A STUDY OF THE INFLUENCE OF DIETARY FATTY ACIDS AND THEIR METABOLITES ON UTERINE LIPID METABOLISM AND FUNCTION

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Declaration

I confirm that the work presented in this thesis was the result of experiments conducted by myself and that the thesis composition is my own.

Studies which were carried out in collaboration with colleagues are fully acknowledged in the text.

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Abstract

Prostaglandins E_2 and $F_{2\alpha}$ play major roles in the initiation and maintenance of parturition. There is evidence that they also prime the uterus prior to parturition. Diets high in n-3 fatty acids have been reported to be associated with impaired parturition, whereas uterine infection by the intracellular pathogen Chlamydia psittaci is associated with abortion and premature labour. In each case the course of disruption of normal parturition is unknown, however, impaired or excessive 2-series prostaglandin production has been postulated to play an important part.

The effect of high dietary n-3 and n-6 fatty acid intake on uterine fatty acid composition and metabolism by desaturase, elongase, phospholipase and cyclooxygenase enzymes was investigated. The effect of C. psittaci infection on 2-series prostaglandin production and its control was also studied. The uterine fatty acid content of rats maintained on diets high in n-6 and n-3 essential fatty acids (EFA) for various periods was analysed and compared with a control group fed a normal pelleted diet. Rapid changes in uterine n-6 and n-3 fatty acid content were observed after three weeks feeding. However, in all three diet groups conservation of arachidonic acid was observed, which was highest in rats fed the n-6 fatty acid diet and lowest in rats fed the n-3 fatty acid diet. The 20C and 22C EFA were incorporated into phospholipids to a greater extent than into neutral lipids.

The distribution of EFA in the individual lipid classes in the three diet groups indicated selective release of arachidonic acid and eicosapentaenoic acid into the free fatty acid pool. Phosphatidylethanolamine arachidonic acid levels were more susceptible to changes in dietary fatty acid content than those of phosphatidycholine and phosphatidylinositol. Analysis of prostaglandins produced by uteri of rats on the three diets, by mass spectrometry, suggested an inhibitory effect of the n-3 fatty acids on total prostaglandin production, and the synthesis of the 3-series prostaglandins E and F.

Pregnant sheep experimentally infected with an ovine abortion strain of C. psittaci were found to prematurely release prostaglandin E_2 (PGE_2) into the amniotic and allantoic fluids. Impaired release of PGE_2 into the utero-ovarian vein was also detected in infected sheep. The plasma oestradiol 17ß also increased earlier than that of control sheep.

This study detected competetive inhibition of uterine n-6 EFA metabolism at the level of esterification, chain elongation, desaturation and cyclooxygenase metabolism by the dietary n-3 EFA. In infectious abortion, abnormalities in PGE_2 and oestradiol 17ß were detected. The first evidence of 3-series prostaglandin production by uterine tissue is presented.

Abbreviations

PI - phosphatidylinositol
PC - phosphatidylcholine
PE - phosphatidylethanolamine
PS - phosphatidylserine
PIP - phosphatidylinositolmonophosphate
PIP_2 - phosphatidylinositolbiphosphate
FO - fish oil
EPO - evening primrose oil
EFA - essential fatty acid
GC - gas chromatograph
MS - mass spectrometer
TIM - total ion monitoring
SIM - single ion monitoring
MID - multiple ion detection
TLC - thin layer chromatography
HPTLC - high performance thin layer chromatography
DARS - donkey anti-rabbit serum
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
TMS - trimethylsilyl
TBDMS - tertbutyldimethylsilyl
MG - monoglyceride
DG - diglyceride
TG - triglyceride
CHOL - cholesterol
Egg LD_{50} - egg lethal dose 50
SEM - standard error of mean
en% - energy supplied by food as % of total energy
n-3 and n-6 have been abbreviated to n3 and n6.

CONTENTS

Chapter 1

General Introduction

1.1 General Background

The importance of fat as an energy source has been recognised for over 200 years, and in an empirical sense, long before. The additional function of a certain class of lipids was identified in the 1930's by G.O. Burr and M.M. Burr (1929, 1930) who described acute deficiency states induced in rats by feeding fat free diets. These states could be reversed only by feeding certain fatty acids in the diet. The fatty acids used by Burr and Burr to correct the deficiency symptoms were linoleic acid and arachidonic acid and the term vitamin F was given to them. This name was later changed to essential fatty acids (EFA) and a number of other fatty acids have since been added to the names of linoleic acid and arachidonic acid in this very important group of lipids.

When an animal is deprived of EFA it develops EFA deficiency symptoms, which have been extensively identified in the rat (table 1.1). The wide range of deficiency symptoms observed are due to disruption of a number of EFA-mediated processes at the cellular and tissue level. Attempts to explain all the symptoms of EFA deficiency by the loss of one of the functions of EFA, for example prostaglandin production, have so far been unsuccessful.

The uterus is dependent on a supply of EFA to maintain normal functional activity. During the life of the mammal the uterus exhibits various states of activity. Before puberty the uterus exhibits a quiescent state, after puberty it has cyclical activity under the control of reproductive hormones. Anabolic and quiescent

Table 1.1
Major symptoms of EFA-deficiency in rats (from Burr and Burr 1929 and 1930).

Scaly skin
Caudal necrosis
Inflamed hind feet
Dandruff
Loss of hair
Haemorrhagic skin sores
Impairment of growth and weight gain
Increased water consumption
Kidney degeneration
Impaired reproductive function and sterility (male and female)

states are observed during pregnancy which change to rapid and coordinated catabolic and expulsive activity at parturition. Normal parturition will not occur in the rat if it is EFA deficient (Deuel et al., 1964) or if n3 fatty acids are supplied as the major EFA source (Leat and Northorp, 1981; Leaver et al., 1986; Quakenbush et al., 1942). Microbial infection of the uterus is another cause of abnormal parturition, with the result often being premature birth and abortion, or delivery of a dead foetus. Although the importance of prostaglandins and EFA in the control of uterine activity are recognised, less is known about uterine EFA composition, and no reports have been published on the effect of n3 EFA on uterine fatty acid composition.

1.2 Fatty Acids: Structure, Structural Variation, Nomenclature and Synthesis

The fatty acid molecule consists of a chain of carbon and hydrogen atoms with a carboxyl group at one end of the molecule and a methyl group at the other end. The chain may contain one or more double bonds between carbon atoms and the double bonds may have either a cis or a trans configuration, the cis type of double bond is more common. Fatty acids containing no double bonds are known as saturated fatty acids, those with one double bond as monounsaturated and those with more than one double bond as polyunsaturated fatty acids. In describing a fatty acid with more than one double bond, the number of carbon atoms before the first double bond counting from the methyl end of the molecule is usually given. For example, arachidonic acid may also be known as eicosatetraenoic acid or 20:4 n6. The last name provides the best description of the fatty acid,

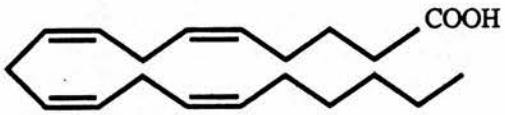
with the number before the colon indicating the number of carbon atoms in the molecule, the number after the colon indicating the number of double bonds in the molecule, and the n6 indicating that the first double bond from the methyl end of the molecule is between the sixth and seventh carbon atoms. Branched chain fatty acids with carbon-hydrogen side chains, or cyclic fatty acids with carbon rings are also found in nature, mainly in plants and microorganisms. This study will only be concerned with the straight chain fatty acids predominant in mammals.

In most of the metabolic reactions of fatty acids, the fatty acid is in an 'activated' form, which is formed by the fatty acid linking to the complex nucleotide, coenzyme A, by a thiol ester bond. Saturated fatty acid chains are synthesised by successive condensations of two carbon units originating from malonyl CoA by fatty acid synthetase enzymes. Saturated fatty acids can be desaturated within animals by desaturase enzymes. In the animal there are three main families of long chain unsaturated fatty acids distinguished by the position of the first double bond from the methyl end of the molecule, these are the n3, n6 and n9 fatty acids (see figure 1.1). Fatty acids of the n3 and n6 series cannot be synthesised by mammalian cells and are termed essential fatty acids. Fatty acids obtained from the diet or synthesised within the animal cell that are unsaturated before the n9 position can be further desaturated towards the carboxyl end of the molecule, but not towards the methyl end. In plants the opposite is usually true with the fatty acid being desaturated towards the methyl end. Fatty acid synthetase and desaturase enzymes will only act on the CoA form of the fatty acid molecule (Fulco, 1974). Due to the inability of the

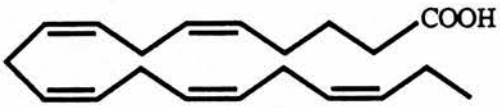
Figure 1.1

Stucture of n3, n6 and n9 20C fatty acids.

20:3 n9



20:4 n6



20:5 n3

animal to desaturate towards the methyl end of the molecule the different series of fatty acids are not interconvertible.

When essential fatty acids are not present in the tissue the n9 fatty acids (18:1 n9 and 20:3 n9) will accumulate. Arachidonic acid is the fatty acid that is usually most effective in clearing EFA deficiency symptoms (Holman et al., 1971). One of the most important functions of arachidonic acid is as a precursor for the 2-series prostaglandins and thromboxanes and 4-series leukotrienes. These locally acting hormones have important effects on many tissues, including the uterus where they are involved in the initiation of parturition (Liggins et al., 1977; Mitchell, 1980). The only EFA activity so far demonstrated for linoleic acid is in the maintenance of the epidermal water barrier (Hansen and Jensen, 1985). In cats, which cannot further desaturate linoleic acid to gamma-linolenic acid or arachidonic acid, there is some dispute as to whether or not administration of linoleic acid will cure EFA deficiency symptoms (MacDonald et al, 1984; Rivers et al, 1975, 1976).

Although the exact properties of the n6 fatty acids that make them essential dietary constituents are still unclear it is established that they are necessary for the maintenance of health in animals. The role of the n3 essential fatty acids, however, is less clear. α-linolenic acid will not cure EFA deficiency symptoms in EFA deficient rats (Greenberg et al., 1950). Studies involving rats on n3 deficient diets have failed to show development of EFA deficiency symptoms (Tinoco et al., 1971; Tinoco et al., 1978). However, some tissues contain high quantities of n3 fatty acids (mainly 22:6n3),

suggesting specific roles for these fatty acids. These tissues include the retinas of a range of animals, including humans (Anderson, 1970), and the brain of humans (Svennerholm, 1968) and rats (Breckinridge et al., 1973). n3 fatty acids, and in particular 22:6n3 are also tenaciously retained by animals even when on fat free diets (Forrest & Futterman., 1972). Whether or not the uterus retains the n3 fatty acids is not known (this has been investigated in chapters 3 and 4). Although typical EFA deficiency symptoms do not appear in mammals on diets lacking n3 fatty acids, some experiments have suggested some symptoms of n3 fatty acid deficiency. These include a lack of learning abilitiy of rats fed low levels of n3 fatty acids (Lamptey & Walker., 1976). In contrast to higher mammals, some fish do exhibit EFA deficiency symptoms when deprived of n3 fatty acids (Yu & Sinnhuber, 1972). Many beneficial effects of n3 fatty acids in mammals have, however, been reported, including those on heart disease (Dyerberg et al., 1978; Wood et al., 1987; Glomset, 1985) and inflammatory conditions such as rheumatoid arthritis (Kremer et al., 1987) and psoriasis (Burton, 1989).

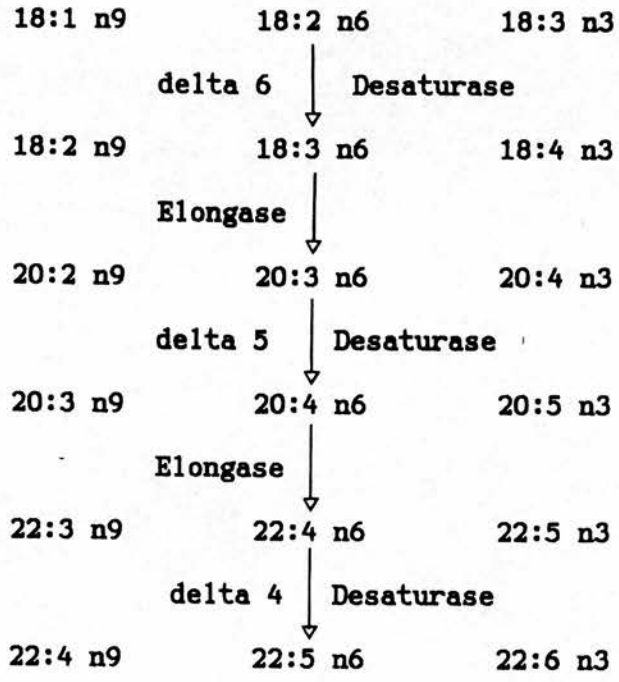
The major sources of the essential fatty acids are from plant oils which provide mainly linoleic acid (18:2n6), but also gamma-linolenic acid (18:3n6) and α-linolenic acid (18:3n3). Fish oils are a major source of the n3 fatty acids, providing predominantly eicosapentaenoic acid (20:5n3) and docosahexaenoic acid (22:6n3). In most animals (except cats, Rivers et al., 1975, 1976) the n3 and n6 essential fatty acids can all be synthesised from their respective eighteen carbon unsaturated fatty acids, which are α-linolenic acid for n3 EFA and linoleic acid for n6 EFA. This

synthesis is carried out by a series of elongase and desaturase enzymes. Figure 1.2 shows the metabolism of the n9, n6 and n3 fatty acids, they are all metabolised by the same or a closely related enzyme sequence. The different fatty acids will compete with each other for desaturation and elongation (Alfin-Slater & Aftergood, 1968; Rahm & Holman, 1964), with the affinity of the desaturase and elongase enzymes being in the order of n3>n6>n9 (Brenner, 1974). Mutual inhibition of the conversion of linoleic acid to gamma-linolenic acid and eicosatrienoic acid to arachidonic acid has also been observed (Brenner, 1969) as well as inhibition by trans fatty acids and saturated fats (Horrobin, 1983; Brenner, 1982). The rate determining step is the D6 desaturase conversion of linoleic acid to gamma-linolenic acid or α-linolenic acid to 18:4n3.

1.3 The Distribution and Metabolism of the Essential Fatty Acids in Cellular Lipids

Lipids constitute at least 50% of the mass of most animal cell membranes. The three major types of lipid present in cell membranes are phospholipids (the most abundant), cholesterol, and glycolipids. Fatty acids are important constituents of phospholipids, cholesterol esters and glycolipids and play an important structural role in the membrane. The hydrophobic nature of the carbon chain on the fatty acids helps to form the membrane into a bilayer with the long hydrophobic chains of glycolipids and phospholipids pointing inward towards each other and the polar head groups on the outside of the bilayer. This discussion shall be mainly concerned with the fatty acid composition of phospholipids.

Figure 1.2
Desaturation and elongation of n9, n6 and n3 fatty acids.



The phospholipids, for many years, were considered metabolically uninteresting compounds playing a purely structural role in the cellular membranes, that once incorporated during the initial growth of the tissue exhibited very little further turnover. Recent studies have, however, indicated the metabolic and regulatory role of the phospholipids. The two main types of phospholipids found in the mammalian cell are the glycerophospholipids and the sphingolipids. Sphingolipids generally have a low EFA content and most research concerning EFA has centred on the glycerophospholipids, so only these will be discused. The structure of a typical glycerophospholipid is shown in figure 1.3.

The most common fatty acids found in animal phospholipids are palmitic (16:0), stearic (18:0), oleic (18:1n9), linoleic (18:2n6) and arachidonic (20:4n6) acids. The main types of phospholipid in the mammalian cell; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), differ in the chemical composition of the head group.

