined for presence or absence of CIN (Brown and Brown (1986). It has been shown that CIN 1 has the potential to progress to
CIN 3. Furthermore, it was reported that 33% of women with mild dyskaryosis had CIN 3 (Campion et al. 1986).

The finding that exfoliated cells from women with HPV infection but without any evidence of CIN gave %MR values between those obtained from normal and CIN 1 cases is consistent with there being a role for HPV in the aetiology of CIN. The %MR values of cervical tissue from women with HPV infection but without any colposcopic evidence of CIN were significantly higher (p<0.01) than normal tissue but significantly lower (p<0.03) than any grade of CIN or cancer. Many reports in the past few years have implicated a major role for HPV in the aetiology of cervical cancer. It has been estimated that 80-90% of cervical carcinomas contain DNA from HPV type 16, HPV type 18, HPV type 33, or untyped HPV (Howley 1986). One study found that 16% of women with HPV infection but without CIN developed CIN 2 or CIN 3, and in one case microinvasive cancer (Evans and Monaghan 1985).

Three factors which are known to hinder the accurate interpretation of the Papanicolaou smear test: the phase of menstrual cycle at the time of specimen collection; method of contraception used; and the presence of cervical erosions had no apparent effect on the estimation of the %MR.

The first collection of cells from eight with CIN has %MR values within the calculated normal range but when retested 2-3 months later seven had abnormally high %MR values. This finding can be explained in three ways. First, the sampling of a normal area is unlikely because all specimens were collected under colposcopic vision. Second, biochemistry at the cellular level may have changed between the first and second smears. Third, and most probable is the inconsistency in the exfoliation of the CIN epithelium.
It was clearly important to extend the study to a 'normal' population (in which the incidence of CIN was believed to be 5%) so that the comparative specificity and positive predictive value of the method could be assessed.

4.2.5. Multicentre studies with cervical epithelial cells.

In contrast to the previous study the findings of the multicentre trial did not support the proposition that the %MR could be used to discriminate between normal and cancerous cells. There was, in fact, no significant difference in %MR between the colposcopically normal women and women with HPV but no CIN, and no trend of increasing %MR with severity of CIN.

The Papanicolaou smear test failed to recognise any cytological abnormalities in 9 cases of HPV infection, 24 cases of CIN 1 and one case of CIN 2. However, the presence of dyskaryosis was reported in all CIN 3 cases. These findings, therefore, confirmed previous observations that the Papanicolaou smear test is specific (Hudson 1985) but lacks the sensitivity that is desirable in a first line screening test (Dunn and Schweitzer 1981; Patterson, Peel and Joslin 1984).

It is possible that in a normal population the estimation of the %MR is not as efficient for discriminating normal from CIN as the preliminary study had suggested. However, it was difficult to interpret with any certainty the findings of a study in which a full set of data was only available on a third of the women recruited to it. The need still remained for a reliable evaluation of the usefulness of the %MR in the detection of cervical cancer.
4.2.6. Concluding studies with cervical epithelial cells.

The study findings showed the Papanicolaou smear test was very specific as no cytological abnormalities were reported in any of the colposcopically normal women. This confirmed the previously reported low false positive rate of the test (Hudson 1985). However, its false negative rate in this study was 32% and of particular concern was the false negative rate for CIN 1 of 42%. The poor sensitivity of the Papanicolaou smear test is well recognised (Bolger and Lewis 1988; Walker, Dodgson and Duncan 1986; Soutter et al. 1986) and its higher sensitivity for the severe conditions does not mitigate its poor performance for CIN 1. It has been shown that CIN 1 can progress to CIN 3 within the currently recommended five year screening interval (Campion et al. 1986).

There was no significant difference in %MR between the colposcopically normal women and women with HPV or CIN. When the CIN group was divided into CIN 1, CIN 2, and CIN 3 there was a significant difference in %MR between the normal and CIN 1 groups. However, it was unlikely that this difference was due to cancerous change as it was only confined to the minor condition. The statistical difference in %MR between the normal and the CIN 1 group was due to a cluster of specimens with particularly high %MR values (>6.00).

When compared with data from the first study the wider spread of the %MR values within the normal group in this study was immediately apparent. It was suggested that one cause of the wide range of %MR values in the normal group was the variable proportion of columnar and squamous cells in the smear specimens. The transformation zone (the interface of the squamous cells of the ectocervix and columnar cells of the endocervix) is
sharply defined in a young women. In later life the transformation zone broadens and the clear distinction between squamous and columnar cells becomes progressively blurred as the ectocervical and endocervical epithelia merge. However, the strong correlation between the %MR's of endocervical and ectocervical smear specimens of each women showed that the %MR is unaffected by the cellular composition of the smear.

Four of the nine women, who were found to have anaerobic vaginosis, had a %MR greater than 6.0. This association suggested bacteria responsible for the vaginal infection might be responsible for the high %MR, possibly by concentrating or producing the 18:2(9c,11t). Since HVS specimens were only collected during colposcopical examination if abnormal or symptomatic discharge was evident it is possible some of the women with minor vaginal infections were not identified. The findings of experiments to determine whether vaginal bacteria could generate 18:2(9c,11t) are discussed in detail later in section 4.3.3.

Successive studies have shown that women who smoke cigarettes run an increased risk of developing cervical cancer (Thomas 1973; Marshall et al. 1983; Trevathan et al. 1983). It was, therefore, surprising to find no difference in %MR between smokers and non-smokers because cigarette smoke contains a host of proven carcinogens and is a rich source of free radicals (Pryor 1983). Nicotine and cotinine are concentrated in the cervix and it has been reported that the concentration of nicotine in cervical fluid can be more than 100 times that in serum (Sasson et al. 1985). Available evidence suggests that smoking products have a direct effect on the local immunology of the cervix (Barton et al. 1988; Tay, Jenkins and Singer 1987; Tay et al. 1987).
In sharp contrast to the preliminary studies these findings do not support the proposition that %MR estimation could be of benefit in the detection of cervical cancer. This was the largest study and the only one to use colposcopy and, where indicated, colposcopically directed biopsy to assess all women. Women for this study unlike the initial studies were drawn from the same population. This might in part explain the difference between the findings of the two studies. In the initial studies the normal group were recruited from a higher socioeconomic group than the CIN group and the vaginal flora of the two groups may differ.

Two other groups have now reported on the use of the %MR in differentiating normal normal women and those with CIN (Green et al. 1988; Coleman, Taylor and Thomas 1988). Neither has been able to demonstrate a difference in %MR between women with and without CIN.

4.2.7. Peroxidisability of cervical epithelial cells

The bulk of available evidence suggests the rate of peroxidation in tumour tissue is reduced compared to normal tissue (Burton et al. 1982). ESR studies of cervical tissue suggest lipid peroxidation is reduced in cancer of the cervix (Benedetto et al. 1981). Peroxidation studies with smear specimens were performed to determine if these changes extended to the precancerous (CIN) stages of cancer of the cervix.

Initial peroxidation experiments showed that although incubation of cervical smears with hydrogen peroxide, cumene hydroperoxide (CHP), and tert-butylhydroperoxide (tBHP) all promote the production of TBA reactive material maximal response was obtained from incubation with the latter. This may be because tBHP was suited for use in suspensions of cells in an
aqueous medium. tBHP not only has greater aqueous solubility than CHP, and is, therefore, more evenly distributed in the suspension but also has greater lipid solubility than hydrogen peroxide and is, therefore, more able to penetrate cell membranes.

It was not possible to calculate the interbatch coefficient of variation because this entailed the storage of aliquots of a specimen over a period of time and it has been shown that the concentration of TBA reactive material in a specimen rises on storage.

The normal and CIN groups each comprised two distinct populations and so the clinical and specimen data was reviewed. These investigations revealed an association between blood contamination of the smear and high concentrations of TBA reactive material. Red blood cells have been shown to contain many compounds apart from lipids which can react with the TBA reagent to give chromogens with similar fluorescence characteristics to the TBA-MDA complex. When the peroxidisability test was performed on aliquots of a cell suspension of pooled cervical smears to which different amounts of red blood cells had been added, it was recognised that the greater the red blood cell contamination the higher the concentration of TBA reactive material measured. Even when red blood cell contamination was only just visible to the naked eye in the sample the effect on the concentration of TBA reactive material was still significant. When the red cell contamination was very high its relationship with the concentrations was no longer linear. This was almost certainly because the concentration of free TBA was limited. Attempts to remove the red blood cells from cervical smears failed, primarily because the procedures led to the loss of a high proportion of the epithelial cells.
There are two possible causes for the failure to demonstrate a difference in susceptibility to peroxidation between cervical cells from women with and without CIN. Either normal and precancerous cells are equally susceptible to peroxidation or the difference in peroxidisability between the two groups was not detected using this technique. However, many biochemical indices which are distinctly altered in cancer are not noticeably changed in precancer; and so it is possible that cancerous but not precancerous cervical epithelial cells may be less susceptible to peroxidation than normal cells.

There are clearly problems with this assay of peroxidisability as it stands. The true ability of cervical epithelial cells in smears cannot be assayed unless any contaminating red blood cells can be removed.
4.3. THE ORIGINS OF 18:2(9c,11t) IN HUMANS.

Further supporting evidence for 18:2(9c,11t) being the major diene conjugated non-peroxide lipid in humans has come from the work of Smith et al. (1992). These workers analysed the Diels-Alder adduct of all four geometrical isomers of 18:2(9,11) by GCMS and concluded that all available evidence points to the isomer present in humans being 18:2(9c,11t).

Though it is now accepted that the isomer is 18:2(9c,11t) there is no consensus over its origin. Throughout the duration of this project the debate over the origin of 18:2(9c,11t) present in humans has continued.

Evidence in support of a free radical origin for 18:2(9c,11t) is derived from two lines of investigation. First, concentrations of 18:2(9c,11t) higher than those found in normal subjects have been detected in blood and body fluids from subjects with diseases or conditions in which increased free radical activity has been reported: alcoholism (Fink et al. 1985, Szebeni et al. 1986), pre-eclamptic toxaemia of pregnancy (Erskine, Iversen, and Davies 1985), and paraquat poisoning (Crump et al. 1985). Second, 18:2(9c,11t) has been produced in vitro from 18:2(9c,12c) using a free radical generating system.

Critics of theory of free radical generation of 18:2(9c,11t) have pointed to the apparent stereospecificity of the reaction (Thompson and Smith 1985) and argued that 18:2(9c,11t) must surely be produced enzymatically. Moreover, it is known that Butyribrio fibrisolvens, a ruminant bacteria, produces 18:2(9c,11t) as an intermediate metabolite. However, since 18:2(9c,11t) is the major component of TDC in human blood (Cawood et al. 1984) any doubt over the free radical origin of 18:2(9c,11t), therefore, has implications for the measurement of TDC in humans.
The possible sources of the 18:2(9c,11t) in the specimens studies in this project are considered in the following pages.

4.3.1. The free radical origin of 18:2(9c,11t).

The finding that the concentration of 18:2(9c,11t) is increased in tumour tissue was unexpected, and initially seemed at odds with the generally accepted view that tumour tissue is less susceptible to lipid peroxidation than normal tissue. A low rate of peroxidation in tumour tissue is consistent with ESR studies which showed normal tissue gave a strong signal characteristic of a lipid peroxy radical but the signal from cancerous tissue was much attenuated or even absent (Benedetto et al. 1981),

One explanation for the discrepancy between these and previous studies is that although classical peroxidation may be reduced in cancer free radical activity might be proceeding through a non-peroxide route. In this scheme hydrogen abstraction produces a carbon centred radical which cannot be easily detected by an ESR spectroscopy method used to study lipid peroxyradicals. Reduction of the carbon centred radical by a protein thiol produces stable non-peroxide 18:2(9c,11t) and might be the cause of the marked decrease in protein thiol groups in cancer tissue (Nöhammer et al. 1986). If this scheme was in operation one would expect to see an increase in 18:2(9c,11t) when cancerous cervical tissue is incubated with hydrogen peroxide. Instead, however, as TBA reactivity rises the concentrations of each unsaturated fatty acid including linoleic acid and 18:2(9c,11t) falls at a rate proportional to its degree of unsaturation.
Studies comparing cervical cells from women with and without CIN could not show a difference in %MR between the two groups. However, there may be artifactual difficulties associated with the study of cervical cells possibly caused by cervical mucus and vaginal flora: in a recent ESR study comparing cervical cells from women with and without CIN could not demonstrate a difference in the ESR signal obtainable from the two groups (Wickens et al. 1989).

4.3.2. Diet as a source of 18:2(9c,11t) in humans.

An enzymatic pathway resulting in the generation of 18:2(9c,11t) is not known to exist in humans. However, bacteria in ruminant animals do have this ability. (Kepler and Tove (1967) showed that 18:2(9c,11t) was the first intermediate in the conversion of 18:2(9c,12c) to 18:0 by Butyrovibrio fibrisolvens, a commensal bacterium of the rumen. In later studies these workers identified the enzyme responsible for converting 18:2(9,12) to 18:2(9c,11t) as linoleate 12-cis, 11-trans isomerase, EC 5.2.1.5. (Kepler, Tucker and Tove 1971)

As conjugated dienes are not normally present in cattle feed the abundance of 18:2(9c,11t) in dairy produce can be attributed to enzymatic conversion by rumen bacteria. Butter and milk have been shown to have high concentrations of 18:2(9c,11t) (Parodi 1977). Hansen and Czochanska (1976) found that up to 0.7% of fat from lamb was 18:2(9c,11t). More recently Fogerty, Ford and Svoronos (1988) showed that fat in lamb, beef and butter (all derived from ruminants) are the richest sources of 18:2(9c,11t) in the human diet. Ha, Grimm and Pariza (1987) have shown that 18:2(9c,11t) and the other diene conjugated isomers of linoleic acid can be produced during the frying of ground beef.

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From the outset of the project it was clear that with 18:2(9c,11t) abundant in dairy produce, beef, and lamb a proportion of the fatty acid in humans is derived from the diet. It felt that this dietary contribution only accounted for background concentrations of 18:2(9c,11t). Studies have since suggested the effect of diet may have been underestimated.

Studies carried out on normal males (18-60) years; n=40) in the UK established a 18:2(9c,11t) concentration of 18.3 ± 5.8μmol/l, a 18:2(9,12) concentration of 933 ± 190μmol/l and a %MR of 2. ±0.6 (PhD thesis of Paul Cawood). However, a study of normal males (44-54 years; n=8) in Arizona, USA found a 18:2(9c,11t) of 11.1 ±3.8μmol/l, a 18:2(9,12) of 813 ± 94μmol/l, and a %MR of 1.36 ± 0.43 (Szebeni et al. 1986). It is possible that this discrepancy is due to differences in national diet and milk consumption in particular, although investigation of this theory require a carefully controlled study. However, if confirmed, the finding would imply that longterm changes in diet can significantly influence these measurements.

The findings of a preliminary study, soon to be published, lends support to the theory that changes in diet can affect the concentration of 18:2(9c,11t) in humans. Switching to a diet with a higher or lower fat content than normal for only three weeks was found to elicit profound changes in 18:2(9c,11t) concentrations, 18:2(9,12) concentrations and %MR values in serum (Britton et al. 1992).
4.3.3. Production of 18:2(9c,11t) by bacteria infecting the vagina.

At the start of the project it was thought that bacterial generation of 18:2(9c,11t) could only influence the content of human specimens through the diet. The finding that cervical smears from women with anaerobic vaginosis typically had high %MR values led to a re-evaluation of this belief.