Phospholipids have distinct fatty acid contents. These differences could arise either through specific incorporation of the fatty acids by the acyl-transferases or through the diacylglycerol transferase with acyl selectivity. Of the four major phospholipids, studies in rat heart, cultured human keratinocytes and guinea pig uterus have shown that PE and PC usually contain the most arachidonic acid (Zijlstra & Vincent, 1985; Punnonen et al., 1987; Leaver & Poyser, 1981; Leaver et al., 1986). Differences in the incorporation of the n3 and n6 fatty acids into specific phospholipids have been

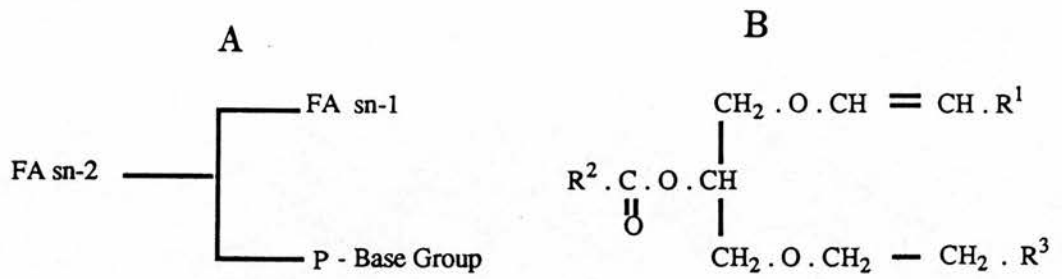


Figure 1.3

Figure A shows a simplified structure of a glycerophospholipid molecule. The phospholipid contains a hydrophilic base group, attached at one end of the glycerol molecule (base groups may be choline, ethanolamine, serine, inositol, inositol monophosphate, inositol biphosphate or inositol triphosphate). Two fatty acid molecules form ether or ester linkages with the other two available hydroxyl groups of the glycerol molecule. Fatty acids bound to glycerol by ester linkages are known as acyl groups, whereas those bound by ether linkages are either alkyl or alkenyl groups. Figure B shows a triacylglycerol molecule with an alkenyl group at R^1, an acyl group at R^2 and an alkyl group at R^3. Phospholipids containing a fatty acid bound by an alkenyl and an acyl linkage are known as plasmalogens. Alkyl or alkenyl linkages are usually found in the 1-position of the phospholipid with an acyl linkage in the 2-position. The diacyl and alkenyl - acyl phospholipids are the most common.

When giving a phospholipid its full name, the identity of the two fatty acid molecules is given, therefore an inositol phospholipid containing palmitic acid and arachidonic acid linked by ester linkages would be named as follows; 1-palmitoyl-2-arachidonoyl-3-sn-phosphatidylinositol, where the sn stands for stereochemical numbering. The carbon atom of the glycerol linked to the phosphate group is always numbered three, and the fatty acids linked to the other two carbons of the glycerol numbered accordingly.

Phospholipids are not essential dietary components and can be synthesised within tissues. The glycerol phospholipid molecule can be divided into five different basic components; the two fatty acid chains, glycerol, the phosphate group, and the base group. Each of the constituents can turnover independently and so there is no one definite route of phospholipid synthesis.

observed; Abeywardena et al, (1987) reported a greater displacement of arachidonic acid by n3 fatty acids in PE of rat myocardial tissue in rats fed on a long term saturated fat diet followed by diets containing n3 and n6 fatty acids than other phospholipids. They also observed preferential incorporation of docosahexaenoic acid compared to eicosapentaenoic acid in PC, diphosphatidylglycerol and PE with the greatest incorporation being in PE. Conservation and specific incorporation of fatty acids in the uterus in response to diets with differing n3 and n6 fatty acid contents is investigated in chapters 3 and 4. Specificity of incorporation of fatty acids into phospholipids with particular types of linkages has been observed. Analysis of the uptake and stimulated release of arachidonic acid from human platelets demonstrated preferential uptake into the di-acyl class compared with the alkyl-acyl class and preferential release from the diacyl class when stimulated by thrombin but equal release with the alkyl-acyl class when stimulated with calcium ionophore A23187 (Purdon & Smith, 1985). Preferential incorporation of docosahexaenoic acid into the alkenyl-acyl (plasmalogen) class of phospholipid above that of the alkyl-acyl class has been reported in P388D$_1$ cells (Blank et al., 1989) cultured in medium containing a 22:6n3 supplement, suggesting a higher turnover rate of the plasmalogens. Further evidence for this comes from studies on the incorporation of the fish oil fatty acids, eicosapentaenoic acid and docosahexaenoic acid, into PE of human platelets. Greater incorporation and replacement of arachidonic acid and adrenic acid was found in the alkenyl-acyl class than the diacyl class (Aukema & Holub, 1989).

The formation of fatty acid - CoA esters may play a role in specific

incorporation. In human platelets a fatty acid - CoA synthetase enzyme specific for arachidonic acid has been identified (Neufeld et al., 1983). In a mutant strain of the mouse fibrosarcoma cell line lacking this enzyme less arachidonic acid was found and prostaglandin synthesis was lower than in the normal cell line which contains the enzyme (Neufeld et al., 1984). Whether this enzyme plays a similar role in other cell types is unknown.

1.4 The Enzymic Release of Phospholipid Bound Essential Fatty Acids

The turnover of fatty acids in phospholipid molecules requires the release of fatty acids from the phospholipid molecule. The enzymes that catalyse this reaction are known as phospholipases and include the following enzymes; phospholipase A1, A2, B, C and D. Phospholipase A_2 and phospholipase C are the phospholipases most involved in fatty acid release for prostaglandin synthesis. Two of the major functions of phospholipases are to regulate the composition of membrane lipids and to release fatty acids for conversion to prostaglandins, thromboxanes, and leukotrienes (known as the eicosanoids). Prostanoids do not occur in stored form in tissues and the availability of the free acid precursors is thought to be the limiting factor in their formation. This will be discussed further in section 1.5. The levels of free fatty acids in resting cells are usually very low (Bills et al., 1977; Marcus et al., 1969) due to their rapid reacylation or metabolism.

Phospholipase A2 is the phospholipase upon which most interest has centred and is usually a membrane bound enzyme, (Victoria et al., 1971; Newkirk & Waite, 1973) although it can be found in soluble

form (De Haas et al., 1968). Phospholipases A_2 release the fatty acid molecules from position 2 of phospholipids. Fatty acids in position 2 are predominantly unsaturated and are often eicosanoid precursors. Therefore phospholipase A_2 activity results in a release of a high proportion of free fatty acids for eicosanoid synthesis. Phospholipase C cleaves between the head group and the glycerol molecule to release diacylglycerol and the phosphorylated head group. Free fatty acids may then be released from the diacylglycerol by diacylglycerol lipase (Bell et al., 1979) and from the resulting monoacylglycerol by monoacylglycerol lipase (Chan and Tai, 1981; Okazaki et al., 1981b).

Phospholipase A_2 is largely specific for PC and PE (Jesse and Franson, 1979; Kanagi and Koizum, 1979; Lagarde et al., 1981), however, it may also hydrolyse PI (Hong and Deykin, 1981; Hong et al., 1985; Emilsson and Sundler, 1984; Billah and Lapetina, 1982). PI is the main substrate of phospholipase C (Banno et al., 1986; Takenawa and Nagai, 1981; Wilson et al., 1984; Allan and Michell., 1978; Kamisaka et al., 1986; Rothenberg et al., 1983), although PC hydrolysing phospholipases C have also been identified in canine myocardium (Wolf and Grass, 1985) and rabbit platelets (Hwang, 1988). Arachidonic acid may also be released from the quantitatively minor, but metabolically active lipids phosphatidylinositol -4-monophosphate (PIP) and phosphatidylinositol -4,5-biphosphate (PIP2). These have been shown to be hydrolysed by phospholipase C (Berridge, 1983, Downes & Wusteman, 1983). However, these polyphosphoinositides are minor cell constituents and the extent of the role they play in releasing arachidonic acid and other eicosanoid precursors is unknown.

Radiotracer studies with [^{14}C] and [^{3}H] labelled arachidonic acid have shown that the main phospholipid sources of arachidonic acid released on agonist stimulation vary between different species and different tissues. In MC5-5 mouse fibroblasts the main sources were PC and PI (Hong & Deykin, 1979), this was also true for human platelets, (Bills et al., 1977; Bills et al., 1976; Rittenhouse-Simmons et al., 1976) human umbilical vein endothelial cells (Hong et al., 1985; Thomas et al., 1984), and human neutrophils (Walsh et al., 1981). However, in porcine aortic endothelial cells (Hong & Deykin, 1981) the main source was PE and PI, in MDCK cells it was PC, PI, PE and PS (Daniel et al., 1981), and in human lymphocytes it was PI (Parker et al., 1979). In human foetal membrane at term PE and PI were found to be the main sources of arachidonic acid (Okita et al, 1982), whereas, in the guinea pig uterus, radiotracer studies suggested PC, PE and triglyceride were the major sources of free arachidonic acid (Ning et al., 1983). In chapter 4 the arachidonic acid content of major lipids in the rat uterus has been investigated.

Phospholipases A_2 specificity for fatty acids in phospholipids has been observed. Bills et al. (1977), using human platelets found that phosphatidylcholine hydrolysis occurred only in arachidonic acid containing molecules and Okazaki et al. (1978) identified phospholipase A_2 specific for PE with arachidonic acid in the sn-2 position in human foetal membranes. Evidence of specificity for ether or diacyl phospholipids has also been found. Jesse and Cohen (1976) analysed the lysophophatidylethanolamine produced from PE in human platelets along with fatty acid release and found it to contain virtually no fatty aldehydes. This led the authors to

believe that phospholipase A_2 was specific for diacyl phospholipids and not plasmalogens. Rittenhouse-Simmons et al. (1976) reported a rise in [^{3}H] radioactivity in alkenyl PE after thrombin activation in human platelets prelabelled with [^{3}H] arachidonic acid. They suggested that arachidonic acid might be transferred to the alkenyl PE from other phospholipids and that the alkenyl PE serves as the donor of arachidonic acid for eicosanoid synthesis. However, such transfer has not been observed in other types of cells (Hong & Deykin, 1979; Weithman et al., 1989; Schremmer et al., 1979).

The disintegration of membrane structure observed in necrotic and other autolytic processes (including parturition) involving the activation of tissue phospholipases indicates the importance of control of phospholipase activity. Factors involved in activation and control of phospholipase activity include; association with G-proteins (Billah and Siegel, 1987; Nakashima et al., 1989), platelet activating factor (Nakashima et al., 1989), protein kinase C (Emilsson et al., 1986) regulation by Ca^{2+} concentration, diacylglycerol and phorbol myristate acetate (Emilsson et al., 1986; Emilsson and Sundler, 1986), influence of hormones, and regulation by changes in membrane structure (Vogt, 1978). Phospholipase A2 can be activated by addition of calcium to isolated membranes (Derksen & Cohen, 1975), or by the addition of calcium ionophore to platelets (Pickett et al., 1977) and uterus (Leaver and Richmond, 1984). Stimulation of prostaglandin production in the rat uterus by the calcium ionophore A23187 is discussed in chapter 5. Hormones have also been shown to stimulate some phospholipases A2 (Haye et al., 1973). In the uterus, steroid hormones are known to regulate

prostaglandin release (Leaver and Seawright, 1982; Roberts et al., 1975), however, it is not known whether or not this is through control of phospholipase activity. Oxytocin, calcium ionophore A23187, endotoxin, and mechanical stimulation of the uterus have also been found to increase prostaglandin release from the uterus (Leaver and Seawright, 1982; Roberts et al., 1975), however, only in the case of A23187 has evidence suggested that this is achieved through the release of substrate for prostaglandin synthesis (the control of prostaglandin release in the uterus is discussed in section 1.8). The hydrolysis of PI-4,5-P_2 by phospholipase C has been suggested to be the initial event in phospholipase activation in stimulated cells (Hong, 1988). This may occur through the activation of phospholipase C by G-proteins (Cockcroft and Gomperts, 1985; Uhing et al., 1985), with the phospholipase C releasing diacylglycerol and inositol phosphates that will in turn increase calcium levels (Berridge, 1983). The calcium and diacylglycerol could then act synergistically to activate phospholipase A2.

1.5 The Prostaglandins : Synthesis and Functions

Prostaglandins were first isolated by Bergstrom and Sjovall in 1957, who isolated PGE_1, and $PGF_{1\alpha}$ in crystalline form. Prostaglandins are synthesised from three different essential fatty acids, giving rise to three distinct series of prostaglandins, depending on the number of double bonds in the molecule. One-series prostaglandins are synthesised from dihomo-gamma-linolenic acid (20:3n6), two-series from arachidonic acid (20:4n6) and three-series from eicosapentaenoic acid (20:5n3). It is also now known that the 22C fatty acids docosatetraenoic acid (22:4n6) and docosahexaenoic acid

(22:6n3) can be converted to prostaglandins (Sprecher et al., 1982; Ferretti and Flanagan, 1986; Mai et al., 1981), however, little is yet known about the activity of these prostaglandins.

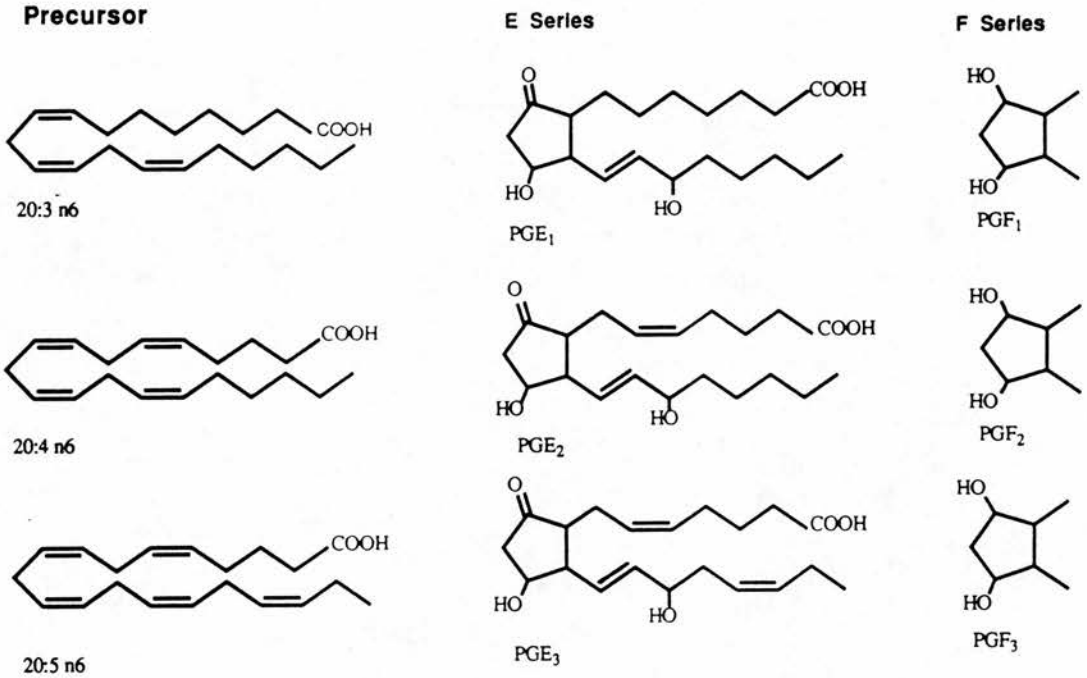
The fatty acids are converted to prostaglandins by cyclooxygenase enzymes (Van Dorp et al., 1964; Ferretti and Flanagan, 1986; Weber et al., 1986). Figure 1.4 shows the structure of the 1, 2, and 3 series E and F prostaglandins produced from the three different precursor fatty acids. PGE and PGF are necessary for the induction of uterine contractions during parturition (Embrey, 1969; Liggins et al., 1973) and impaired prostaglandin production may delay or prevent normal parturition (Mitchell, 1980; Leaver et al., 1986; Olsen et al., 1986).

Eicosanoids are involved in the activity of blood cells such as platelets (Marcus, 1978), monocytes, macrophages, and neutrophils and they have effects on aspects such as vasal tone (Needleman et al., 1979; Knapp et al., 1986), inflammation (Pelus and Strausser, 1977; Burton, 1989; Tate et al., 1989; Kremer et al., 1987; Leslie et al., 1985), and immunosuppression (Erickson, 1986). Many of the effects of the 1,2 and 3-series prostaglandins oppose each other and this principle has been used to try and cure or alleviate the symptoms of a number of diseases partly or wholly caused by imbalanced prostaglandin production.

The n3 fatty acids will inhibit the desaturation and elongation and the uptake into phospholipids of n6 fatty acids (see sections 1.2 and 1.3). This is also true with the production of prostaglandins, where the three different fatty acid precursors will compete with each other for the cyclooxygenase enzymes. The non-essential fatty

FIGURE 1.4

Structure of the 1,2 and 3 series E and F prostaglandins and their precursors.