Further investigations provided compelling evidence that bacterial infection of the vagina was responsible for the very high %MR's. Most of the 18:2(9c,11t) in cervical smears from women with anaerobic vaginosis was present as the free fatty acid and not esterified and so unlikely to have originated from the cervical cells themselves. More feasible was the release of the 18:2(9c,11t) by bacterial cells in the region immediately surrounding the infected area. Culture of the bacteria isolated from high vaginal swabs from women with microbiologically confirmed anaerobic vaginosis showed the colonies to contain large quantities of a fatty acid which co-chromatographed on HPLC with reference 18:2(9c,11t). Individual colonies of bacteria were taken from these mixed cultures and subcultured in an attempt to identify the species responsible for 18:2(9c,11t) production. Although co-chromatography with reference 18:2(9c,11t) strongly suggested the bacteria were producing 18:2(9c,11t) this could not be considered overwhelming evidence. However, the similarity of the GCMS pattern of the fatty acid produced by the bacteria with that of the reference 18:2(9c,11t) provides further compelling evidence.

In several instances although the primary mixed culture produced 18:2(9c,11t) this ability was not demonstrable in any of the subcultures. One explanation of this observation is that the conversion of 18:2(9,12) to 18:2(9c,11t) requires the cooperation of more than one species or strain of
bacteria. More likely, however, is that the bacteria responsible for the production of 18:2(9c,11t) was lost during the subculture stage.

Three bacterial strains, which produced 18:2(9c,11t), were identified:
1. *Lactobacillus brevis*;
2. *Propinibacterium acnes*;
3. *Coryneform* species.

It was unfortunate that, despite referring the *Coryneform* species to a national reference centre, the strain could not be identified further. *Lactobacillus brevis* was also found to produce trans-vaccenic acid, 18:1(11t), perhaps using the same mechanism as *Butyrivibrio fibrisolvens* described by Hughes and Tove (1980).

The finding that bacterial infection of the vagina can have such a profound influence on the %MR of cervical smear specimens makes it clear that the estimation of the %MR as a screening method for cervical cancer is of little practical use.
4.4. GENERAL CONCLUSIONS.

The method for the measurement of phospholipid esterified diene conjugated and non-diene conjugated fatty acids in serum was successfully adapted for the investigation of tissue and cells. The increased sensitivity enabled analysis of small tissue biopsies and individual cervical smear specimens. The automated sample injection together with the computerised calculation of results using dedicated software enabled the rapid and reliable analysis of large numbers of tissue and cell specimens.

A study of biopsies from the cervix uteri suggested a significant difference in 18:2(9c,11t) concentration and %MR between tissue from women with and without CIN although some overlap between the groups was evident. Initial studies of cervical smear specimens showed a similar difference in %MR. However, in a larger and carefully controlled subsequent study there was no significant difference in %MR between women with and without CIN. The most likely explanation of the contrast between these studies of cervical smears is that the initial study compared women referred to a colposcopy clinic with women from a different population attending a general gynaecology clinic. Findings of the larger study are in agreement with two other independent studies investigating the use of %MR determination for the diagnosis of CIN. It was, therefore, concluded that the %MR can not be used in the screening for cervical precancer.

Further investigations showed that production of 18:2(9c,11t) by commensal bacteria in the vagina could greatly increase the %MR of cervical smear specimens and the %MR cannot be used as a marker of free radical activity in these specimens.
4.5. FURTHER STUDIES.

The findings of this series of studies do not support the use of 18:2(9c,11t) or the %MR as a specific marker for free radical damage to lipids in humans unless the proportion derived from other sources, principally diet and bacterial infection, can be quantitated and subtracted from the whole. It is also clear that the assay of 18:2(9c,11t) and %MR as it stands cannot assist the detection of cervical cancer.

In order to assess the contribution of 18:2(9c,11t) produced by commensal bacteria to the 18:2(9c,11t) content of human blood and tissues it is important to establish whether intestinal bacteria can carry out the conversion. It would also be useful to determine whether 18:2(9c,11t) produced by bacteria in the vagina can be absorbed into the body and influence the serum concentration of 18:2(9c,11t) and %MR. In the light of the recent work by Britton et al. (1992) it is clear that diet accounts for a greater proportion of serum 18:2(9c,11t) than previously thought. A more extensive study of the relationship between diet and serum and tissue 18:2(9c,11t) is indicated.

If some of the 18:2(9c,11t) in human blood and tissues does arise as a result of free radical attack of linoleic acid it still remains to be established exactly how a free radical reaction can produce a stereospecific product. Theories suggesting that 18:2(9c,11t) arises from a radical reaction within the constraints of an organised membrane structure between phospholipid esterified linoleic acid and a protein thiol group still require experimental confirmation.

The physiological role of 18:2(9c,11t) and the other three diene conjugated isomers of linoleic acid, however is worthy of further investigation; in particular the reported anticarcinogenic activity of diene conjugated isomers
of linoleic acid (Ha, Grimm, and Pariza 1987; Pariza 1987). These isomers are produced during the frying of ground beef and can inhibit tumour formation. Evidence to date suggests that they act at least in part, by inhibiting the cytochrome P₄₅₀ enzyme system, which is responsible for the activation of many carcinogens.

It must, therefore, be concluded that changes in 18:2(9c,11t) concentration and %MR in man are not attributable to free radical activity alone. This has wider implications for free radical research because 18:2(9c,11t) is the principal diene conjugated component in man. Total diene conjugation should not, therefore, be considered to be a simple function of free radical damage to lipids.
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SUPPLEMENTARY REFERENCES


The following publications resulted from work presented in this thesis.

Griffin JFA, Wickens DG, Tay SK, Singer A and Dormandy TL. (1987)
Recognition of cervical neoplasia by the estimation of a free-radical reaction product (octadeca-9,11-dienoic acid) in biopsy material.

Tay SK, Singer A, Griffin JFA, Wickens DG and Dormandy TL. (1987)
Recognition of cervical neoplasia by the estimation of a free-radical reaction product (octadeca-9,11-dienoic acid in exfoliated cells.
Clin Chim Acta 163: 149-152.

Diene conjugation in cervical precancer.
In: Free Radicals, Oxidant stress and drug action. (ed: Rice-Evans C.)

Singer A, Tay SK, Griffin JFA, Wickens DG and Dormandy TL. (1987)
Diagnosis of cervical neoplasia by the estimation of octadeca-9,11-dienoic acid.
Lancet 1: 537-539.

Octadeca-9,11-dienoic acid in the diagnosis of cervical intraepithelial neoplasia.
Lancet 2: 329 (letter)

Octadeca-9,11-dienoic acid in cervical intraepithelial neoplasia: a colposcopic study.
APPENDIX 1.

The development of the computer program to calculate the results of HPLC analysis was a significant achievement during this project. The use of the program in conjunction with the autoinjector allowed fully automated analysis, which freed the analyst to prepare more specimens or examine results. A brief explanation of the different sections of the program is given below.

Lines 25-490 Display on the monitor an introduction to the program and prompt for input of the name of experiment, the samples of which, are to be analysed.