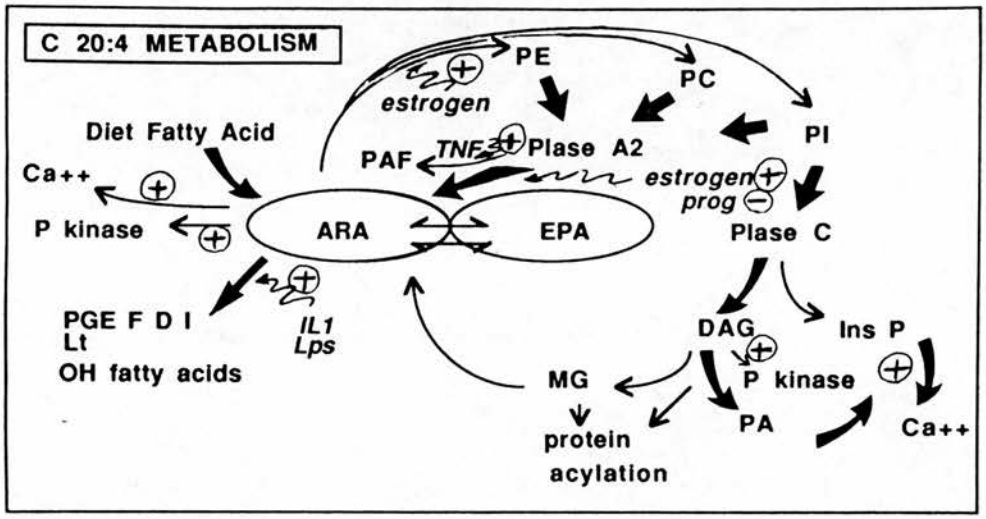
acid, 20:3 n9, which accumulates in essential fatty acid deficiency, is also known to block prostaglandin production (Okazaki & Araki, 1974; Okazaki & Araki, 1978).

Eicosapentaenoic acid has been shown to block oxidation of arachidonic acid by the cyclooxygenase enzymes in human platelets (Culp et al., 1979; Morita et al., 1983b), cultured vascular smooth muscle cells (Morita et al., 1983a) and uterus (Leaver et al., 1986). Species difference in the inhibition caused by EPA have also been observed (Morita et al., 1983b), with much greater inhibition in human platelets than in rat platelets. There are also species differences in the ability to convert EPA to 3-series prostaglandins. In human and rabbit smooth muscle cells and bovine endothelial cells EPA was converted to 3-series PG, but in murine and porcine smooth muscle cells, and rat platelets no conversion to 3-series prostaglandins was observed (Morita et al., 1983a). In view of the inhibitory effects of the n3 fatty acids on parturition it is important to know to what extent the uterus will produce the 3-series prostaglandins as this may be one of the causes of the inhibition. In chapter 5, 3-series prostaglandin production by the uteri of rats maintained on diets with varying n3 and n6 fatty acid contents has been analysed.

1.6 The Role of Essential Fatty Acids in Parturition

The metabolism of arachidonic acid during early parturition is shown in figure 1.5. The importance of EFA and their metabolites in parturition has been illustrated by the inability of EFA deficient rats to undergo normal parturition (Deuel et al., 1964, Houtsmuller, 1981). The major role of fatty acids in parturition is probably in

Figure 1.5
The metabolism of arachidonic acid during early parturition, indicating quantitatively major pathways and the site of extracellular signals.



Key
P Kinase - protein kinase
PGE F D I - prostaglandins E, F, D, and I
Lt - leucotrienes
OH fatty acids - hydroxy fatty acids
ARA - arachidonic acid
EPA - eicosapentaenoic acid
PAF - platelet activating factor
TNF - tumour necrosis factor
Plase - phospholipase
PA - phosphatidic acid
DAG - diacylglycerol
Ins P - inositol phosphate
MG - monoglyceride
IL1 - interleukin 1

prostaglandin synthesis, demonstrated by the inhibition of parturition caused by cyclooxygenase inhibiting drugs such as indomethacin. This demonstrates that even in the presence of normal cellular levels and normal release of free fatty acids from intracellular stores, if prostaglandin synthesis is inhibited, normal parturition will not occur. Columbinic acid (18:3; 5 trans, 9 cis, 12 cis) is not converted to prostaglandins but it will alleviate many of the symptoms of EFA deficiency, however rats maintained on an EFA deficient diet supplemented with columbinic acid are unable to undergo normal parturition (Houtsmuller, 1981).

The effects of the n3 fatty acids on parturition were first demonstrated by Quackenbush et al. in 1942, who found that linoleic acid and arachidonic acid would support normal parturition. However, if α-linolenic acid was given as the only source of essential fatty acid the rats had major difficulties in initiating and sustaining labour, despite only slight abnormalities in weight, implantation and gestation being observed in the rats. The uteri of the n3 fed rats were found to be thin, flacid and lacking tone, and vaginal bleeding was observed. Foetal mortality was high in n3 fed animals, but if Caesarian section was carried out early in labour live animals were delivered (Leat & Northrop, 1980). Leat and Northrop (1979) suggested that the inhibitory effect of α-linolenic acid on parturition was due to inhibition of conversion of linoleic acid to arachidonic acid, thus inhibiting production of the prostaglandin initiators of parturition, PGE_2 and $PGF_{2\alpha}$. Olsen et al. (1986) have suggested that the high intake of marine fat by Faroese women inhibits 2-series prostaglandin production, resulting in increased gestation time compared to women from Denmark. Fish

oil diets have also been found to inhibit parturition in rats (Leaver et al., 1986; Leaver et al., 1989) and will cause prolonged gestation, foetal death, extended duration of labour and vaginal bleeding. Showing that even when n6 fatty acids are present, inhibition of their metabolism by high levels of the n3 fatty acids will still prevent normal birth, this was found in a fish oil diet having an n3/n6 ratio of 6.28 (Leaver et al., 1989). PGE_2 measurements made on intrauterine tissues obtained during parturition showed significantly lower levels ($P < 0.05$) in fish oil fed rats compared to those fed on evening primrose oil or a control pelleted diet (Leaver et al., 1986). The inhibition caused by the fish oil diet suggested that the n3 inhibition was not due to the total loss of n6 fatty acids but was more likely to be due to inhibition of processes such as incorporation into phospholipids, desaturation, elongation, release from esterified stores, and prostaglandin formation by the n6 fatty acids. These studies indicate the importance of studying replacement of n6 fatty acids by n3 fatty acids in uterine tissue in relation to dietary fatty acid content and parturition.

Mating, pregnancy rate and number of pups per litter were unaffected by a fish oil diet (Leaver et al., 1986), despite the fact that prostaglandins are thought to play a role in ovulation and implantation (Poyser, 1981). The fact that the n3 fatty acids did not affect these processes may suggest that they required lower levels of 2-series prostaglandins for their normal functioning or that 3-series prostaglandins produced from the n3 fatty acids were able to substitute for the 2-series prostaglandins in regulating

these processes. Arachidonic acid and prostaglandins are released in quantities 2 to 3 times greater at parturition than at ovulation, implantation or lactation (MacDonald et al., 1974; Poyser, 1981; Bussman & Deis, 1979; Ogburn et al., 1980; Leaver et al., 1987). Measurements of 3-series prostaglandins have not previously been made in the uterus of the rat, however, renal tissue in the rat is known to synthesise 3-series prostaglandins (Kivits and Nugteren, 1988; Gallon and Barcelli, 1986). Their activity in stimulating uterine contractions and other processes necessary for normal parturition are unknown. The profound effects of the n3 fatty acids on parturition illustrate the importance of studying their incorporation into the uterus and their effect on levels of n6 fatty acids, and in particular arachidonic acid.

Essential fatty acids may have further roles to play in parturition apart from acting as precursors for prostaglandin synthesis (see table 1.2). Large amounts of arachidonic acid are released at parturition and these quantities are far in excess of the quantities required for prostaglandin production. Protein kinase C is known to control certain contractile proteins and enzyme activities (Preiss et al., 1987; Bell, 1986) and arachidonic acid and diacylglycerols can activate protein kinase C (Sekiguchi et al., 1987). The lyso-phospholipid, platelet activating factor (PAF), has potent contractile effects on smooth muscle (Findlay et al., 1981) and an increase in PAF in human amniotic fluid at parturition has been reported (Billah & Johnston, 1983). PAF availability is regulated by a phospholipase A_2 with arachidonate selectivity (Hanahan, 1986). PAF can also stimulate Ins-1,4,5-P3 release in platelets and leucocytes (Shukla et al., 1987), and if it has a similar effect on

Table 1.2
Functions of essential fatty acid in the uterus.

Proposed Molecular Activity	References
Prostaglandins	Thorburn (1979); Leat (1981).
PAF Synthesis	Billah (1983); Findlay (1981); Hanahan (1986)
Protein Kinase Activation	Nishizuka (1988)
Diacylglycerol	Okazaki (1981); Ning (1983); Leaver (1983); MacDonald (1987)
Phosphoinositides	DiRenzo (1981); Leaver (1981, 1985)

uterine tissue, the Ins-1,4,5-P3 induced release of calcium may in turn activate phospholipases, resulting in increased prostaglandin production.

1.7 Control of Prostaglandin Production in the Uterus of the Sheep, the Rat and the Woman.

During pregnancy the uterus synthesises increased quantities of prostaglandins from precursor fatty acids (Dray and Friedman, 1976; Keirse et al., 1983). Two prostaglandins have been identified to have an important role in the control of parturition, these are prostaglandin E_2 and prostaglandin $F_{2\alpha}$ (Dray and Friedman, 1976). In the sheep, the rat and man increased levels of PGE_2 and $PGF_{2\alpha}$ are thought to be the main stimulus for the initiation of parturition, but in each case the mechanisms controlling prostaglandin production differ.

1.7.1 Control of Prostaglandin Production in the Uterus of the Sheep

In the sheep the main sites of prostaglandin production are maternal cotyledons, where production is thought to be under the control of the steroid hormones; progesterone and oestrogen, oxytocin may also exert its effect on parturition through prostaglandins (Roberts et al, 1975). Oestrogen is thought to stimulate prostaglandin synthesis (Roberts et al, 1975), whereas progesterone is thought to inhibit its release (Liggins et al, 1973). Oestrogen levels increase sharply at term (Challis, 1971), whereas progesterone levels decline towards term (Thorburn et al, 1972).

The key role of prostaglandins in mediating the action of oestrogen and progesterone has been illustrated by Mitchell and Flint (1978).

The administration of meclofenamate, an inhibitor of prostaglandin synthesis, to pregnant sheep prevented the premature delivery of a live foetus in response to intrafoetal dexamethasone infusion. Despite the anticipated rise in oestradiol and fall in progesterone, no increase in uterine activity occurred, indicating that changes in steroid hormones are inadequate to cause delivery if prostaglandin synthesis is blocked.

In a reciprocal study, $PGF_{2\alpha}$ infused into the aorta of pregnant sheep, at rates comparable to the production rates at term, stimulated uterine contractions similar to those normally seen at term (Liggins et al., 1973). Maternal plasma progesterone and oestradiol levels were unaltered during the infusion, again indicating that the major role of the steroids is to modulate prostaglandin synthesis, and that this role can be bypassed with exogenous $PGF_{2\alpha}$. The ability of oxytocin to stimulate uterine contractions is also thought to be mediated through prostaglandins. Oxytocin has been shown to stimulate prostaglandin production (Flint et al, 1975; Roberts et al, 1975).

In conclusion, progesterone, oestrogen and oxytocin, in the sheep, control levels of prostaglandins during parturition, and either an increase in oestrogen or a decrease in progesterone can initiate parturition through stimulation of prostaglandin production. However, it seems most likely that the stimulus required to induce production of sufficient levels of prostaglandin to initiate parturition is a lowering of the progesterone: oestrogen ratio below a critical level and possibly also for a critical period of time (Taylor et al, 1982; Mitchell et al, 1983; Roberts et al, 1975).

However, little is yet known about the cellular mechanisms by which the hormones control ovine prostaglandin release.

1.7.2 Control of Prostaglandin Production in the Uterus of the Rat

In the rat uterus the major site of prostaglandin synthesis is the endometrium (Williams et al., 1974). The control of prostaglandin production in the uterus of the rat resembles that of the sheep in its oestradiol and progesterone dependence (Thorburn, 1979 for review). As in the sheep, oestradiol stimulates prostaglandin production in the uterus, whereas progesterone prevents this stimulation (Ham et al., 1975; Castracane & Jordan, 1976). However, in contrast to the sheep, progesterone does not appear to inhibit prostaglandin release or myometrium stimulation (Fuchs, 1974). The mechanism of action of oestrogen and progesterone on prostaglandin synthesis and release in the rat uterus is unknown, however, an inhibitory effect of oestrogen and a stimulatory effect of progesterone on prostaglandin degradation by 15-hydroxyprostaglandin dehydrogenase (PGDH) has been observed (Flower, 1977). Little information is, as yet, available on phospholipase activity in the uterus of the rat. Evidence has suggested that prostaglandin synthetase may play a role in controlling the level of prostaglandins in the rat uterus, with increasing prostaglandin synthetase activity being observed with advancing pregnancy (Williams et al., 1974; Williams & Vane, 1975). It has been suggested that lysosomal lability may be increased by the newly formed prostaglandins, leading to a rise in phospholipase A2 activity. Therefore, increasing the synthesis of prostaglandins may initiate a positive feedback system stimulating a further increase

in prostaglandin release (Thorburn and Challis, 1979).

Oxytocin will also stimulate prostaglandin production in the rat uterus, however, as with oestrogen and progesterone little is known about the mode of stimulation. The role of phospholipase and cyclooxygenase activity in control of prostaglandin production in the rat uterus requires investigation. In chapter 5 the effect of phospholipase activation by intracellular calcium on rat uterine prostaglandin production has been investigated.

1.7.3 Control of Prostaglandin Production in the Uterus of the Woman

Although much research has been carried out on the control of prostaglandin production in the uterus of the woman, it is possibly the least understood of the three species discussed here. Progesterone and oestrogen appear to play a lesser part in controlling prostaglandin production in humans than in the rat and the sheep. No abrupt changes of either progesterone or oestrogen are observed at the onset of labour (Turnbull et al., 1974), and human labour cannot be induced by oestrogen treatment (Pinto et al., 1967; Larsen et al., 1973). There is evidence that prostaglandins have an important place in the physiology of human labour, both PGE_2 and $PGF_{2\alpha}$ will stimulate uterine contractions and they are also known to soften and ripen the cervix prior to parturition (Liggins et al., 1977).

In humans the main sites of prostaglandin synthesis are thought to be the foetal membranes and the deciduum and the main control mechanisms appear to be local mechanisms acting on these tissues. The foetal membranes and decidua contain glycerophospholipids that

are highly enriched with arachidonic acid (Olund and Lunell, 1979; Okita et al., 1982; Okazaki et al., 1978; Okazaki et al., 1981b) and the specific incorporation of arachidonic acid into the foetal membranes has been detected late in pregnancy (Das et al, 1975), increasing the potential for prostaglandin synthesis.

The release of the fatty acid prostaglandin precursors from cellular stores (mainly membrane phospholipids) is thought to be the major controlling factor in prostaglandin synthesis (section 1.5). This is probably also true of prostaglandin synthesis by uterine and foetal tissues. MacDonald et al (1974) found that women injected with arachidonic acid into the amniotic sac underwent abortion, suggesting that the major control of prostaglandin synthesis was not at the level of the cyclooxygenase, at least during late gestation.

In many tissues, phospholipases are lysosomal enzymes and their activity depends on release from the lysosome. This led Gustavii (1972) to propose a 'lysosomal theory' of parturition in which a key role in the initiation of parturition is attributed to lysosomes of the deciduum. According to Gustavii, the deciduum becomes rich in lysosomes which are maintained in a stable state by the presence of stabilisers, particularly progesterone. The onset of labour is precipitated by labilising influences that cause leakage of lysosomal enzymes, including phospholipase A2, into the cytoplasm. The increased activity of phospholipase A2 causes accelerated deacylation of phospholipids at the sn-2 position which leads in turn to the release of fatty acids, including arachidonic acid resulting in increased prostaglandin production. In support of this

hypothesis, Brunk and Gustavii (1973) found that the lysosomes of decidual cells are unusually fragile and leak their contents when subjected, in vitro, to slight physical stress that has no discernable effect on other tissues. Also, decidual cells obtained at elective caesarian section at term showed marked degenerative changes and signs of release of the lysosomal enzyme, acid phosphatase into the cytoplasm (Gustavii, 1975).

The Gustavii theory was further extended by Schwarz et al., (1974) who found a progesterone binding protein in the cytosol of foetal membranes, the concentration of which increased towards term. This led to the proposal that a specific progesterone binding protein appears in the cytosol of foetal membranes near term and competes with lysosomes for progesterone. As a consequence, the lysosomes become more unstable and their contents leak out. The cause for the rapid accumulation of progesterone binding protein is unknown.

Phospholipase A2 activity with specificity for arachidonic acid in human foetal membranes has been identified by Okazaki et al (1978). Phosphatidylinositol specific phospholipase C activity has also been demonstrated in human foetal memranes and decidua vera (Di Renzo et al, 1981) and diacylglycerol lipase and monoacylglycerol lipase enzymes with specificity for glycerol with arachidonate in the sn-2 position have also been found (Okazaki et al, 1981b). These results are consistent with those of Okita et al (1982), who found that at term, arachidonic acid was specifically released from PE and PI. The specificity of these enzymes for arachidonate containing lipids may account for the increases in arachidonic acid concentration observed in amniotic fluid of women during labour (MacDonald et al,

1974), which coincided with increases in $PGF_{2\alpha}$ concentrations. Other fatty acids were found to increase less strikingly.

There is some evidence for control of prostaglandin production at other levels than the phospholipase. A prostaglandin synthetase inhibitor has been found in human amniotic fluid (Saeed et al, 1982). Potency of the inhibitor was significantly less during labour than it was at term before the onset of labour. This suggests that, throughout gestation, substances in amniotic fluid may suppress the production of prostaglandins by foetal membranes. The substances may be secreted into the amniotic fluid in foetal urine (amniotic fluid consists mainly of foetal urine). The inhibitory activity of these substances may be overcome at term by the foetus secreting substances into the amniotic fluid that stimulate prostaglandin synthesis. A substance in human foetal urine that will stimulate bovine prostaglandin synthetase activity has been found (Strickland et al., 1983). Samples from foetuses after spontaneous vaginal delivery were found to have more stimulant activity than those from foetuses at term before the onset of labour. These results could explain the decrease of prostaglandin inhibitory activity in the amniotic fluid observed during labour. Foetal urine has also been found to stimulate PGE_2 synthesis in cultured human amnion cells, but not in endometrial stromal cells, glandular epithelium, endometrial carcinoma cells, myometrial cells, or adipose tissue stromal cells (Casey et al., 1983). This suggests that the stimulant activity may be specific for tissues involved in prostaglandin production at parturition. Prostaglandin synthetase activity has also been found to be significantly higher in amnion

from women obtained after the spontaneous onset of labour compared to amnion from women obtained at elective Caesarian section before the onset of labour (Okazaki et al., 1981a).

One other possible level at which prostaglandin synthesis may be controlled is at the level of fatty acid incorporation into phospholipids. Specific incorporation of arachidonic acid into phosphatidylethanolamine and phosphatidylcholine has been observed in human amnion (Schwartz et al., 1977). Arachidonic acid levels have been observed to increase with gestation in cholesterol esters of amniotic fluid (Das et al., 1975), and it has been suggested that the specific incorporation into PE and PC may also occur late in pregnancy (Liggins et al., 1977). Since these may be major sources of arachidonic acid released for the stimulation of parturition, the incorporation of arachidonic acid into these phospholipids in sufficient levels could be an important factor in the production of sufficient quantities of prostaglandin for the stimulation of parturition.

In parturition the prostaglandins must travel a much greater distance from their sites of synthesis to the target tissues (the foetal membranes and uterine decidua to the myometrium and uterine cervix respectively) than in other aspects of placentation (Wickland et al., 1984; Ellwood, 1980). Therefore, at parturition prostaglandin E and F appear to act in a more 'hormonal' capacity than in other prostaglandin mediated processes, diffusing locally into the myometrium and cervix, and coordinating, and possibly inducing, labour by an extracellular, rather than an intracellular, mode of action. This may allow greater diversity in modes of

control of prostaglandin levels reaching their target tissues than in other processes involving prostaglandins.

1.8 Chlamydia, Infectious Abortion and Premature Labour

Microbial infection is a common cause of abortion and premature labour (Silver et al., 1986) and one of the infectious agents known to cause abortion in a number of species, including sheep (Studdert, 1968; Novilla & Jensen, 1970) and man. (Johnson et al., 1985) are the microorganisms of the genus Chlamydia. Cross - species infectivity from sheep to man can also occur (Roberts et al., 1967).

There is widespread distribution of Chlamydiae in both sheep (McEwen et al., 1951; Studdert & Mckercher, 1968) and man (Sweet et al., 1987) and Chlamydiae are now known to be the most common sexually transmitted organism in the USA, with more than three million infections occurring each year (Schachter, 1978). There are two different species of the genus Chlamydia: C. trachomatis and C. psittaci. Chlamydiae are spread mainly by three methods; by direct contact by uninfected persons with eye secretions from infected carriers, sexual intercourse, or by infection of an infant during passage through an infected mothers birth canal. Hosts can be infected by Chlamydiae for long periods of time without any apparent ill effects. The Chlamydiae also have a remarkable ability to evade the hosts' immune system.

Chlamydiae are intracellular parasites that have two distinct forms during their life cycle; the elementary and reticulate bodies. The elementary body is spherical and surrounded by a rigid trilaminar cell envelope similar in composition to those of gram-negative

bacteria, except that the cell walls do not exhibit characteristic endotoxic properties. The elementary body is specially adapted to extracellular survival and is the infectious form of the organism, entering new cells by phagocytosis. The elementary bodies remain within the phagosome where they somehow manage to prevent lysosome fusion. Within 6 to 8 hours after phagosome formation, the elementary bodies undergo a conversion to the second form of the organism, the reticulate body. After 12 hours, binary fision of reticulate bodies begins and from 20 to 24 hours reticulate bodies begin to convert back into elementary bodies. After about 40 hours the host cells begin to die and host cell lysis occurs, releasing the Chlamydiae. It has been suggested that host cell lysosomes release into the cytoplasm hydrolytic enzymes that digest host cell constituents with consequent membrane lysis and release of Chlamydiae (Todd & Storz, 1975).

Although cell envelopes of the elementary bodies do not have endotoxic properties, live elementary bodies exhibit toxicity, the source of which is unknown. The reticulate body is non-toxic, and this may account for the organisms ability to live for long periods in the host without causing any apparent ill effects. Signs of infection often only manifest themselves when the host organism is subjected to stress, nutritional deprivation, or other traumatic events, indicating a delicate balance between host defence and chlamydial pathogenicity. This is also observed during pregnancy, Chlamydiae being latent until late pregnancy. Treatment of chlamydial infection is usually with the drugs chloramphenicol, tetracycline, and rifamcin.

Although infection by Chlamydiae is now known to cause abortion (Studdert, 1968; Studdert and Mckercher, 1968; Aitken, 1986; Johnson et al., 1985) little is known about how it causes this. One likely route is by altering the balance of hormones, such as progesterone, oestrogen, oxytocin, and prostaglandins, which control the onset of parturition under normal conditions. However, very little research has, as yet, been conducted on the effect of chlamydial infection on these hormones (Martel et al., 1983; Rank et al., 1982), although a recent report suggested elevated $PGF_{2\alpha}$ metabolite concentrations in the plasma of four sheep infected with C. psittaci (Fredriksson et al., 1988). In chapter 6 the effects of infection by C. psittaci on PGE_2, progesterone and oestradiol 17ß production in pregnant ewes has been investigated.

1.9 Aims of the Project

The project attempted to answer the following questions:

a) What effects do dietary n3 and n6 fatty acids have on the fatty acid composition of the uterus?

b) To what extent will n3 fatty acids replace n6 fatty acids in uterine tissue when given as the predominant dietary fatty acid source?

c) Do the 20C and 22C n3 fatty acids replace arachidonic acid in the uterus?

d) How quickly does the uterine fatty acid composition respond to changes in dietary fatty acid content and do rats of different ages have different fatty acid composition and metabolism?

e) Is there selective incorporation of fatty acids into specific lipid pools?

f) Does the uterus specifically incorporate, and/or synthesise arachidonic acid?

g) Does the uterine fatty acid composition and response to dietary fatty acid intake change with age?

h) Does the uterus produce the 3-series prostaglandins?

i) What effect does infection by an ovine abortion strain of Chlamydia psittaci have on the release of prostaglandins and hormones important for the control of parturition in pregnant sheep?

Chapter 2

Materials and Methods

2.1 Rats and Diet

Female Sprague - Dawley rats were used in all experiments. Animals were fed either a normal pelleted rat diet (CRM Diet, BSS Ness, Edinburgh, Scotland) or a semisynthetic diet supplemented with one of five different essential fatty acid preparations, which were administered orally at a dose of 1.5 µl/g body wt/day. Animals were divided into different age groups. Adult rats were maintained on the diet from weaning (21 days) until they were between 151 and 586 days old. Young rats were maintained on the diet from weaning to 42-49 days old. The normal pelleted control diet consisted of fat, 8 en%; protein, 20 en%; carbohydrate, 72 en%. The essential fatty acid content of the control pelleted diet consisted of approximately 0.8 en% of n6 fatty acids and 0.01 en% of n3 fatty acids as a proportion of the total calorific value of the diet. The semi-synthetic diet had the following composition: fat approximately 16.5 en%, of which 13.5 en% was saturated fat (hydrogenated coconut oil, Pilsbury's, Birmingham, UK) and approximately 3 en% was essential fatty acid supplement; protein 15.7 en% (fat-free casein, BDH, Poole, UK); Carbohydrate 68.7 en% (sucrose; British Sugar PLC, Peterborough, England) with non-digestible fibre (cellulose 11.02 g/kcal, kaolin 5.5 g/kcal; both Special Diet Services, Cambridge, UK); DL-methionine (2.6 g/kcal; BDH, Poole, England); Vitamin premix (2.76 g/kcal) and mineral premix (12.9 g/kcal) (both Special Diet Services, Cambridge, UK). The percentage fatty acid composition of the hydrogenated coconut oil was C12:0, 55.0%; C14:0, 18.9%; C14:1, 0.17%; C16:0, 7.94%; C16:1, 0.322%; C18:0, 7.0%; C18:1, 2.4%; C18:2n6, 0.76%; C18:3n3, 0.034%; C18:3n6, <0.01%; C18:4n3, <0.01%; C20:0, 0.016%; C20:1, 0.058%; C20:4n6, 0.058%; C20:5n3, <0.01%; C22:0, <0.01%; C22:5n3, <0.01%; C22:6n3, <0.01%.

The five different essential fatty acid supplements were; MaxEPA (Seven Seas Health Care Ltd, Marfleet, Hull), Hi-EPA, evening primrose oil (both Efamol Ltd, Guildford, Surrey), linoleic acid ethyl ester and α-linolenic acid ethyl ester (both >99% pure, supplied by Nu-Chek Prep, Elysian, MN, USA). Supplements were stored under nitrogen to avoid oxidation. Energy content of EFA in the different diets were as follows: Linoleic ethyl ester supplemented diet contained 3.11 en% n6 fatty acids and 0.006 en% n3 fatty acid; linolenic ethyl ester supplemented diet contained 0.11 en% n6 fatty acid and 3.006 en% n3 fatty acid. Fish oil (FO) and evening primrose oil (EPO) supplemented diets contained approximately 3 en% essential fatty acids.

The growth rate and consumption of diet was not significantly different in rats fed the semisynthetic diets with different EFA supplements. There was also no significant difference in weight between rats on the control and the semisynthetic diets.

2.2 Killing of Rats and Uterus Excision

After being on a diet for the required time, rats were killed by cervical dislocation. One of two procedures was then adopted. If the uteri were to be used for measurements of prostaglandin production they were excised under sterile conditions (care being taken to remove any adipose tissue from the uterus) and placed separately into pre-weighed vials containing 5 mls of sterile medium 199 with Hanks salts (Flow Laboratories, Irvine, Scotland) and uterine weights were recorded. If uteri were only to be used for fatty acid analysis then sterile conditions were not used for uterine excision and excised uteri were placed in a pre-weighed vial

containing 5 mls analar methanol (BDH Ltd, Poole, England) and sealed under nitrogen and stored at -30°C. The weight of each uterus was recorded by weighing the uterus in the pre-weighed vial then subtracting the weight of the vial.

2.3 Tissue Culture

Excised uteri in medium 199 (as described above) were transferred to 60 x 15 mm sterile culture dishes (Sterilin, Feltham, UK) containing 3 mls of sterile medium 199 and the tissue cut into 2 mm slices under sterile conditions. Some of the uteri were also incubated with culture medium containing 5 ug/ml of the calcium ionophore A23187 (Sigma Chemical Co., Poole, England). Tissue explants were cultured in a CO_2 incubator at 37°C for 15 hours with the medium renewed at 30 mins, 3 h and 15 h.

2.4 Extraction, Purification and Separation of Prostaglandins

The prostaglandins released into the medium were extracted immediately by adjusting the aqueous phase to pH4 and extracting the acidic prostanoids into ethyl acetate (4 fold volume) (Rathburn Chemicals, Walkerburn, Scotland). The extraction was carried out three times and the ethyl acetate was evaporated. The prostaglandin extract was resuspended in water:methanol (20:1, v:v) and absorbed on C18 Sep-Pak columns (Waters, Millford, Mass., U.S.A.) pre-washed with 20 mls of methanol, then 20 mls distilled water. Polar material was eluted using 10 mls of ethanol:water (15:100, v:v). Prostaglandins were eluted with 5 mls of ethyl acetate. Samples were taken to dryness under a vacuum and resuspended in a mixture of analar chloroform (BDH Ltd, Poole, England):methanol (2:1, v:v) and applied in a line to 20x20 cm thin layer chromatography (t.l.c.)

aluminium sheets coated with silica gel 60 (E. Merck, Darmstadt, West Germany). Prostaglandin standards were also suspended in chloroform:methanol (2:1, v:v) and were applied as spots on one side of the t.l.c. plate. PGE_2 and $F_{2\alpha}$ standards were from Upjohn Ltd, Crawley, England, E_3 and $F_{3\alpha}$ standards were from Cayman Chemical Co., Ann Arbor, Michigan, U.S.A. The solvent system used was Acetone : Ethyl Acetate: Acetic Acid (75:25:1, v:v). The solvent was allowed to move up the plate until it almost reached the top (approximately 1 hour).

The strip of the t.l.c. plate containing the standards was cut from the plate and developed in iodine vapour. R_F values were calculated for the standards (E_2/E_3, 0.81; $F_{2\alpha}/F_{3\alpha}$, 0.69). Areas corresponding to these R_F values on the part of the t.l.c. plate containing the sample were scraped into glass vials containing 6 mls chloroform-methanol (2:1, v:v), the vials shaken thoroughly and then centrifuged at 300 xg for 5 mins in an M.S.E. benchtop centrifuge (Fisons Scientific Equipment, Crawley, England). The supernatants were decanted into pear shape flasks and the procedure repeated twice more. Extracted prostaglandins were sealed under nitrogen and stored at -30°C.

2.5 Conversion of PGE to PGB

Conversion of PGE samples and standards to PGB was carried out by incubation at room temperature with 2 mls of aqueous 1M KOH (BDH, Poole, England) for 45 mins (Bergstrom et al., 1963). The reaction mixture was then adjusted to pH 3-4 using acetate buffer and the PGB extracted twice with glass distilled grade diethyl ether (Rathburn Chemicals Ltd, Walkerburn, Scotland). To ensure that the E to B

conversion was taking place the reaction was monitored using a Cary model 118 U.V. spectrophotometer (Palo Alto , California, USA) at 280 nm absorption. Maximum conversion of E to B was observed after 45 mins of reaction time.

2.6 HPLC of PGB

Some of the PGE samples, after conversion to PGB, were further purified by high pressure liquid chromatograohy (HPLC). The HPLC used was a Gilson model 303 pump and Gilson model 802 manometric module controlled by an Apple II E computer (Apple Comp. Ltd, Cupertino, CA, USA), with a Partisil Px5 10/25 PAC column (Whatman Inc, Clifton, New Jersey, USA), equilibrated overnight with HPLC grade dichloroethane (Rathburn Chemical Co., Walkerburn, Scotland): methanol (100:1, v:v) at a flow rate of 0.5 mls/min. PGB samples were injected and eluted in the same solvent at a flow rate of 1 ml/min. A Perkin Elmer LC55 Spectrophotometer (Beaconsfield, Bucks, England) at 278 nm was used to detect the elution of the PGB, along with a Servoscribe 1s chart recorder (Belmont Instruments, Glasgow, Scotland). PGB standards were run prior to samples to obtain elution times of PGB (figure 2.1 shows HPLC of PGB_2).