25 DIM T(5,4)
35 GOSUB 7300
40 PRINT TAB(5,5)"A PROGRAM TO CALCULATE THE SAMPLE ";
45 PRINT "CONCENTRATIONS OF"
50 PRINT TAB(6,5)"20:4, 18:2, 18:2(9C,11T) & 18:1; MOLAR ";
55 PRINT "RATIOS 18:1 / 20:4"
60 PRINT TAB(7,5)"& 18:2(9C,11T) / 18:2; AND EXTRACTION ";
65 PRINT "EFFICIENCY."
70 PRINT TAB(9,5)"THE PROGRAMME WAS DEVELOPED BY :-"
75 PRINT TAB(11,8)"JOHN GRIFFIN"
80 PRINT TAB(12,8)"DEPARTMENT OF CHEMICAL PATHOLOGY,"
85 PRINT TAB(13,8)"WHITTINGTON HOSPITAL,"
90 PRINT TAB(14,8)"LONDON N19 5NF. TEL. 01-272 3070 EXT4798"
95 PRINT TAB(18,15)"PRESS RETURN TO CONTINUE";
100 INPUT T$
300 GOSUB 7300
320 PRINT TAB(6,5)"* CALIBRATION INFORMATION *"
340 PRINT TAB(8,5)"SYSTEM CALIBRATION, WHICH REQUIRES THE ";
350 PRINT "INJECTION ".
360 PRINT TAB(9,5)"OF EXT STD FOLLOWED BY INT STD, IS ";
370 PRINT "CARRIED OUT";
380 PRINT TAB(10,5)"AT THE START OF A RUN AND AT INTERVALS ";
390 PRINT "DURING IT."
400 PRINT TAB(12,5)"PLEASE NOTE THAT THE 18:2(9T,11T) ISOMER";
410 PRINT ", INT STD,"
420 PRINT TAB(13,5)"IS ALSO USED IN THE EXT STD BECAUSE ";
430 PRINT "18:2(9C,11T),"
440 PRINT TAB(14,5)"THE BIOLOGICAL ISOMER IS NOT ";
450 PRINT "COMMERCIALY AVAILABLE."
480 PRINT TAB(16,5)"ENTER RUN ID (UP TO 32 CHARACTERS)"
490 INPUT T$
Prompt input of calibration data including the concentration of the fatty acids in the external standard, which fatty acids are present in the external standard and details of the sample preparation.
Lines 2300-2900 Permit input of any further dilution factors required and selection of the format required for the calculation of the fatty acid concentrations.

```
2300 REM * CONSTANTS FOR USE IN CONC N CALCS *
2310 REM *********************************************
2320 GOSUB 7300
2330 PRINT TAB(5,1)"THE CONCENTRATION OF EACH FATTY ACID MAY ";
2340 PRINT "MULTIPLIED BY A PRESET CONSTANT."
2350 PRINT TAB(6,19)"IF NO FACTOR REQUIRED ENTER 1."
2360 PRINT TAB(8,10)"ENTER CONSTANT FOR 18:2(9,11) CONC. :-" 
2370 PRINT TAB(10,10)"ENTER CONSTANT FOR 18:2(9,12) CONC. :-"
2380 IF C<(1,1)"Y" THEN 2450 
2390 PRINT TAB(12,10)"ENTER CONSTANT FOR 20:4 CONC. :-"
2400 PRINT TAB(14,10)"ENTER CONSTANT FOR 18:1 CONC. :-" 
2410 PRINT TAB(8,50)"
2420 INPUT K(1)
2430 PRINT TAB(10,50)"
2440 INPUT K(2)
2450 IF C$(1,1)"Y" THEN 2450 
2460 PRINT TAB(12,10)"
2470 INPUT K(4)
2480 PRINT TAB(14,50)"
2490 PRINT TAB(17,1)"SELECT FORMAT FOR 18:2(9,11) AND ";
2500 PRINT "18:2(9,12) CALCULATIONS :- " 
2510 PRINT TAB(19,1)"1 CONCENTRATIONS"TAB(19,30)"2 MOLES ";
2520 PRINT "PER SAMPLE "K(3)
2530 IF K(3)=1 THEN K$="UMOL/L"
2540 IF K(3)=2 THEN K$="NMOL/SAMP"
2550 GOSUB 9400
2560 K(1)=K(1)*W(2)*100,K(2)=K(2)*W(1)*10 
2570 W(6)=W(3)*W(4)/W(5),W(7)=W(3)*W(4)/1000 
2580 IF THEN 2877 
2590 K(4)=K(4)*V(1)*10,K(5)=K(5)*V(2)*10 
2600 PRINT #P,K(1)" "K(2)" "K(4)" "K(5)
2610 X=VAL(S<(1,4)"-1 
2620 C(1)=C(1)+2,S(2)=1 
2630 IF $<1,1)="Y" THEN 9000 
2640 GOTO 4000
```
Print out the peak heights of the fatty acids in the external and internal standards after analysis. Response factors are calculated following the analysis of external or internal standards.

```
3000 REM * CALIBRATION *
3010 REM *******************
3015 FOR Y=1 TO 10
3020 NEXT Y
3025 IF C(2)=1 THEN I$="EXT STD"
3030 IF C(2)=2 THEN I$="INT STD"
3032 IF C(2)=3 THEN I$="EXT MULTISTD"
3035 PRINT #P,"SAMPLE "S(1)" "I$
3037 PRINT #K,"SWOP"
3040 PRINT #P,"SAMPLE "S(1)" "I$
3042 PRINT #K,"SWOP"
3045 PRINT #P,"18:2(9,11) "H(4)"
3050 PRINT #P,TAB(32)"18:2(9,12) "H(2)"
3055 PRINT #P,TAB(63)"INT STD "H(6)
3056 IF C$="Y" THEN 3060
3057 PRINT #P,"20:4 "I(2);
3058 PRINT #P,TAB(32)"18:1 "I(4)
3060 IF C(2)=0 THEN 3200
3067 IF C(2)=2 THEN 3102
3068 Q(1)=K(1)/H(6),Q(2)=K(2)/H(2)
3069 IF C$(1,1)<="Y" THEN 3100
3070 IF C(2)=0 THEN 3200
3070 IF C(2)=2 THEN 3102
3078 Q(4)=K(4)/I(2),Q(5)=K(5)/I(4)
3079 PRINT #P,"CALIBRATION 1 ACCEPTED"
3082 C(2)=0
3085 IF W(4)<0 THEN C(2)=2
3100 RETURN
3102 REM *CALCS FOR CALIBRATION 2*
3105 IF K(3)=2 THEN 3125
3110 R(1)=Q(1)*W(6)*H(6),R(2)=Q(2)*W(6)*H(6)
3115 IF C$(1,1)<="Y" THEN 3140
3120 R(4)=Q(4)*W(6)*H(6),R(5)=Q(5)*W(6)*H(6)
3122 GOTO 3140
3125 R(1)=Q(1)*W(7)*H(6),R(2)=Q(2)*W(7)*H(6)
3130 IF C$(1,1)<="Y" THEN 3140
3135 R(4)=Q(4)*W(7)*H(6),R(5)=Q(5)*W(7)*H(6)
3140 R(3)=10000/(W(6)*H(6))
3145 PRINT #P,"CALIBRATION 2 ACCEPTED:=";
3150 PRINT #P,TAB(30)"18:2(9,11) RESPONSE FACTOR = "R(1)
3160 PRINT #P,TAB(30)"18:2(9,12) RESPONSE FACTOR = "R(2)
3162 IF C$(1,1)<="Y" THEN 3170
3165 PRINT #P,TAB(30)"20:4 RESPONSE FACTOR = "R(4)
3166 PRINT #K,TAB(30)"18:1 RESPONSE FACTOR = "R(5)
3170 PRINT #P,TAB(30)"RECOVERY CONSTANT = "R(3)
3185 C(2)=0
3190 RETURN
```
Print out the peak heights of fatty acids in the samples. The concentrations of the fatty acids, the %MR and percentage recovery are printed.

```
3200 REM * SAMPLE DETERMINATION *
3210 REM ****************************************
3300 REM * SAMPLE CALC *
3310 REM ****************************************
3320 IF W(4)=0 THEN H(6)=0
3325 IF H(6)=0 THEN F(2)=F(3)
3330 IF F(4)<0 THEN F(2)=F(4)
3340 F(2)=F(2)/100
3350 IF C$="Y" THEN 3450
3360 N(1)=0,N(2)=0,N(3)=0,N(4)=0,M(3)=0,M(4)=0
3370 IF F(2)<0 THEN 3400
3380 N(1)=R(4)*I(2)/H(6),N(3)=R(5)*I(4)/H(6)
3390 GOTO 3410
3400 N(2)=INT(N(1)),N(4)=INT(N(3))
3415 IF H(1)-N(2)<0.5 THEN 3425
3420 H(2)=H(2)+1
3425 IF N(3)-N(4)<0.5 THEN 3432
3430 N(4)=INT(N(3))
3435 N(1)=INT(N(1))
3440 N(2)=INT(N(2))
3445 IF N(1)-N(2)<0.5 THEN 3450
3450 M(4)=M(4)+1
3455 L(1)=0,L(2)=0,L(3)=0,L(4)=0,L(5)=0,L(6)=0,M(1)=0,M(2)=0
3460 IF F(2)>0 THEN 3430
3470 L(1)=R(1)*H(4)/H(6),L(3)=R(2)*H(2)/H(6)
3475 GOTO 3485
3480 L(1)=R(1)*H(4)/F(2),L(3)=R(2)*H(2)/F(2),L(5)=F(2)*10000
3485 L(2)=INT(L(1)),L(4)=INT(L(3))
3490 IF L(1)-L(2)<0.5 THEN 3510
3500 L(2)=L(2)+1
3510 IF L(3)-L(4)<0.5 THEN 3530
3520 L(4)=L(4)+1
3530 IF F(2)>0 THEN 3550
3540 L(5)=H(6)*R(3)
3550 L(6)=INT(L(5))
3560 IF L(5)-L(6)<0.5 THEN 3590
3570 L(6)=L(6)+1
3590 IF H(2)<0 THEN 3640
3600 M(1)=(Q(1)*H(4)*1000)/(Q(2)*H(2))
3610 M(2)=INT(M(1))
3620 IF M(1)-M(2)<0.5 THEN 3640
3630 M(2)=M(2)+1
3640 L(2)=L(2)/100,L(4)=L(4)/10,L(6)=L(6)/100,M(2)=M(2)/100
3650 H(2)=N(2)/10,N(4)=N(4)/10,M(4)=M(4)/100
3700 REM * PRINT OUT RESULTS *
3710 PRINT #p,";18:2(9,11) = "L(2)" "K$;
3715 PRINT #p,Tab(32)"9.11/9.12 %MR = "M(2)
3720 PRINT #p,";18:2(9,12) = "L(4)" "K$;
3730 IF C$="Y" THEN 3760
3735 PRINT #p,
```