2.7 Derivatisation of Prostaglandins

(a) Trimethylsilyl (TMS) derivatives

Prostaglandin standards and samples were methylated by addition of a few drops of diazomethane (generated from "Diazold" - Aldrich Chemical Co. Ltd, Gillingham, Dorset, England) in ether to the dry prostaglandin in an eppendorf tube (Brinkmann Instruments Co., Westbury, N.Y., U.S.A.). The tube was shaken and the diazomethane-

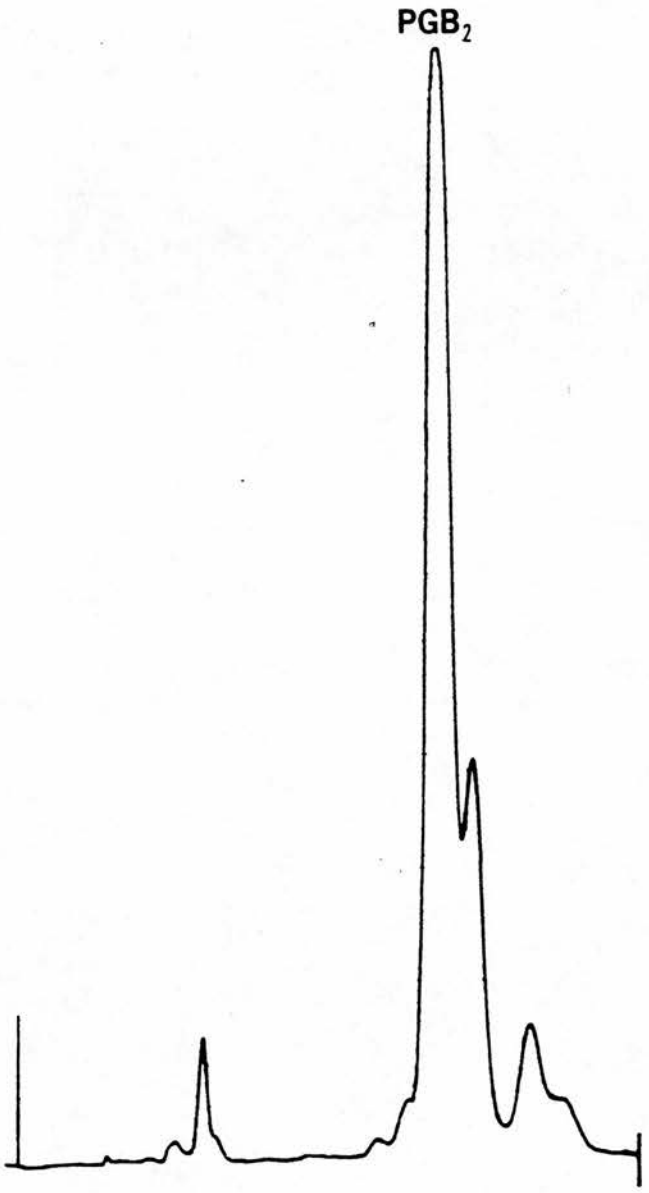


Figure 2.1
HPLC of 20 µg of standard PGB_2. Conditions were as described in section 2.6. Chart paper speed was 0.5 cms/min.

ether evaporated by a stream of nitrogen. Ketone groups of E prostaglandins were converted to butoxime groups by addition of 5 drops of butoxylamine (prepared by Dr N. H. Wilson by the method of Fujii et al, 1967) in pyridine (5 mgs/ml) (Aldrich Chemical Co, Gillingham, Dorset, England) follwed by incubation for 1½ hours at 60°C. Pyridine was then evaporated under a stream of nitrogen. 25 µls of bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (Sigma Chemical Co., Poole, England) was added to all prostaglandin samples and standards and incubated for 15 minutes at 60°C. Figure 2.2 shows an outline of the preparation of prostaglandin TMS derivatives.

(b) Tertbutyldimethylsilyl (TBDMS) Derivatives

TBDMS derivatives were prepared as for TMS derivatives, except that instead of adding TMS, the TBDMS derivative was created by the addition of 2 drops of 4M immidazole (Aldrich Chemical Co. Ltd, Gillingham, Dorset, England) in dimethylformamide (DMF) (Aldrich Chemical Co. Ltd, Gillingham, Dorset, England), followed by 2 drops of 2M tertbutyldimethylsilyl chloride (Aldrich Chemical Co. Ltd, Gillingham, Dorset, England) in DMF and the mixture incubated at 100°C for 1 hour. The derivatised prostaglandin was removed from the DMF by shaking the solution with 0.5 mls of distilled water and 0.5 mls of glass distilled grade ether (Rathburn Chemicals Ltd, Walkerburn, Scotland). The top layer of ether, containing the derivatives, was aspirated and the ether extraction repeated once more. Before injection into the gas chromatograph (GC), the ether was evaporated under a stream of N_2 and the sample resuspended in 50 mls HPLC grade hexane (Rathburn Chemicals Ltd, Walkerburn, Scotland). Butylboronate derivatives were formed when using some

Figure 2.2
Formation of Me,TMS and Me,BuO,TMS Derivatives

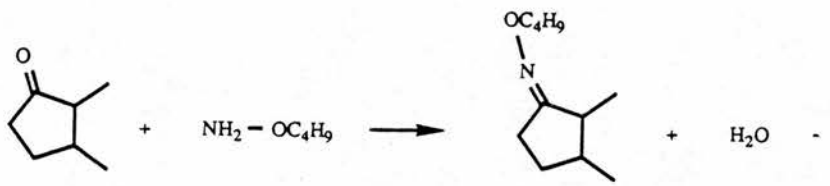
It is essential to form a derivative of the prostaglandin which has the correct volatility and a high thermal stability.

(a) Methylation

The PG is treated with diazomethane to produce the methyl ester.

(b) Butoxime formation

Prostaglandins with ketone groups (such as PGE) usually have the ketone group converted to an alkyloxime group prior to mass spectroscopy. In our experiments we created the butoxime group.



(c) Addition of TMS

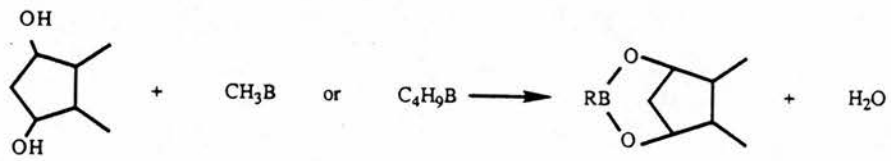
Diazomethane does not attack hydroxyl groups to form the methyl ester in the absence of a catalyst. However, a number of compounds (known as silylating agents) readily form trimethylsilyl esters with free hydroxyl groups. One of these compounds is bis. (trimethylsilyl) trifluoroacetamide (BSTFA).

(d) Addition of TBDMS

The TBDMS creates a heavier derivative than TMS which gives it a greater retention time in the GC. This may allow for greater separation of the prostaglandin from contaminating material in a biological sample.

(e) Formation of Butylboronate Derivatives

F prostaglandins may form very high mass derivatives with TBDMS-Cl due to their having three hydroxyl groups. This often makes GC retention times impractically long. If lighter derivatives are desirable then the two cis-hydroxyl groups of the cyclo-pentane may be converted to the cyclic boronate.



PGF samples to reduce the retention time of the sample in the GC. The boron conjugates were formed by the addition of 5 drops of n-butyl boronic acid (Aldrich Chemical Co. Ltd, Gillinghamm, Dorset, England) before addition of TBDMS at a concentration of 5 mgs/ml in 2,2 dimethoxypropane (Aldrich Chemical Company Ltd, Gillingham, Dorset, England) and the sample incubated at 60°C for 1-2 hours. Figure 2.2 shows an outline of the preparation of prostaglandin Me, n-butylboronate TBDMS derivatives.

2.8 Analysis of Prostaglandins by Combined Gas Chromatography - Mass Spectometry

Analysis was carried out using a Pye Unicam 2000M gas chromatogram and a VG Analytical Micromass 7070F mass spectrometer (MS). The GC was fitted with a fused silica DB1 capillary column (J and W Scientific, Rancho Cordova, California, USA). GC conditions were as follows: Injector temperature - 250°C; detector temperature, 250°C; column temperature, 180° - 280°C with 1 min initial time then rising at 4°C/min. Three different types of detection were used with the mass spectrometer, these were total ion detection (TID), single ion monitoring (SIM) and multiple ion detection (MID).

(a) TID

TID monitors all the ions produced in a sample at any one time. This system is often not sensitive enough for use with biological samples as the substance for analysis is often lost in contaminating material eluting at the same time.

(b) SIM

SIM allows the mass spectrometer to monitor for one single ion or a

small range of ions. This means that the recorder will only show a peak when the particular ion that the mass spectrometer is set for is detected, this allows for greater sensistivity. When monitoring with either TID or SIM, prostaglandin standards were run first to give a retention time for the different prostaglandins. Samples were then run and spectra taken when peaks appeared at the correct time. Spectra were taken as the pen reached the top of the peak. Ions monitored for under SIM were as follows:

(1) Me,TMS derivatives

PGE_2 - 512, 295
PGE_3 - 510, 295
$PGF_{2\alpha}$ - 423
$PGF_{3\alpha}$ - 421

(2) Me,TBDMS derivatives

PGB_2 - 405
PGB_3 - 403
$PGF_{2\alpha}$ - 653
$PGF_{3\alpha}$ - 651

(c) MID

The MID system allows for very accurate detection of a number of different single ions simultaneously. It operates by locking a channel onto a known ion, either produced by one of the samples being analysed or produced by a standard that may be injected directly into the M.S. detector. In the case of PGB (Me,TBDMS) derivatives the 405 PGB_2 ion was used in the lock channel, whereas for PGF (Me,TBDMS) derivatives either the $PGF_{2\alpha}$ 653 ion or the 614 ion of heptacosafluorotributylamine (Aldrich Chemical Co. Ltd., Gillingham, England) was used in the lock channel. Other channels were then set to monitor ions produced by the sample. As the ions produced by the sample are detected, the M.S. locks itself onto the

signal, using the standard substance as a reference, to produce a very accurate measurement of the quantity of sample present. By monitoring for more than one ion, ratios of one ion to another can be obtained to give evidence for the presence of different substances. For example, the procedure for detecting the presence of $PGF_{2\alpha}$ and $PGF_{3\alpha}$ was as follows. After setting the channels appropriately and injecting the standard substance a known quantity of $PGF_{2\alpha}$ (Me,TBDMS) was injected. The channels were set to monitor the 653 ($F_{2\alpha}$) and the 651 ($F_{3\alpha}$) ions. When these ions were detected a multi-penned chart recorder recorded the size of each signal with a separate pen for each ion. Although the 653 ion is the major ion for $F_{2\alpha}$ there is also a small 651 ion produced. A retention time was obtained for standard $F_{2\alpha}$ (which was the same for $F_{3\alpha}$) and a proportion obtained of 651/653 ions taking the 653 ion as 100%. If the calculated proportion for the 651 ion in the sample was the same as that for standard $F_{2\alpha}$ then this suggests there is no $F_{3\alpha}$ present, however, if the 651 proportion increased then this provides evidence for the presence of $PGF_{3\alpha}$.

The main advantages of MID above SIM are that more than one ion can be monitored very accurately without interference from other ions. The main disadvantage is that full mass spectra cannot be taken when MID is in operation.

2.9 Uterine Lipid Extraction

Uteri were placed in a mortar and pestel containing C17:0 internal standard (Sigma Chemical Co., Poole, Dorset, England) and chloroform: methanol (2:1, v:v). The uteri were then pulverised and the chloroform - methanol mixture poured through a glass wool filter

into a pear-shape flask. The mortar and pestel were washed four times with chloroform: methanol (2:1, v:v) and filtered into the flask.

2.10 Neutral Lipid and Phospholipid Separation by Silicic Acid Column Chromatography

Glass columns were packed with 4g Unisil silicic acid (Clarkson Chemical Co. Inc., Williamsport, USA) suspended in ether. Columns were then dehydrated with 20 mls ether, followed by 20 mls ether : acetone (1:1, v:v) (glass distilled grade - Rathburn Chemicals Ltd, Walkerburn, Scotland), followed by a further 60 mls of ether. The sample was added to the top of the column in 1 ml of ether. The tube that contained the sample was washed twice with 1 ml of ether and the ether added to the column. Neutral lipids were washed from the column with 30 mls of ether, 30 mls of methanol were then used to wash the phospholipids from the column.

The efficiency of the separation was tested as follows: 20 mgs of standards of both dioleic PC (approximately 15 mgs of which was fatty acid) and free oleic acid (both Sigma Chemical Co. Poole, Dorset, England) were run on separate columns and neutral lipid and phospholipid fractions collected from both columns. Fractions were then dried under a vacuum and hydrolysed and methylated as described in section 2.15. 4 mgs of heptadecanoic acid methyl ester (Sigma Chemical Co. Poole, Dorset, England) were then added to each fraction and gas chromatography carried out on the samples by the method described in section 2.17. The quantity of phosphatidylcholine recovered was calculated from the quantity of fatty acid detected by the GC, assuming that 15 mgs of the 20 mgs of

phosphatidylcholine was fatty acid, and using the C17:0 internal standard to calculate the total quantity of lipid recovered in each fraction. The results are shown in table 2.1.

Due to the poor recovery of phospholipid from the column two further experiments were tried to see if any improvement could be made. The first involved attempting to wash the phospholipids from the column with a 0.5% acetic acid (BDH Ltd, Poole, England) - methanol mixture instead of 100% methanol. The second experiment involved removing the silicic acid from the column after the neutral lipid fraction had been collected and attempting to hydrolyse the phospholipids from the silicic acid by incubating it in 8 mls 0.5M Aristar grade KOH (BDH Ltd, Poole, England) in methanol for 1 hour at 60°C. However, neither of these methods significantly improved phospholipid recovery.

2.11 Two Phase System for Separation of Neutral Lipids and Phospholipids

A two phase system was also used for the separation of neutral lipids and phospholipids. The two phases were 10 mls 66% HPLC grade ethanol (Rathburn Chemicals Ltd, Walkerburn, Scotland) in distilled water with 10 mls hexane, mixed in a pear-shaped flask. The lipids were added in 1 ml of the ethanol - water mixture and the flasks shaken thoroughly. The phospholipids separated into the ethanol - water layer and the neutral lipids into the hexane layer. The hexane layer was then aspirated into another pear shape flask and a further 10mls hexane added to the ethanol - water mixture and the extraction repeated. The neutral lipids were extracted into hexane a total of four times.

Lipid Fraction	mgs f.a. recovered	% of total f.a. added to column
Phosphatidylcholine phospholipid fraction	3.66	24.4
Phosphatidylcholine neutral lipid fraction	0.68	4.5
Oleic acid phospholipid fraction	0.26	1.3
Oleic acid neutral lipid fraction	18.76	93.8

Table 2.1
Recovery of phospholipids and neutral lipids after silicic acid chromatography. 20 mgs of dioleoyl phosphatidylcholine and 20 mgs of oleic acid were separated in a glass column packed with 4 g of silicic acid. Oleic acid was eluted with 30 mls of diethyl ether and phosphatidylcholine was eluted with 30 mls of methanol. Eluted phosphatidylcholine was hydrolysed with alkali and fatty acids methylated with boron trifluoride in methanol. Fatty acids recovered from the column were identified and quantified by GC analysis using heptadecanoic acid as an internal standard.

The separation of the neutral lipids and phospholipids was tested using [1-^{14}C] stearic acid (neutral lipid) or 1-palmitoyl -2- [1-^{14}C] palmitoyl phosphatidylcholine (phospholipid). Both were from Amersham International PLC, Amersham, Bucks, England. 1.85 KBq ^{14}C Stearate or 1.16 KBq of ^{14}C phosphatidylcholine (PC) were added to the two phase system and the extraction completed as described above. The two phases were then taken to dryness under a vacuum and resuspended in 4 mls NE266 scintillation fluid (Nuclear Enterprises, Edinburgh, Scotland) and radioactivity measured in an LKB Wallac 1216 Rackbeta II beta counter (Turku, Finland) (see table 2.2).

The results show that the separation was successful except for approximately 10% of the phospholipids separating into the hexane fraction. This would clearly contaminate the fatty acid measurements made of the neutral lipids so it was decided to repeat the extraction but this time the ethanol - water phase contained 0.5% analar acetic acid (BDH, Poole, England) to enhance phospholipid ionisation and so decrease solubility in the hexane fraction. The results from this experiment are shown in table 2.3.