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Lines 4000-4770  Determine whether an external standard, internal standard or sample is to be injected and prompt for its injection.

4000 REM * SYSTEM RUN - CHECK CALCULATION CALIB OR SAMPLE *
4010 REM **********************************************
4030 GOSUB 7300
4050 S(1)=VAL(S%(1,4))-X
4065 IF C(2)=2 THEN 4450
4090 IF S(1)<S(2) THEN 4300
4100 C(2)=1
4110 IF C$(1,1)="Y" THEN C(2)=3
4300 REM * WAITING FOR INJECTION *
4310 REM **************************
4315 U=0
4320 IF C(2)>0 THEN 4410
4330 IF C(4)=1 THEN 4380
4340 PRINT TAB(9,1)"(SPECIAL FUNCTION CODE = SF$)"
4350 PRINT TAB(7,1)"ENTER NAME / ID NO. OF SAMPLE TO BE ";
4360 PRINT "INJECTED :-";
4370 INPUT I$
4375 IF I$="THEN 4350
4380 IF C(4)=1 THEN I$=""
4383 IF I$(1,3)="SF$"THEN GOSUB 8000
4384 IF I$(1,1)="#"THEN 4490
4389 IF U>0 THEN 4300
4390 PRINT TAB(15,1)"INJECT "I$" WHEN READY"
4400 GOTO 4460
4410 IF C(2)=2 THEN 4450
4415 IF C(2)=0 THEN 4460
4420 PRINT TAB(7,15)"INJECT EXTERNAL STANDARD WHEN READY"
4440 GOTO 4460
4450 PRINT TAB(7,15)"INJECT INTERNAL STANDARD WHEN READY"
4460 PRINT #K,CHR$(27)
4465 IF S(1)=1 THEN 4490
4470 IF S%(15,19)="" THEN 4470
4480 IF S%(15,19)<"" THEN 4480
4490 GOSUB 7300
4500 PRINT TAB(5,18)"SAMPLE NO. :-"S(1)
4510 IF C(2)=1 THEN 4550
4520 IF C(2)=2 THEN 4570
4525 IF C(2)=3 THEN 4552

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Lines 5000-5040  These five lines of programming are an important feature of the program: enabling the program to update the time windows as the retention times fluctuate with temperature.

5000 REM * SETTING TIME WINDOWS FOR PEAKS *
5010 FOR Y=1 TO 5
5020 T(Y,2)=T(Y,1)-(T(Y,1)*F(1)/100)
5030 T(Y,3)=T(Y,1)+(T(Y,1)*F(1)/100)
5040 NEXT Y
Extract from the 'Data Files' the peak heights of the fatty acids detected within the preset time windows for use in the calculation of the fatty acid concentrations, the %MR and percentage recovery.
Lines 6000-6500 Prompt for input of the retention times of the fatty acids of interest to enable time windows for detecting those fatty acids to be set.

6000 REM * INPUT RETENTION TIMES *
6010 REM ******************************
6050 GOSUB 7300
6060 PRINT "RETENTION TIMES"
6070 PRINT TAB(6,1)"ENTER RET.TIME 18:2(9,11)"TAB(54)"(7.5)"
6080 PRINT TAB(8,1)"ENTER RET.TIME INT STD"TAB(54)"(8.3)"
6090 IF C$(1,1)<"Y" THEN 6100
6100 PRINT TAB(12,1)"ENTER RET.TIME 20:4"TAB(54)"(6.0)"
6110 PRINT TAB(14,1)"ENTER RET.TIME 18:1"TAB(54)"(11.0)"
6120 PRINT TAB(16,1)"ENTER TOLERANCE FACTOR (%)"TAB(54)"(4 )"
6130 PRINT TAB(18,1)"VALUES IN BRACKETS USED AT WHITTINGTON ";
6140 PRINT "HOSPITAL."
6150 PRINT TAB(6,40)"";
6160 INPUT T(4,1)
6170 PRINT TAB(8,40)"";
6180 INPUT T(5,1)
6190 PRINT TAB(10,40)"";
6200 INPUT T(2,1)
6210 PRINT TAB(12,40)"";
6220 INPUT T(1,1)
6230 PRINT TAB(14,40)"";
6240 INPUT T(3,1)
6250 PRINT TAB(16,40)"";
6260 INPUT F(1)
6400 IF S(1)=1 THEN RETURN
PUBLICATIONS:

The following publications resulted from work presented in this thesis.

Griffin JFA, Wickens DG, Tay SK, Singer A and Dormandy TL. (1987)
Recognition of cervical neoplasia by the estimation of a free-radical reaction product (octadeca-9,11-dienoic acid) in biopsy material.

Tay SK, Singer A, Griffin JFA, Wickens DG and Dormandy TL. (1987)
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Diene conjugation in cervical precancer.
In: Free Radicals, Oxidant stress and drug action. (ed: Rice-Evans C.)

Singer A, Tay SK, Griffin JFA, Wickens DG and Dormandy TL. (1987)
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Lancet 1: 537-539.