The acetic acid decreased the quantity of phospholipid in the hexane fraction to around 8%, to further reduce this, silicic acid chromatography, as described in section 2.1, was used. To test the purification after silicic acid chromatography, an experiment using the two phase system was set up as before, except that only ^{14}C PC was used and after separation the hexane layer was evaporated under a vacuum and resuspended in ether and applied to a column as previously described. Neutral lipids were then washed from the column as before. The ether containing the neutral lipids was

Sample description	cpm	% of total sample recovered
PC No-1 hexane fraction	702	10.4
PC No-2 hexane fraction	733	12
PC No-1 ethanol-water fraction	5716	89.1
PC No-2 ethanol-water fraction	5389	88
Stearate No-1 hexane fraction	17848	98.4
Stearate No-2 hexane fraction	20892	98.5
Stearate No-1 ethanol-water fraction	294	1.6
Stearate No-2 ethanol-water fraction	322	1.5

Table 2.2
Recovery of phospholipids and neutral lipids after two phase separation. 1.85 KBq of ^{14}C stearate and 1.16 KBq ^{14}C labelled phosphatidylcholine were added individually to two phase separation systems containing 10 mls 66% ethanol in distilled water and 10 mls hexane. Stearate was extracted into the hexane fraction four times. Radioactivity in each phase for both samples was measured in a liquid scintillation counter. The experiment was carried out in duplicate.

Sample description	cpm	% of total sample recovered
PC No-1 hexane fraction	962	7.5
PC No-2 hexane fraction	1131	7.9
PC No-1 ethanol-water fraction	11867	92.5
PC NO-2 ethanol-water fraction	13164	92.1
Stearate No-1 hexane fraction	16138	99.7
Stearate No-2 hexane fraction	16799	99.8
Stearate No-1 ethanol-water fraction	54	0.3
Stearate No-2 ethanol-water fraction	31	0.18

Table 2.3
As for table 2.2, except that the ethanol - water fraction contained 0.5% acetic acid.

evaporated under a vacuum and the sample resuspended in 4 mls scintillant and cpm measured. The column removed nearly all the phospholipid that was present in the hexane fraction with greater than 99% of the recovered radiolabel being in the ethanol-water-acetic acid fraction (the results are shown in table 2.4).

The recovery of the phospholipid and neutral lipid from the different fractions was tested by measuring the cpm for 1.16 KBq ^{14}C PC and 1.85 KBq ^{14}C stearate after they had been extracted by the two phase separation followed by silicic acid chromatography for the neutral lipid, and comparing this with the same quantities of ^{14}C stearate and ^{14}C PC placed directly into scintillant and counted on the beta counter. The recovery was found to be 85-90% for the phospholipid and 90-95% for the neutral lipid.

2.12 Separation of Neutral Lipids by Thin Layer Chromatography

A solvent system of petroleum spirit : ether : acetic acid (75:25:1, v:v) was used for separating the neutral lipids. Uterine neutral lipids that had been isolated by the two phase system followed by silicic acid chromatography were suspended in chloroform : methanol (2:1, v:v) and applied to 0.2 mm thick silica gel 60 t.l.c. plates in a line as described in section 2.3. Standards of cholesterol, triglyceride, free fatty acid, diglyceride and monoglyceride were applied as spots on one side of the plate. After developing the plate the different neutral lipids were identified and extracted by the procedure described in section 2.3. Neutral lipids were separated into the following four fractions; cholesterol ester and triglyceride, diglyceride, monoglyceride and a free fatty acid fraction (R_F values are shown in table 2.5).

Sample description	cpm	% of total sample recovered
PC No-1 ethanol-water-acetate fraction	10000	99.7
PC No-2 ethanol-water-acetate fraction	9250	99.6
PC No-1 hexane fraction after chromatography	30	0.3
PC No-2 hexane fraction after chromatography	37	0.4

Table 2.4

Phospholipid recovered in the ethanol water fraction after two phase separation and in the neutral lipid fraction after silicic acid chromatography. The two phase separation was carried as described in the legend for table 2.2. The hexane fraction was dried in a vacuum and resuspended in ether and added to a glass column containing 4 g silicic acid. The column was then washed with 30 mls diethyl ether and radioactivity in this fraction measured in a liquid scintillation counter. The experiment was carried out in duplicate.

Lipid		R_F
Monoglyceride:	Monopalmitin	0.05
	Monoolein	0.05
	Monostearin	0.05
Diglyceride:	Dipalmitin	0.28
	Diolein	0.36
	Distearin	0.36
Free Fatty Acid:	Tricosanoic Acid	0.49
	Arachidonic Acid	0.57
	Myristic Acid	0.62
Triglyceride:	Trilinolein	0.81
	Trilinolenin	0.81
	Triolein	0.87
	Tristearin	0.91
Cholesteryl Stearate		0.95

Table 2.5

R_F values for monoglycerides, diglycerides, triglycerides, free fatty acids and cholesterol esters. Lipids were separated on an aluminium t.l.c. plate coated with silica gel 60 using a solvent system of petroleum spirit : ether : acetic acid (75:25:1, v:v). Lipids were visualised with iodine vapour.

2.13 Separation of Phospholipids by Thin Layer Chromatography

A solvent system was required that would separate phophatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylinositol monophosphate (PIP) and phosphatidylinositol biphosphate (PIP_2). Standards of the above phospholipids (all from Sigma Chemical Co. Ltd, Poole, Dorset, England) were run on t.l.c. plates using different solvent systems to try and obtain the best separation of the phospholipids. Aluminium backed silica gel 60 high performance t.l.c. (h.p.t.l.c.) plates (E. Merck, Darmstadt, West Germany) were used for the phospholipid separation. These were sprayed with a methanol - water mixture (3:2, v:v) containing 1% 'analar' potassium oxalate (BDH Chemicals Ltd, Poole, Dorset) then dried overnight in a drying oven. Standards were spotted onto the plate and separated in a solvent system of chloroform : acetone : methanol : acetic acid : water (40:15:13:12:8, v:v, Jolles et al., 1981). When the solvent front had nearly reached the top of the plate the plate was removed from the solvent and phospholipids visualised in iodine vaspour. The solvent system gave a reasonable separation of all the phospholipids except for PC and PI which ran a similar distance on the plate. To solve this problem it was decided to try and first separate the major phospholipids (PC, PE, PI and PS) using a solvent system of chloroform : methanol : ammonia (Analar; BDH, Poole, England) (65:35:5, v:v). Then to separate the PIP and PIP_2 with a different solvent system. Normal t.l.c. plates that had not been treated with the potassium oxalate solution were used for the first separation and h.p.t.l.c. plates that had been treated with potassium oxalate were used for the second separation. The

chloroform-methanol-ammonia solvent system succesfully separated PC and PE, however, PS and PI did not separate (R_F values are shown in table 2.6). Therefore, PS and PI would have to be separated on the 2nd t.l.c. along with the PIP and PIP_2. A number of slightly different solvent systems were then tested for their ability to separate the four phospholipids. The solvent systems are shown in table 2.7.

K and L were found to adequately separate all the phospholipids, with K giving slightly better separation than L. It was therefore decided to use K for the separation of uterine phospholipids (R_F values for phospholipids using solvent system K are shown in table 2.8). The uterine phospholipids were applied to the t.l.c. plates as previously described and were separated alongside standards as before, first on the chloroform - methanol - ammonia system, after which the PC and PE were detected and removed as in section 2.4. The PI, PS, PIP and PIP_2 were then separated, on the h.p.t.l.c. plates that had been treated with potassium oxalate, using solvent system K and were extracted from the plates.

2.14 Lipid Hydrolysis and Methylation

All lipid samples (except free fatty acids) that were to be analysed by gas chromatography were hydrolysed and methylated according to the procedure of Leaver and Poyser (1981). This was as follows: Solvents were vacuum evaporated from samples and 3 mls 0.5 M KOH in methanol added and the flasks purged with nitrogen and stoppered. Flasks were shaken and incubated at 60°C for 10 mins. Heptadecanoic acid methyl ester was added at this point as an internal standard in the following quantities depending on the lipid fraction: Total

Phospholipid	R_F
Phosphatidylinositol	0.12
Phosphatidylinositol Monophosphate	0.12
Phosphatidylinositol Biphosphate	0.12
Phosphatidylcholine	0.38
Phosphatidylethanolamine	0.56

Table 2.6
R_F values for phospholipids separated on an aluminium backed t.l.c. plate coated with silica gel 60, using a solvent system of chloroform : methanol : ammonia (65:35:5, v:v). Phopholipids were visualised with iodine vapour.

Solvent system	Chloroform	Acetone	Methanol	Acetic acid	Water
A	25	0	15	4	2
B	40	15	13	12	8
C	40	16	8	8	4
D	40	20	10	10	6
E	40	18	10	8	6
F	40	23	10	10	8
G	40	26	10	12	8
H	40	20	10	10	8
I	40	21	10	10	10
J	40	20	10	11	6
K	40	20	12	11	7
L	40	21	13	12	8

Table 2.7
Solvent systems tested for separation of PS, PI, PIP and PIP_2 by t.l.c. Aluminium backed silica gel 60 high performance t.l.c. plates pretreated with 1% potassium oxalate were used for separation. Samples were applied in chloroform - methanol (2:1, v:v). After development phospholipids were visualised with iodine vapour.

Phospholipid	R_F
Phosphatidylinositol Biphosphate	0.22
Phosphatidylinositol Monophosphate	0.33
Phosphatidylinositol	0.55
Phosphatidylserine	0.73

Table 2.8

R_F values for phospholipids separated on aluminium backed silica gel 60 high performance t.l.c. plates, pre-treated with 1% potassium oxalate in a methanol-water mixture (3:2, v:v), using a solvent system of chloroform : acetone : methanol : acetic acid : water (40:20:12:11:7, v:v). Iodine vapour was used to visualise phospholipids.

lipid, 0.6 mgs; phospholipid, 0.5 mgs; neutral lipid, 0.1 mgs; PE, 0.2 mgs; PC, 0.2 mgs; PI, 0.05 mgs; triglyceride-cholesterol ester, 0.1 mgs; diglyceride, 0.05 mgs; monoglyceride, 0.05 mgs; free fatty acid, 0.01 mgs. 3.5 mls boron trifluoride (BDH, Poole, England) was then added, the flasks purged with nitrogen again and stoppered and shaken. After incubation at 60°C for a further 5 mins, 3 mls saturated sodium chloride solution and 10 mls 40-60° petroleum spirit (glass distilled grade, Rathburn Chemicals Ltd, Walkerburn, Scotland) were added. The flasks were shaken thoroughly and the petroleum spirit, containing the fatty acids, aspirated into pear shape flasks. Petroleum spirit extraction was repeated a further three times. Samples were then taken to dryness in a vacuum and resuspended in a small volume (approximately 250 µl) of h.p.l.c. grade hexane (Rathburn Chemicals Ltd, Walkerburn, Scotland).

2.15 Free Fatty Acid Methylation

Free fatty acid samples were placed in small glass test tubes (volume 5 mls) and taken to dryness under a stream of nitrogen and five drops of diazomethane added. The tubes were gently shaken for approximately 20 seconds and the diazomethane evaporated under a stream of nitrogen. The samples were then resuspended in 250 µl of hexane.

2.16 Gas Chromatography

Samples in hexane were injected into a Pye 204 gas chromatograph with flame ionisation detector fitted with a 15 metre DB wax bonded carbowax capillary column (J and W. Scientific, Rancho Cordova, California, USA). Helium (British Oxygen Corporation, Guildford, England) was used as a carrier gas (5 ml/min). Injector and

detector temperatures were 175 and 250°C respectively. Oven temperature was programmed at 180°C for 1 min, then rising to 230°C at 4°/min, where the temperature was maintained until the sample, was fully eluted. Fatty acids detected were recorded on a Venture Servoscribe 1s chart recorder (Belmont Instruments, Glasgow) and a supergrator 1A integrator (Kentronix UK Ltd, Compton, Berkshire, England). Fatty acid methyl esters from samples were identified by comparison with retention times of standards (some short chain fatty acids that were commercially unobtainable as standards were tentatively identified by extrapolation from graphs of carbon number against log retention time of standard fatty acids). Each sample was analysed in duplicate and fatty acid percentages and quantities taken as the mean of the two results obtained. The mean variation for a large peak (6-12%) measured ten times in duplicate was 0.8% ± 0.6, the mean variation for a small peak (0.1-1%) was 10.5% ± 6. Figure 2.3 shows the GC separation of fatty acid standards.

2.17 Experimental Infection of Sheep

Twenty-two Scottish Blackface ewes were infected by subcutaneous innoculation with between 0.45×10^6 and 0.5×10^6 egg LD_{50}(ELD_{50}) of an ovine abortion strain (no. S26/3) of C. psittaci between day 90 to 115 of pregnancy. Uninfected pregnant ewes were used as controls. The number of foetuses was determined in all animals between day 60 to 80 of gestation using ultrasonography, and at birth. Infection and sampling of sheep was carried out by I.D. Aitken, B.W. Appleyard, I.E. Anderson, G. Jones, L.A. Hay, G.E. Williams and D. Buxton of the Moredun Research Institute.

2.18 Amniotic, Allantoic and Utero-Ovarian Vein Samples for PGE_2 Assays

Twelve Scottish Blackface ewes were implanted with indwelling catheters into the amniotic and allantoic cavities and into the utero-ovarian vein on day 113 of gestation (Mellor DJ, 1970; Mellor DJ, 1980). Amniotic and allantoic sacs of each foetus were catheterised using Folex two-way balloon catheters (size 12 Ch, with

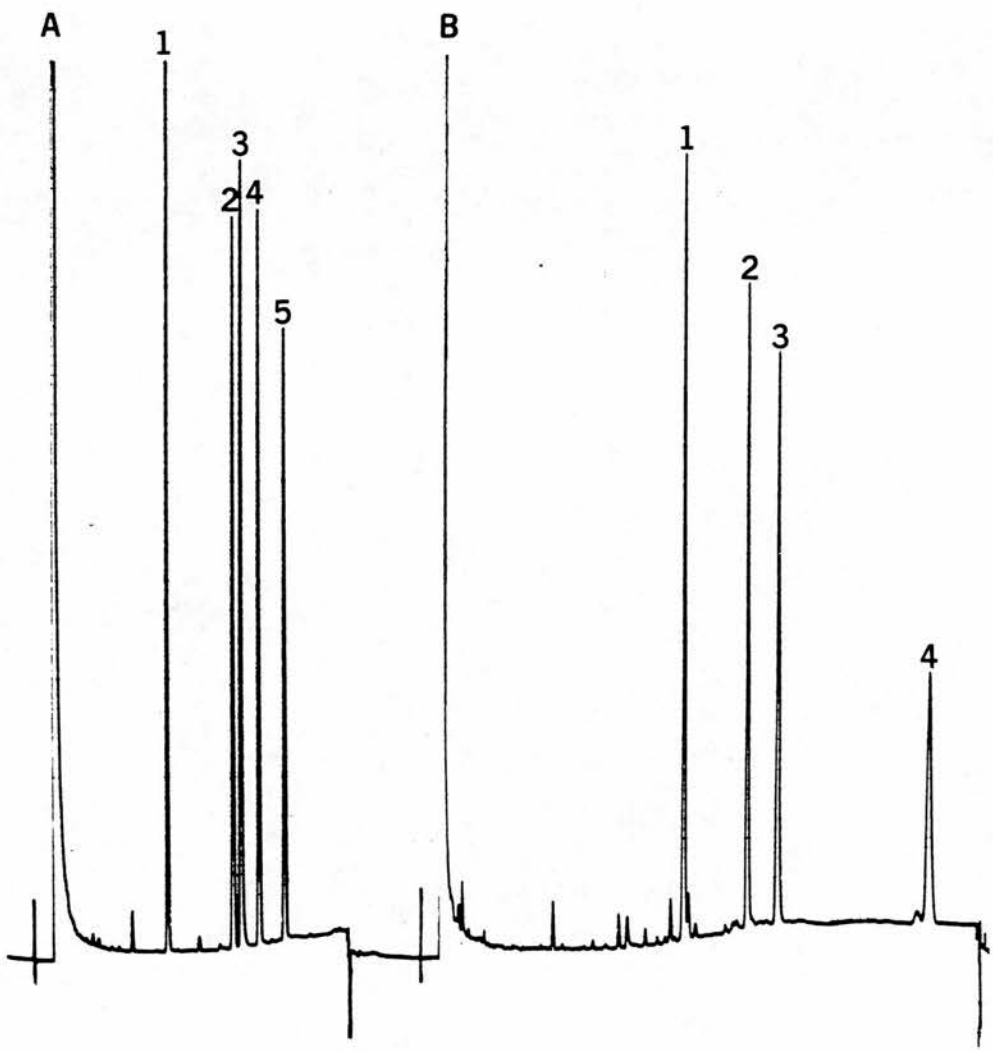


Figure 2.3
Separation of methyl ester fatty acid standards by gas chromatography. See section 2.16 for GC conditions. Chart speed was 0.5 cms/ min.
Peaks numbered in A and B correspond to the following fatty acids:
A- peak 1, 16:0; peak 2, 18:0; peak 3, 18:1n9; peak 4, 18;2n6; peak 5, 18:3n3.
B- peak 1, 18:4n3; peak 2, 20:4n6; peak 3, 20:5n3; peak 4, 22:6n3.
Column number of theoretical plates = 58774.