Octadeca-9,11-dienoic acid in the diagnosis of cervical intraepithelial neoplasia.
Lancet 2: 329 (letter)

Octadeca-9,11-dienoic acid in cervical intraepithelial neoplasia: a colposcopic study.
Control the selection of special functions (lines 8200-9300) which are available when injections are made manually.

lines 8000-9300

8000 REM * SPECIAL FUNCTIONS **************************
8010 GOSUB 7300
8020 PRINT TAB(4,1)"SPECIAL FUNCTIONS"
8030 PRINT "SAMPLE NO./ID SELECTED CODES FOR SPECIAL FUNCTION"
8040 PRINT "IT CANNOT BE USED TO NAME A SAMPLE "
8050 PRINT TAB(10,1)"1 IMMEDIATE CALIBRATION"
8060 PRINT TAB(12,1)"2 RECALCULATION OF RESULTS FOR "
8070 PRINT "PREVIOUS SAMPLE "
8080 PRINT TAB(13,1)"3 RESET PROGRAMMED RETENTION TIMES"
8090 PRINT TAB(16,1)"4 EXIT SPECIAL FUNCTIONS"
8110 PRINT TAB(18,1)"ENTER CHOICE THEN PRESS RETURN"
8120 INPUT U
8130 IF U<1 THEN 8000
8135 IF U>4 THEN 8000
8140 IF U=2 THEN 8600
8150 IF U=3 THEN 8800
8180 IF U=4 THEN RETURN

lines 8200-8300

Allow for immediate calibration of the system regardless of how many samples have still to be injected before the next scheduled calibration sequence.

8200 REM * IMMEDIATE CALIBRATION *
8210 GOSUB 7300
8220 PRINT TAB(6,1)"IMMEDIATE CALIBRATION NOW REQUIRED"
8230 PRINT TAB(8,1)"AFTER CALIBRATION HOW MANY SAMPLES BEFORE"
8235 PRINT "RECALIBRATION"
8240 PRINT TAB(10,1)"IF YOU ARE NOT SURE OVERESTIMATE AND "
8250 PRINT "REQUEST CALIBRATION"
8260 PRINT "WHEN REQUIRED USING SPECIAL FUNCTIONS"
8270 PRINT TAB(8,58)"
8280 INPUT C(1)
8290 C(1)=C(1)+2,C(2)=1,S(2)=S(1)
8292 IF W(4)=0 THEN C(1)=C(1)-1
8295 IF C*(1,1)="Y" THEN C(2)=3
8300 GOSUB 7300
8310 GOTO 4420
8500 END
Allow the recalculation of the results of a previous sample by manual input of fatty acid peak heights.

8600 REM * RECALCULATION OF PREVIOUS SAMPLE *
8610 GOSUB 7300
8620 S(1)=S(1)-1
8630 PRINT TAB(8,1)"ENTER PH 18:2(9,11) :- ";
8640 INPUT H(4)
8650 PRINT TAB(10,1)"ENTER PH 18:2(9,12) :- ";
8660 INPUT H(2)
8665 IF W(4)=0 THEN 8690
8670 PRINT TAB(12,1)"ENTER PH INT STD :- ";
8675 INPUT H(6)
8680 IF H(6)<90 THEN 8700
8690 PRINT TAB(12,1)"ENTER RECOVERY TO BE ASSUMED (%) :- ";
8695 INPUT F(4)
8700 IF C$<>"y" THEN 8745
8725 PRINT TAB(14,1)"ENTER PH 20:4 :- ";
8730 INPUT I(2)
8735 PRINT TAB(16,1)"ENTER PH 18:1 :- ";
8740 INPUT I(4)
8745 PRINT TAB(18,1)"ENTER SAMP ID OF PREVIOUS SAMPLE ";
8750 INPUT U$
8770 I$=U$
8775 S(1)=S(1)+1
8780 GOSUB 7300
8785 GOSUB 3000
8790 GOTO 8000

Allow the resetting of the set retentions times.

8800 REM * RESETTING RTS *
8810 GOSUB 6000
8820 GOTO 8000

Enable this calculation program to be run (started) after injection of the external and internal standards.

9000 REM *** CALIBRATION AFTER STD(S) RUN ***
9005 GOSUB 7300
9010 PRINT "CALIBRATION AFTER STD(S) HAVE RUN ";
9020 S(1)=1,C(2)=1
9025 IF C$(1,1)="y" THEN C(2)=3
9030 GOSUB 6000
9035 GOSUB 6900
9040 GOSUB 6500
9110 GOSUB 7300
9120 PRINT TAB(8,1)"ENTER PH 18:2(9,12) IN EXT STD ";
9130 INPUT H(2)
9140 PRINT TAB(10,1)"ENTER PH INT STD IN EXT STD ";
9150 INPUT H(6)
9155 IF C$(1,1)<>"y" THEN 9190
9160 PRINT TAB(12,1)"ENTER PH OF 20:4 IN EXT MULTISTD ";
9165 INPUT I(2)
9170 PRINT TAB(14,1)"ENTER PH OF 18:1 IN EXT MULTISTD ";
9175 INPUT I(4)
9190 GOSUB 3000
9195 IF W(4)=0 THEN 9230
9200 GOSUB 7300
9210 PRINT TAB(0,1)"ENTER PH INT STD ON DIRECT INJECTION ";
9220 INPUT H(6)
9230 S(1)=2,S(2)=S(2)+C(1),H(2)=0,I(2)=0,I(4)=0,X=X-2
9235 IF W(4)>0 THEN 9280
9240 GOSUB 3000
9245 GOTO 4000
9280 C(2)=0
9300 GOTO 4000

Lines 9400-9900  Print out all run settings after input of the data, such as
the concentrations of the standards, the frequency of
calibration, any dilution factors used during sample
preparation, whether manual or automatic injection is

9400 REM * RUN SETTINGS PRINT OUT *
9410 REM ****************************************
9450 FOR Y=1 TO 2
9460 PRINT #P,CHR$(27)"1"
9470 PRINT #P,TAB(3)T$
9475 PRINT #P,TAB(3)"DATE: "&D(4,6)&D(1,3)&D(7,8)" TIME: ":T%1
9480 PRINT #P
9490 PRINT #P
9495 PRINT #P,CHR$(27)"0"
9500 PRINT #K,"SWOP"CHR$(27)
9530 NEXT Y
9560 PRINT #P,TAB(10)"RUN SETTINGS"
9565 PRINT #P
9570 IF C(4)=1 THEN 9600
9580 PRINT #P,"MANUAL INJECTIONS"
9590 GOTO 9600
9600 PRINT #P, "INJECTIONS BY AUTO SAMPLER"
9605 PRINT #P,"RECALIBRATION AFTER EVERY "C(1)" SAMPLES"
9610 PRINT #P,"CONCN 18:2(9,11) = "W(2)" UMOL/L"
9620 PRINT #P,"CONCN 18:2(9,12) = "W(1)" UMOL/L"
9622 IF C$(1,1)<"Y" THEN 9630
9624 PRINT #P,"CONCN 20:4 = "V(1)" UMOL/L"
9626 PRINT #P,"CONCN 18:1 = "V(2)" UMOL/L"
9630 IF W(3)>1 THEN 9660
9640 PRINT #P,"INTERNAL STD = WORKING CONCN"
9650 GOTO 9670
9660 PRINT #P,"INTERNAL STD = WORKING CONCN/W(3)
9670 PRINT #P,"VOL. INT STD ADDED = "W(4)" UL"
9680 PRINT #P,"RESUSPENSION VOL. = "W(5)" UL"
9690 PRINT #P,"18:2(9,11) CONCN ADDITIONAL CONSTANT = "K(1)
9700 PRINT #P,"18:2(9,12) CONCN ADDITIONAL CONSTANT = "K(2)
9702 IF C$(1,1)<"Y" THEN 9708
9704 PRINT #P,"20:4 CONCN ADDITIONAL CONSTANT = "K(4)
9706 PRINT #P,"18:1 CONCN ADDITIONAL CONSTANT = "K(5)
9708 PRINT #P,"BACKUP RECOVERY FACTOR "F(3)" %"
9710 IF K(3)=2 THEN 9740
9720 PRINT #P,"RESULTS AS UMOL/L"
9730 GOTO 9750
9740 PRINT #P,"RESULTS AS NMOL/SAMPLE"
9750 PRINT #P
9800 RETURN
9900 CLEAR
9901 PRINT #K,"TIME09:10:55"CHR$(13)
9902 END

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A typical sequence of print-outs during an analytical run.