30-40 ml balloon; Eschmann, Sussex, England). A utero-ovarian vein was also catheterised using 1.4 mm external diameter vinyl tubing (Portex Ltd, Kent, England). A two-way luer stopcock was attached to each catheter. Vascular catheters were sampled daily and flushed with a heparin saline solution (80 I.U. ml^{-1} preservative-free heparin; Evans Medical, Dunstable, Beds) twice daily. Blood was placed into tubes containing 2 $I.U.ml^{-1}$ preservative-free heparin, and plasma was prepared by centrifugation of the heparinised sample at 2000xg for 10 minutes. All plasma samples were stored at -40°C. Peripheral plasma samples were taken from the jugular vein and prepared and stored as for the utero-ovarian vein samples. Small samples (0.5-2 ml) of amniotic and allantoic fluids were withdrawn using minimal suction. Sterility within each 2-way tap was maintained by twice daily flushing with thiomersal solution, consisting of thiomersal 0.1% alcohol (British Drug Houses, Poole, Dorset): acetone: ethanol (1:500:500, v:v). On day 115 of gestation, six ewes were infected by subcutaneous injection of 4.5×10^{9} ELD_{50} of an ovine abortion strain of C. psittaci, and six controls injected with sterile saline. Single samples of amniotic fluid, allantoic fluid or utero-ovarian vein were taken from each sheep on alternate days up to day 130 of gestation, and daily from day 130 until parturition. Occasionally, it was not possible to withdraw samples on the required day, particularly from the allantoic cavity, due to catheter blockage. Amniotic fluid and allantoic fluid was placed immediately into 10 mls of analytical reagent grade methanol, and stored at -40°C. Samples which were stained with blood or meconium (Leaver et al., 1988) were discarded. Utero-ovarian plasma (0.2 mls) was immediately extracted using 2 mls

of redistilled diethyl ether, vortex mixed, then immersed in acetone/dry ice until the aqueous layer was frozen. The organic layer was decanted, and the extraction was repeated three times. The extraction of PGE_2 was 94-99% using this technique. Samples were stored at -40°C before radioimmunoassay. The mean duration of gestation was 144.5 ± 1.1 days in the control group, and 141.3 ± 1.8 days in the infected group, and the difference between these gestation periods was not significant. In the control group, three of the ewes had twins and three had single lambs, and in the infected group, four of the ewes bore twins and two bore single lambs. In the Chlamydia-infected group, two out of ten lambs were born dead: one of twin lambs was dead on delivery, and another, single lamb, died of asphyxia during delivery. In the control group, all nine lambs survived.

2.19 Peripheral Plasma for Progesterone Assays

Six ewes were infected on day 90 of gestation by subcutaneous injection with 5×10^5 ELD_{50} of C. psittaci strain no. 26/3, and six control ewes were injected with sterile saline. The mean duration of gestation ± SE in control sheep was 144.7 ± 1.08 d (n=6), range 144-149 d, and in C. psittaci-infected sheep, 137 ± 1.6 d (n=6), range 132-142 d. Only sheep with single lambs were used, as twin pregnancies are associated with higher progesterone levels, which are not directly proportional to the number of lambs (Bedford et al., 1972; H.A. Leaver, unpublished observations). Blood was withdrawn at 3 day intervals from the jugular vein of infected and control sheep up to day 110 of gestation and at 2 day intervals after day 110, and placed into tubes containing 2 I.U. ml^{-1}

preservative-free heparin (Evans Medical, Dunstable, Beds). Plasma was prepared by centrifugation of the heparinized sample at 5000 r.p.m. for 10 min. All plasma samples were stored at -40°C. Progesterone concentrations were expressed as the means ± SE in samples of plasma taken from 3 to 6 individual control or infected sheep on the same day of gestation.

2.20 Peripheral Plasma Samples for Oestradiol 17β Assays

Oestradiol 17β was analysed in peripheral plasma samples collected from sheep used for progesterone and PGE_2 samples (see above). In the sheep used for PGE_2 sampling, blood was withdrawn from the jugular vein at 24 h intervals during the last three days of gestation, and placed into tubes containing 2 I.U. ml^{-1} preservative-free heparin (Evans Medical, Dunstable, Beds). Plasma was prepared by centrifugation (see above) and stored at -40°C. Oestradiol 17β concentrations were expressed as pg ml^{-1} ± SE in the plasma of control or infected sheep, sampled on separate days before parturition.

2.21 Utero-Ovarian Venous Plasma, Amniotic Fluid and Allantoic Fluid Samples from Catheterised Animals for Oestradiol 17β Assays

The intrauterine distribution of oestradiol 17β during chlamydial infection was analysed in the six control sheep injected with saline, and in the six sheep infected with $5x10^5$ ELD_{50} of *C. psittaci* strain S26/3 on day 115 of gestation that were used for PGE_2 samples (see section 2.19).

2.22 Prostaglandin E_2 Radioimmunoassay

A double antibody radioimmunoassay was used, the procedure for which was as follows. Standard PGE_2 (Upjohn, Crawley, England) was prepared in HEPES buffer (pH 7.4) in triplicate at doubling concentrations from 0.0025 ng/ml to 2.56 ng/ml. These standards were used for production of the standard curve. Samples suspended in solvent (methanol for amniotic and allantoic samples, ethyl acetate for utero ovarian vein) were added in triplicate to 3 ml plastic tubes (Sarstedt, Numbrecht, West Germany - also used for standards) and the solvent dried under a stream of nitrogen. The sample was resuspended in 250 µl of buffer. To each tube was added 50 µl of rabbit anti-PGE_2 antibody (Institut Pasteur, Paris) followed by 50µl of ^3H PGE_2 tracer [5,6,8,11,12,14,15 (n)-3H] (Amersham International PLC, Buchinghamshire, England) which had been diluted in buffer to give a concentration that would provide approximately 1500 cpm per 50 µl aliquot in 3 mls scintillation fluid. The tubes were vortexed and left to incubate at room temperature. After 2 hours incubation 50 µl of a 1/140 dilution of normal rabbit serum (Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland) and 50 µl of donkey anti-rabbit serum (also Scottish Antibody Production Unit) were added. After overnight incubation at 4°C the tubes were centrifuged at 4°C for 30 mins at 1800xg in an M.S.E. Coolspin Centrifuge (Fisons Scientific Equipment, Crawley, England), the supernatant discarded and the pellet resuspended in 3 mls NE 266 scintillation fluid (Nuclear Enterprises, Edinburgh, Scotland). Each tube was counted for 10 minutes in an LKB Wallac Rackbeta II beta counter (Turku, Finland).

In addition to the Pasteur anti-PGE_2 antibody one other anti-PGE_2

antibody was tried in the assay, which was a monoclonal antibody donated by Dr Ivanyi, Wellcome Laboratories. However, this antibody was found to have very high cross reactivity and produced very high estimates of PGE_2 in the samples so it was decided only to use the Pasteur antibody (Cross reactivity of the Pasteur antibody is shown in table 2.9). Problems also arose, however, with the Pasteur antibody. Different batches of the Pasteur antibody were found to have widely differing binding activities with twice the quantity of antibody being required from some batches compared to others to maintain a 50% bound figure for the assay zero standard. Of the different batches of lyophilised Pasteur PGE_2 antibody used, lots D_2 and D_7 were suspended in 11 mls buffer and lots D12 and D13, due to reduced binding activity, were suspended in only 5.5 mls buffer.

Different dilutions of donkey anti-rabbit serum (DARS) were tested to find the concentration that gave approximately 50% binding of ^3H PGE_2 in the zero standard, again of two different batches used different activities were observed, as shown in table 2.10. A 1/10 dilution was used for batch 0907J and a 1/8 dilution for batch 5104L.

Two different buffers, HEPES and phosphate, both at pH 7.4 were tested. 100 mls HEPES buffer contained 0.48 g HEPES powder (94% pure; Aldrich Chemical Co. Gillingham, England), 0.01 g analar sodium azide (BDH Chemicals Ltd, Poole, England) and 0.1g gelatin. 100 mls phosphate buffer contained 0.69 g disodium hydrogenorthophosphate, 0.17 g sodium dihydrogen orthophosphate (both analar grade; BDH Chemicals Ltd, Poole, Dorset, England), 0.01 g sodium azide and 0.1 g gelatin. These were tested to see which

Compound	% Cross Reactivity	Compound	% Cross Reactivity
PGA_1	0.04%	PGA_2	0.3%
PGB_1	<0.01%	PGB_2	<0.01%
PGD_1	<0.01%	PGD_2	<0.01%
PGE_1	6.5%	PGE_2	100%
DH PGE_1	0.01%	DH PGE_2	2.1%
K PGE_1	0.16%	K E_2	13.2%
DHK PGE_1	0.03%	DHK E_2	0.6%
$PGF_{1\alpha}$	0.01%	$PGF_{2\alpha}$	0.11%
K $PGF_{1\alpha}$	<0.01%	DH $PGF_{2\alpha}$	<0.01%
DHK $PGF_{1\alpha}$	<0.01%	K $PGF_{2\alpha}$	<0.01%
19OH PGE_1	<0.01%	DHK $PGF_{2\alpha}$	<0.01%
6 keto $PGF_{1\alpha}$	<0.01%	19OH $PGF_{2\alpha}$	0.019%
		Thromboxane B_2	<0.01%
		6, 15 diketo PGE_2	<0.01%

Table 2.9

Cross reactivity for B/Bo = 0.5 of rabbit anti-PGE_2 antibody.
(DH = 13, 14 Dihydro; K = 15 keto)
3-series prostaglandins were not available for cross reactivity measurements.

	DARS Stock Dilution				
	1/5	1/6	1/8	1/10	1/15
DARS Batch 0907J	30%-40%	-	-	40%-50%	40%-50%
DARS Batch 5104L	-	50%-60%	50%-60%	45%-55%	35%-40%

Table 2.10
The effect of different donkey anti - rabbit serum (DARS) batches and dilutions on the percentage of $^3HPGE_2$ bound in the PGE_2 radioimmunoassay zero standard. HEPES buffer was used for dilution of antibody stock solutions.

one would provide the highest % bound figures for zero standards, HEPES was found generally to give better results and so was used in the assays (see table 2.11).

Included in each assay were three tubes to measure non-specific binding (NSB) of tracer in the assay. These tubes contained all the normal ingredients of the assay except for the anti-PGE_2 antibody. An extra 50 µl of buffer was added to make up the volume. The addition of an extra 0.5 mls of buffer to some non-specific binding tubes was tested to see if a greater total volume would reduce non-specific binding, however, no significant difference was observed (see table 2.12).

Intra-assay variability was measured by repeating standards from the middle of the standard curve (10-40 pg/tube) at the end of the assay and comparing cpm with the standards from the beginning of the assay. Inter-assay variability was monitored by repeating the assay of two samples that had been assayed previously and the estimates from the different assays compared. The mean inter-assay coefficient of variation was 13.9% $\pm$ 1.98 (n=7) and the mean intra-assay coefficient of variation was 10.8% $\pm$ 1.04 (n=15). The sensitivity of the assay was 0.28 pg PGE_2 ml^{-1} of plasma in utero-ovarian vein samples and 0.401 pg PGE_2 ml^{-1} in amniotic and allantoic samples.

2.23 Progesterone Radioimmunoassay

Peripheral plasma progesterone was extracted using ethyl acetate (efficiency 71 $\pm$ 3%). Progesterone was determined by a radioimmunoassay using the antiserum and technique of Scaramuzzi et

	HEPES	Phosphate
RIA No-1	69±1.4	62±1.5
RIA No-2	38±5	50±3
RIA No-3	50±3	52±2
RIA No-4	66±1	41±13

Table 2.11

Comparison of percentage ^{3}HPGE$_2$ bound in zero standards of the PGE$_2$ radioimmunoassay. Rabbit anti-PGE$_2$ antibody, donkey anti-rabbit serum, normal rabbit serum, and ^{3}HPGE$_2$ were diluted in HEPES or phosphate buffers at pH 7.4 and the percentage ^{3}HPGE$_2$ bound in zero standards for four different radioimmunoassays was determined. Results are expressed as means ± standard deviation for three determinations.

	0.5 ml buffer added % bound ± S.D.	0.5 ml buffer not added % bound ± S.D.
RIA 1	6.2±4	4.6±0.2
RIA 2	3.6±0.7	3.4±0.8
RIA 3	2.0±0.3	3.3±0.3
RIA 4	2.9±1.3	5.2±0
RIA 5	2.2±0.6	4.5±2.8

Table 2.12
The effect of adding 0.5 mls of buffer to reaction tubes prior to centrifugation on non-specific binding in the PGE_2 radioimmunoassay. Non-specific binding was tested by adding all the normal ingredients of the radioimmunoassay with the exception of the anti-PGE_2 antibody. Therefore any residual radioactivity after centrifugation and removal of the supernatant was a result of non-specific binding. Results show the mean percentage of $^3H PGE_2$ bound ± standard deviation for three determinations in five radioimmunoassays.

al. (1974), and (1,2,6,7,16,17-^{3}H) progesterone radiotracer (Amersham, Bucks, batch no. 10/H/4723). Antibody-bound progesterone was precipitated using dextran charcoal. The precision of progesterone determination was 10.2% for within-assay duplication (intra-assay coefficient of variation), and 9.6% for between-assay replication (inter-assay coefficient of variation), for two plasma samples analysed six times within the same assay (n=6), and two plasma samples analysed in six different assays (n=6), respectively (Hunter, 1978). The progesterone radioimmunoassay was carried out by Dr H.A. Leaver.

2.24 Oestradiol 17β Extraction from Plasma

Prior to assay of some of the samples, oestradiol was extracted and concentrated five times by the following method: 250 mls of plasma was placed in a glass tube and 2 mls of glass distilled grade ether added. The tubes were shaken for 5 minutes to extract the oestradiol into the ether and the aqueous and organic layers allowed to separate. Card-ice and acetone were mixed in equal quantities and the aqueous layer frozen by touching the bottom of the tube in the card-ice-acetone mixture for a few seconds. The organic layer (containing the oestradiol 17β) was then poured off into another glass test tube, the ether blown off with a stream of air and the oestradiol 17β resuspended in 50 µl of zero standard plasma.

The efficiency of extraction of oestradiol was measured using tritiated oestradiol 17β as follows, 50 µl of tritiated oestradiol (Amersham International PLC, Amersham, Bucks, England) in analytical grade methanol was dried under a stream of air and resuspended in 250 µl of sheep plasma. The extraction was then carried out as

above and the extracted oestradiol resuspended in scintillant and cpm measured in a beta counter. 10 separate extractions were carried out and the cpm obtained compared with that for two 50µl tritiated oestradiol samples that had not undergone the extraction procedure. The results are shown in Table 2.13. The mean extraction efficiency was 80.01 ± 0.52% (S.E.).

2.25 Oestradiol 17β Radioimmunoassay

Utero-ovarian vein plasma, peripheral plasma, and amniotic fluid samples were all assayed for oestradiol 17β. Utero-ovarian vein and peripheral plasma samples were either assayed directly or extracted and concentrated as described. Amniotic and allantoic samples were in methanol so they were taken to dryness under a stream of air and resuspended in 50 µl zero standard plasma. The assay involved a solid phase double antibody method and was supplied as a kit from Steranti Research Ltd, St. Albans, Herts, England. Each assay included both intra- and inter-assay quality controls. Intra-assay variability was tested by assaying the same sample both at the beginning and at the end of the assay and comparing the results. The same sample was then assayed in the next assay for inter-assay precision. Assay accuracy was also measured by assaying three samples taken from three different pools of plasma of known oestradiol concentration. The pools were low (32.4 pg oestradiol/ml), medium (84.5 pg/ml) and high (244.1 pg/ml). Inter-assay and intra-assay coefficient of variation were 11.4% and 6.83% respectively (n=6). Assay sensitivity was 3.1 pg oestradiol ml^{-1} at 2.5 standard deviations from the zero standard value. Figure 2.4 shows an oestradiol 17β radioimmunoassay standard curve.