Print-out of a list of the settings for the analytical run.

**RUN SETTINGS**

**MANUAL INJECTIONS**

**RECALIBRATION AFTER EVERY 12 SAMPLES**

**CONCN 18:2(9,11) = 4.167 UMOL/L**

**CONCN 18:2(9,12) = 166.7 UMOL/L**

**CONCN 20:4 = 41 UMOL/L**

**CONCN 18:1 = 500 UMOL/L**

**INTERNAL STD = WORKING CONCN**

**VOL. INT STD ADDED = 100 UL**

**RESUSPENSION VOL. = 100 UL**

**18:2(9,11) CONCN ADDITIONAL CONSTANT = 1**

**18:2(9,12) CONCN ADDITIONAL CONSTANT = 1**

**20:4 CONCN ADDITIONAL CONSTANT = 1**

**18:1 CONCN ADDITIONAL CONSTANT = 1**

**BACKUP RECOVERY FACTOR 80 %**

**RESULTS AS UMOL/L**

Print-out after analysis of the external standard: The programmed retention times of the peaks of interest and heights of those peaks.

**RETENTION TIME OF 18:2(9,11) = 6.44**

**RETENTION TIME OF INT STD = 7.44**

**RETENTION TIME OF 20:4 = 5.41**

**RETENTION TIME OF 18:2(9,12) = 6.35**

**RETENTION TIME OF 18:1 = 9.7**

**TOLERANCE FACTOR (%) = 4**

**SAMPLE 1 EXT MULTISTD**

**18:2(9,11) 0  18:2(9,12) 3974  INT STD 3770**

**20:4 2670  18:1 2168**

**CALIBRATION 1 ACCEPTED**

Print-out after analysis of the internal standard: The peak height of the internal standard and calculated response factor for each measurement.

**SAMPLE 2 INT STD**

**18:2(9,11) 0  18:2(9,12) 0  INT STD 7879**

**20:4 0  18:1 0**

**CALIBRATION 2 ACCEPTED:-**

**18:2(9,11) RESPONSE FACTOR = 1741.73967**

**18:2(9,12) RESPONSE FACTOR = 6610.11222**

**20:4 RESPONSE FACTOR = 2419.76778**

**18:1 RESPONSE FACTOR = 36342.2503**

**RECOVERY CONSTANT = 1.26919659**

Print-out after analysis of a specimen: The specimen name, height of the peaks of interest, fatty acid concentrations, %MR and percentage recovery (fatty acid extraction efficiency).

**SAMPLE 4 #CONTROL 1**

**18:2(9,11) 5473  18:2(9,12) 5948  INT STD 6785**

**20:4 6938  18:1 715**

**18:2(9,11) = 14.05 UMOL/L**

**18:2(9,12) = 579.5 UMOL/L**

**20:4 = 247.4 UMOL/L**

**18:1 = 383 UMOL/L**

**RECOVERY (%) = 86.11**

280
The data resulting from the study of *post mortem* tissues was too great for inclusion in section 3.2.1. and so the full results of these studies is given below.

Subject 1.
Male; aged 78 years. Examination carried out 17 hours *post mortem*.
Cause of death:
1. Disseminated tumour in lymph nodes, liver, adrenals (bilateral) and spleen. Other tissues normal.
2. Pulmonary congestion and oedema.

Tissues obtained: Brain Heart Lung Liver Kidney Spleen Adrenal

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lipid type</th>
<th>18:2(9c,11t) nmol/g</th>
<th>18:2(9c,12c) nmol/g</th>
<th>%MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td>43.9</td>
<td>3176</td>
<td>1.38</td>
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<tr>
<td>Adrenal</td>
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<td>10.4</td>
<td>567</td>
<td>1.83</td>
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<tr>
<td>Heart</td>
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<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Lipid type</td>
<td>HEART</td>
<td>LUNG</td>
<td>LIVER</td>
<td>KIDNEY</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------------</td>
<td>--------------------------------</td>
<td>--------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>18:2(9c,11t)</td>
<td>18:2(9c,12c)</td>
<td>18:2(9c,11t)</td>
<td>18:2(9c,12c)</td>
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<td>nmol/g</td>
<td>nmol/g</td>
<td>nmol/g</td>
<td>nmol/g</td>
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<tr>
<td>Free fatty acids</td>
<td>36.2</td>
<td>4110</td>
<td>14.2</td>
<td>1132</td>
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<tr>
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<td>18.4</td>
<td>3024</td>
<td>11.4</td>
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<tr>
<td>Triglycerides</td>
<td>32.2</td>
<td>2363</td>
<td>13.5</td>
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<table>
<thead>
<tr>
<th>Lipid type</th>
<th>HEART</th>
<th>LUNG</th>
<th>LIVER</th>
<th>KIDNEY</th>
</tr>
</thead>
<tbody>
<tr>
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<td>18:2(9c,12c)</td>
<td>18:2(9c,11t)</td>
<td>18:2(9c,12c)</td>
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<tr>
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<td>nmol/g</td>
<td>nmol/g</td>
<td>nmol/g</td>
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<td>Triglycerides</td>
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<table>
<thead>
<tr>
<th>%MR</th>
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</thead>
<tbody>
<tr>
<td>HEART</td>
<td>0.88</td>
<td>0.61</td>
<td>1.36</td>
<td>1.25</td>
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<tr>
<td>LUNG</td>
<td>1.25</td>
<td>1.12</td>
<td>0.96</td>
<td>0.94</td>
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<tr>
<td>LIVER</td>
<td>0.94</td>
<td>2.40</td>
<td>3.05</td>
<td>1.13</td>
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<tr>
<td>KIDNEY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ADRENAL LYMPHOMA

Lipid type | 18:2(9c,11t) nmol/g | 18:2(9c,12c) nmol/g | %MR
--- | --- | --- | ---
Free fatty acids | 41.2 | 3804 | 1.08
Phospholipids | 3.9 | 356 | 1.09
Triglycerides | 162.9 | 7390 | 2.20

Subject 2.
Male; aged 55. Examination carried out 10 hours post mortem.
Cause of death:
1. Ruptured abdominal atherosclerotic aneurysm.

Tissues studied:
Brain, Kidney,
Lung, Spleen,
Liver

BRAIN

Lipid type | 18:2(9c,11t) nmol/g | 18:2(9c,12c) nmol/g | %MR
--- | --- | --- | ---
Free fatty acids | Not detected | Not detected | Not detected
Phospholipids | 32.6 | 2742 | 1.19
Triglycerides | Not detected | Not detected | Not detected

LUNG

Lipid type | 18:2(9c,11t) nmol/g | 18:2(9c,12c) nmol/g | %MR
--- | --- | --- | ---
Free fatty acids | 13.3 | 184 | 7.22
Phospholipids | 17.7 | 1056 | 1.68
Triglycerides | 45.7 | 2851 | 1.60
<table>
<thead>
<tr>
<th>Lipid Type</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>%MR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LIVER</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>59.9</td>
<td>2584</td>
<td>2.32</td>
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<tr>
<td>Phospholipids</td>
<td>105.7</td>
<td>6065</td>
<td>1.74</td>
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<td>Triglycerides</td>
<td>147.6</td>
<td>5759</td>
<td>2.56</td>
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<table>
<thead>
<tr>
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<th>18:2(9c,12c)</th>
<th>%MR</th>
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<tbody>
<tr>
<td><strong>KIDNEY</strong></td>
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<tr>
<td>Free fatty acids</td>
<td>50.3</td>
<td>2652</td>
<td>1.90</td>
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<tr>
<td>Phospholipids</td>
<td>16.2</td>
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<td>1.32</td>
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<td>Triglycerides</td>
<td>17.5</td>
<td>1472</td>
<td>1.19</td>
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<table>
<thead>
<tr>
<th>Lipid Type</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>%MR</th>
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</thead>
<tbody>
<tr>
<td><strong>SPLNE</strong></td>
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<tr>
<td>Free fatty acids</td>
<td>71.5</td>
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<td>10.4</td>
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<td>4.16.4</td>
<td>15531</td>
<td>2.68</td>
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</table>
Subject 3.
Male; aged 81. Examination carried out 82 hours post mortem.

Cause of death:
1. Acute heart failure.
2. Coronary artery thrombosis and atherosclerosis
3. Chronic renal failure and renal ischaemia.

Tissues studied: Brain Liver
Heart Kidney
Lung Spleen

<table>
<thead>
<tr>
<th>BRAIN</th>
<th>Lipid type</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>%MR</th>
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<tbody>
<tr>
<td></td>
<td>nmol/g</td>
<td>nmol/g</td>
<td></td>
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</tr>
<tr>
<td>Free fatty acids</td>
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<td>51.8</td>
<td>802</td>
<td>6.46</td>
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<tr>
<td>Triglycerides</td>
<td>17.3</td>
<td>417</td>
<td>4.15</td>
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<table>
<thead>
<tr>
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<th>Lipid type</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>%MR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g</td>
<td>nmol/g</td>
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<tr>
<td>Free fatty acids</td>
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<td>2.04</td>
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<td>46.1</td>
<td>4456</td>
<td>1.03</td>
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<td>8559</td>
<td>3.18</td>
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<tr>
<td></td>
<td>LUNG</td>
<td>LIVER</td>
<td>KIDNEY</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
<td>--------------------------</td>
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<tr>
<td>Lipid type</td>
<td>18:2(9c,11t)</td>
<td>18:2(9c,12c)</td>
<td>18:2(9c,11t)</td>
<td>18:2(9c,12c)</td>
</tr>
<tr>
<td></td>
<td>nmol/g</td>
<td>nmol/g</td>
<td>nmol/g</td>
<td>nmol/g</td>
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<tr>
<td>Free fatty acids</td>
<td>9.7</td>
<td>236</td>
<td>71.3</td>
<td>4692</td>
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<tr>
<td>Phospholipids</td>
<td>25.4</td>
<td>1021</td>
<td>44.2</td>
<td>4079</td>
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<tr>
<td>Triglycerides</td>
<td>11.7</td>
<td>757</td>
<td>206.1</td>
<td>7263</td>
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<td></td>
<td>286</td>
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</table>
### Spleen

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>%MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>12.7</td>
<td>658</td>
<td>1.93</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>21.5</td>
<td>1487</td>
<td>1.45</td>
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<tr>
<td>Triglycerides</td>
<td>5.7</td>
<td>646</td>
<td>0.88</td>
</tr>
</tbody>
</table>

**Subject 4.**

Male.

*Post mortem* report not available.

<table>
<thead>
<tr>
<th>Tissues studied:</th>
<th>Brain</th>
<th>Liver</th>
<th>Heart</th>
<th>Kidney</th>
<th>Lung</th>
<th>Spleen</th>
</tr>
</thead>
</table>

### Brain

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>%MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>6.5</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>94.2</td>
<td>2403</td>
<td>3.92</td>
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<tr>
<td>Triglycerides</td>
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<td>Not detected</td>
<td>Not detected</td>
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</table>

### Heart

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>18:2(9c,11t)</th>
<th>18:2(9c,12c)</th>
<th>%MR</th>
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</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>19.3</td>
<td>205</td>
<td>9.41</td>
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<tr>
<td>Phospholipids</td>
<td>126</td>
<td>3201</td>
<td>3.94</td>
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<td>Triglycerides</td>
<td>322.4</td>
<td>2316</td>
<td>13.92</td>
</tr>
</tbody>
</table>
| Lipid type   | 18:2(9c,11t) nmol/g | 18:2(9c,12c) nmol/g | %MR  
|-------------|---------------------|---------------------|------
| Free fatty acids | 10.3 | 77 | 13.38 |
| Phospholipids  | 68.3 | 647 | 10.56 |
| Triglycerides  | 24.7 | 432 | 5.72 |

**LIVER**

| Lipid type   | 18:2(9c,11t) nmol/g | 18:2(9c,12c) nmol/g | %MR  
|-------------|---------------------|---------------------|------
| Free fatty acids | 40.0 | 926 | 4.32 |
| Phospholipids  | 180.7 | 5809 | 3.11 |
| Triglycerides  | 135.7 | 2164 | 6.27 |

**KIDNEY**

| Lipid type   | 18:2(9c,11t) nmol/g | 18:2(9c,12c) nmol/g | %MR  
|-------------|---------------------|---------------------|------
| Free fatty acids | 37.3 | 728 | 5.12 |
| Phospholipids  | 91.2 | 2365 | 3.86 |
| Triglycerides  | 70.0 | 1400 | 5.00 |

**SPLEEN**

| Lipid type   | 18:2(9c,11t) nmol/g | 18:2(9c,12c) nmol/g | %MR  
|-------------|---------------------|---------------------|------
| Free fatty acids | 12.0 | 137 | 8.76 |
| Phospholipids  | 59.0 | 948 | 6.22 |
| Triglycerides  | 17.7 | 255 | 6.94 |
Subject 5.
Female.

Cause of death:

1. Rectal tumour obstruction with metastases in liver.

<table>
<thead>
<tr>
<th>Tissues studied:</th>
<th>Heart</th>
<th>Liver (interface of normal and metastases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
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<td>Liver metastases</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>Rectal tumour</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (normal)</td>
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<td>Skin tumour (from nose)</td>
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</tbody>
</table>

**HEART**

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>18:2(9c,11t) nmol/g</th>
<th>18:2(9c,12c) nmol/g</th>
<th>%MR</th>
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</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
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<td>9.42</td>
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<tr>
<td>Triglycerides</td>
<td>723.5</td>
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<td>18.83</td>
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**LUNG**

<table>
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<th>18:2(9c,12c) nmol/g</th>
<th>%MR</th>
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<td>Triglycerides</td>
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<td>18:2(9c,12c)</td>
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<tr>
<td>----------------</td>
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<tr>
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<td>nmol/g</td>
<td>nmol/g</td>
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<tr>
<td><strong>KIDNEY</strong></td>
<td>Free fatty acids</td>
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<td>Triglycerides</td>
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<td><strong>SPLAEN</strong></td>
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<td></td>
<td>Phospholipids</td>
<td>24.8</td>
<td>419</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>11.8</td>
<td>305</td>
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<tr>
<td><strong>LIVER (normal tissue)</strong></td>
<td>Free fatty acids</td>
<td>43.0</td>
<td>1167</td>
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<td>Phospholipids</td>
<td>11.7</td>
<td>1370</td>
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<tr>
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<td>Triglycerides</td>
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<td>1127</td>
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<tr>
<td><strong>LIVER (interface of normal and metastatic tissues)</strong></td>
<td>Free fatty acids</td>
<td>64.3</td>
<td>1511</td>
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<td>Phospholipids</td>
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<td>3143</td>
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<td>Triglycerides</td>
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<td>LIVER (metastatic tissue)</td>
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<tr>
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<td>--------------------------</td>
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<td>----------------------</td>
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<tr>
<td>Lipid type</td>
<td></td>
<td>18:2(9c,11t)</td>
<td>18:2(9c,12c)</td>
</tr>
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<td>nmol/g</td>
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<tr>
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<tr>
<td>Lipid type</td>
<td></td>
<td>18:2(9c,11t)</td>
<td>18:2(9c,12c)</td>
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<tr>
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<td>Free fatty acids</td>
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<tr>
<td>Triglycerides</td>
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<td>2764</td>
<td>4.61</td>
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