Tube Contents	cpm	Oestradiol recovery as % of mean counting standard	Mean % recovery ± S.E.
Extracted ^{3}H-Oestradiol No-1	7360.3	82.6	80.01±0.52
Extracted ^{3}H-Oestradiol No-2	7343.7	82.4	
Extracted ^{3}H-Oestradiol No-3	6955.7	78.1	
Extracted ^{3}H-Oestradiol No-4	7084.7	79.5	
Extracted ^{3}H-Oestradiol No-5	7044.7	79.1	
Extracted ^{3}H-Oestradiol No-6	7270.3	81.6	
Extracted ^{3}H-Oestradiol No-7	7033.3	78.9	
Extracted ^{3}H-Oestradiol No-8	7048.0	79.1	
Extracted ^{3}H-Oestradiol No-9	7160.0	80.4	
Extracted ^{3}H-Oestradiol No-10	6988.7	78.4	
^{3}H-Oestradiol counting standard No-1	8760.3		
^{3}H-Oestradiol counting standard No-2	9058.3		
Background	11.7		

Table 2.13

Extraction efficiency of ^{3}H oestradiol 17β into diethyl ether. ^{3}H oestradiol 17β in 250 µl of sheep plasma was shaken with 2 mls of acetone and the mixture allowed to separate into two layers. The aqueous layer was frozen in an acetone - card-ice mixture and the acetone poured off. ^{3}H oestradiol 17β present in the acetone layer was measured in a liquid scintillation counter.

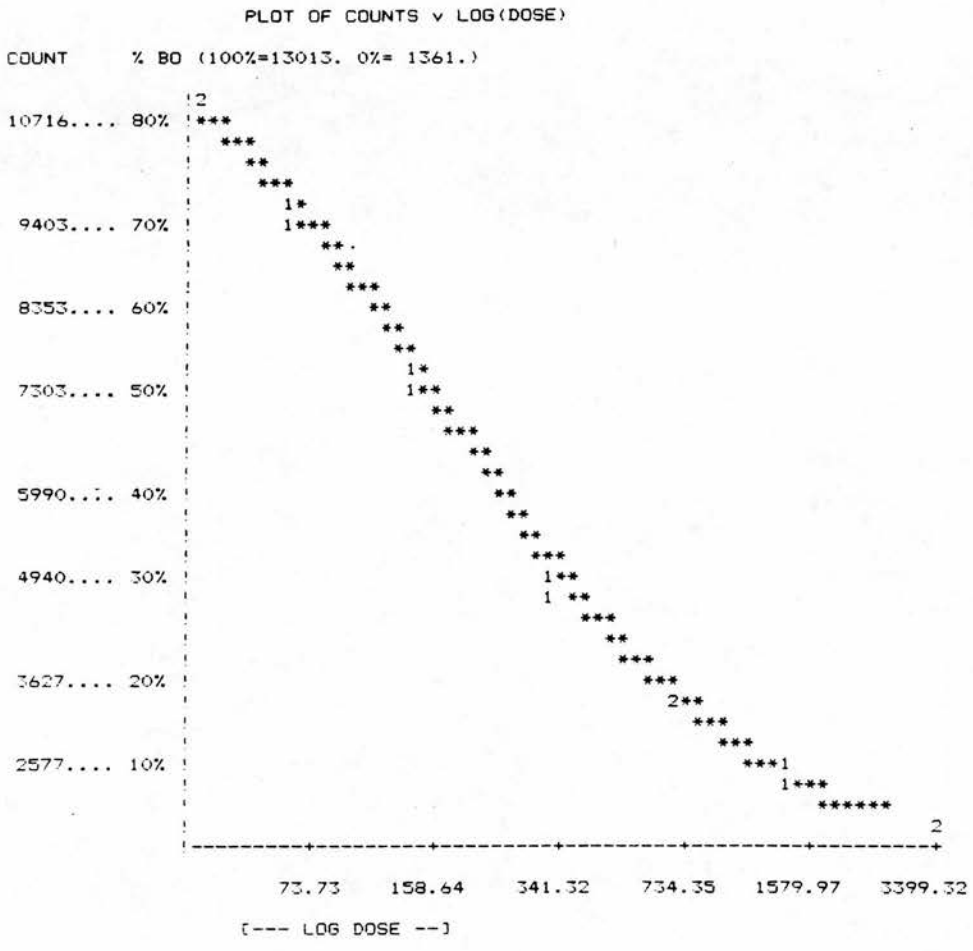


Figure 2.4
Standard curve for oestradiol 17ß radioimmunoassay. See section 2.25 for description of assay technique.

Chapter 3

The Influence of Dietary n3 and n6 Fatty Acids on the Uterine Fatty Acid Composition of Rats of Different Ages

3.1 Introduction

Arachidonic acid and prostaglandins are released from the uterus at parturition (Leaver et al., 1987; MacDonald et al., 1974; Ogburn et al., 1980; Olund & Lunell, 1980). Arachidonic acid is the precursor of $PGF_{2\alpha}$ and PGE_2 which have a crucial role in the initiation of parturition (Thorburn & Challis, 1979). Inhibitors of prostaglandin production will block parturition in almost every species tested to date, whereas infusions of $PGF_{2\alpha}$ and PGE_2 will initiate it (Aitken, 1972; Lewis & Schulman, 1973; Thorburn & Challis, 1979). Quackenbush et al., (1942) were the first to demonstrate the role of the n6 EFA in parturition, it was found that a diet high in n3 fatty acids caused inhibition of the initiation of parturition (Quackenbush et al., 1942; Leaver et al., 1986). However, in animals fed a diet high in n3 fatty acids, normal foetal development was observed, and if Caesarian section was carried out early in labour live animals were delivered (Leat & Northrop, 1981), suggesting that only labour was inhibited by high n3 fatty acid content.

In the rat the main site of fatty acid elongation and desaturation is the liver. Studies on this tissue have illustrated a competetive inhibition between n3 and n6 EFA for the desaturase and elongase enzymes (Aitken, 1972; Bernert & Sprecher, 1975; Brenner & Peluffo, 1966; Brenner & Peluffo, 1967; Brenner, 1974; Nassar et al., 1986; Rahm & Holman, 1964). A similar competition has been observed in

the production of prostaglandins from n3 and n6 EFA by enzymes of the cyclooxygenase system (Magrum & Johnston, 1983; Lokesh & Kinsella, 1987; Hwang & Carrol, 1980; Hwang et al., 1988; Corey et al., 1983). n3 fatty acids are not readily converted to prostaglandins by cyclooxygenase enzymes, so in a diet containing high quantities of n3 fatty acids there may be low prostaglandin production resulting in impairment of parturition.

The rate determining step of the essential fatty acid elongase - desaturase enzyme system is the D6 desaturase step, which in n6 fatty acids converts linoleic acid to gamma-linolenic acid. Evening primrose oil contains relatively high quantities of gamma-linolenic acid which may form a good source for production of arachidonic acid in the uterus due to the rate determining D6 desaturase step not being required for arachidonate production. D5 desaturase activity is equally important for arachidonic acid production. A number of tissues of different species have also been shown to accumulate 20:3n6, suggesting low D5 desaturase activity, including the testes (Ayala et al., 1973), thymus (Horrobin, 1980), vesicular glands (Lands & Samuelsson, 1968) and adrenals (Willis, 1981; Moore & Williams, 1966, Takayasu et al, 1970). It has also been suggested that although some cells, such as platelets, contain far greater quantities of arachidonic acid than 20:3n6, in the free fatty acid fraction, the difference may be much less (Marcus et al., 1969), suggesting that, at least in the resting state, much of the arachidonic acid is not available for prostaglandin synthesis.

The uterine production of PGE_{2} and $PGF_{2\alpha}$ from arachidonic acid is

very important at the time of parturition (Leaver et al., 1987; Lewis & Schulman, 1973; Ogburn et al., 1980; Olund & Lunell, 1980). Inhibition of either arachidonic acid synthesis or conversion to prostaglandins by high n3 concentrations in the uterus could cause impairment of parturition. Also, if levels of 20:3n6 accumulate in the uterus this may cause competition between 20:3n6 and arachidonic acid for cyclooxygenase enzymes, although PGE_1 formed from 20:3n6 will induce uterine contractions (Villar et al., 1985). Cellular lipid composition and enzymes involved in lipid metabolism have been shown to change with age. In the liver of rats HMG-CoA reductase activity decreases with age (Choi & Sugano, 1988) and in rabbit alveolar macrophages increases in unsaturated to saturated fatty acid ratios were observed as well as increases in the neutral lipid fatty acid pool, during the first month of life. After this no further changes were observed up to 150 days of life (Ricardo et al., 1986). Plasma membranes of the macrophages were also found to be more fluid in rabbits of 1 to 14 days old compared to older animals and during the first 30 days of life increases in membrane content of cholesterol and sphingomyelin were observed (Ricardo et al., 1986). These increases may account at least in part for the observed decreases in fluidity.

Differences in fatty acid desaturase activity in rats of different ages have also been observed. In the rat foetus and placenta low D6 desaturase or D5 desaturase activity has been reported, and there is evidence that young animals seem to aquire their long chain essential fatty acids by transport from the maternal circulation (Mercuri et al., 1979). In the rat foetal brain some D6 desaturase

activity has been detected, which increased rapidly after birth, but this fell to very low levels by 4 weeks of age and remained very low in the adult (Cook, 1978). In the liver, however, the opposite was found. Low levels of D6 desaturase were detected at birth, rising sharply on weaning (Cook, 1978), but even in the liver levels were very low by one year of age, Choi and Sugano (1988) also reported a reduction in liver D6 desaturase activity from 8 week old rats to adults fed on an EPO containing diet. In the testes D6 desaturase activity has also been found to decline rapidly at 3-6 weeks after birth in the young rat (Peluffo et al., 1970; Ayala et al., 1973). Reductions in D6 desaturase activity have been suggested to be one of the causes of ageing (Horrobin, 1981).

We studied the effect of diet and age on the metabolism of fatty acids in the uterus of the rat, using rats of three different ages; newly weaned, young, and adult, fed on three different diets; a control diet, a semisynthetic diet supplemented with evening primrose oil (EPO) and a semisynthetic diet supplemented with fish oil (FO).

3.2 Materials and Methods

Female Sprague - Dawley rats were divided into three age groups; Adult (151 - 586 days, mean age 231 days, n=39), young (42 - 49 days, n=15) and newly weaned (21 - 25 days, n=10). Rats from adult and young groups were randomly divided into three diet groups and were fed either a pelleted diet, or a semisynthetic diet supplemented with evening primrose oil or fish oil (see section 2.1 for composition of pelleted and semi-synthetic diets). Adult rats were used for further experiments not reported here, this resulted in my having no control over rat age or number in diet groups. There was no significant difference between rats aged 181 days, 290-368 days and 568-586 days within diet groups. Adult rats were not grouped according to stage of oestrous cycle. The effect of the oestrous cycle on rat uterine fatty acid is not yet known. Young rats had not yet entered the oestrous cycle. Numbers in adult rat groups were; control n=14, evening primrose oil n=4, fish

oil n=21. Numbers in young rat groups were; control n=4, evening primrose oil n=5, fish oil n=6. Adult rats received Maxepa as a fish oil supplement whereas young rats were given Hi-EPA. This was used in preference to Maxepa because of its high eicosapentaenoic acid content (21.45% in Hi-EPA as compared to 15.62% in Maxepa). An analysis of the fatty acid intake of rats on each diet is shown in table 3.1 (taking into account presence of coconut oil in semi-synthetic diets). 90-93% of EFA in the EPO supplemented diets was provided by the EPO supplement and 80-82% of EFA in the FO supplemented diets was provided by the FO supplement. The essential fatty acid composition of the EPO diet consisted of 99.5% n6 and 0.5% of n3 fatty acid; the pelleted diet, 84% n6 and 16% n3; and the FO diet, 25% n6 and 75% n3 fatty acid. Administration of oil supplements to adult rats was carried out by Dr H.A. Leaver. Rats of the newly weaned group were born from mothers of the three different diet grops (pelleted, evening primrose oil and fish oil).

Rats were killed and uteri removed by the method described in section 2.2, lipids were extracted and separated into neutral and phospholipid fractions by the two phase separation system described in section 2.11 followed by silicic acid column chromatography described in sections 2.10 and 2.11. Lipid hydrolysis and fatty acid methylation was carried out as described in section 2.14. GC analysis of fatty acid methyl esters was as described in section 2.16. Significance of differences between groups was analysed using the Wilcoxon Rank Sum Test for unpaired data (Cohen and Holliday, 1984).

F.A.	Control	Adult EPO	Young EPO	Maxepa	Hi-EPA
U		2.86	2.72	2.8	2.84
12:0		51.7	49.18	50.4	51.35
13:0		0.23	0.28	0.21	0.16
13:1		0.14	0.18	0.14	0.09
14:0	0.4	17.84	17.02	18.72	19.1
14:1		0.31	0.38	0.34	0.21
14:2		0.08	0.07	0.08	0.1
15:0		0.06	0.06	0.06	0.1
15:1		0.06	0.06	0.06	0.09
16:0	17	8.19	8.16	9.51	8.98
16:1	0.7	0.3	0.29	1.54	1.36
U				0.19	0.13
17:1				0.14	0.13
17:2n6				0.24	0.32
18:0	2.1	6.8	6.59	6.8	6.86
18:1n9	20.6	3.03	3.27	3.91	3.33
18:2n6	45.6	7.23	10.19	0.98	0.8
18:3n6	0.8	0.84	1.22		
18:3n3	2.0			0.12	0.08
18:4n3				0.24	0.14
20:0	0.4				0.12
20:1n9	0.5	0.05	0.05	0.25	0.15
20:4n6	0.1	0.05	0.05	0.11	0.08
20:4n3					0.06
20:5n3	2.4			1.85	2.19
22:1n9	0.2				
22:2n6	0.07				
22:4n6	0.1				
22:5n6	0.24				
22:5n3	0.06			0.18	0.21
22:6n3	4.1			0.77	0.42

Table 3.1
Percentage fatty acid intake of rats on control pelleted diets or semi-synthetic diets supplemented with evening primrose oil or fish oil. Calculations for rats on semi-synthetic diets included both fatty acid intake from oil supplements and from coconut oil. Fatty acids at <0.05% are not shown.

3.3 Results

3.3.1 The effect of dietary fatty acids on uterine fatty acid composition in adult rats

The effect of dietary evening primrose oil, fish oil and a normal pelleted diet on the uterine fatty acid composition of the adult rats is shown in tables 3.2 and 3.3. The major differences between diet groups arose in the essential fatty acids. The proportion of n6 EFA in uteri of the EPO and control groups was higher than in the FO group. 20:3 n6, arachidonic acid, 22:4 n6 and 22:5 n6 were all significantly higher in the EPO group than in the FO group (all $P<0.05$, except 22:5 n6 which was $P<0.001$) and 18:2 n6, 20:2 n6, 22:4 n6 and 22:5 n6 were significantly higher in the control group than in the FO group ($P<0.05$, $P<0.05$, $P<0.001$ and $P<0.001$ respectively). The n3 fatty acids 20:5 n3, 22:5 n3 and 22:6 n3 were all significantly higher in the FO groups than in both the control ($P<0.001$, $P<0.01$ and $P<0.05$ respectively) and EPO groups ($P<0.05$, $P<0.05$ and $P<0.001$ respectively). α-linolenic acid, however, was significantly higher in the control group than the FO group ($P<0.001$).

3.3.2 The effect of dietary fatty acids on the neutral lipid and phospholipid fatty acid composition of the uterus in young rats

The effect of dietary evening primrose oil, fish oil and a normal pelleted diet on the uterine fatty acid composition of young rats is shown in tables 3.4, 3.5 and 3.6. The fatty acid proportions of the phospholipids of young rats were similar to those of the adult total lipids, although fewer significant differences were detected this may be due to the smaller sample size of this group (table 3.3). Arachidonic acid and 22:4 n6 were significantly higher in the EPO

F.A.	Control	EPO	Maxepa
12:0	0.578 ± 0.502[a,b]	2.53 ± 0.696[b]	2.165 ± 0.399[a]
13:0	0.39 ± 0.112	0.596 ± 0.34	0.559 ± 0.116
13:1	0.144 ± 0.052	0.083 ± 0.033	0.154 ± 0.039
14:0	2.683 ± 1.087[a,b]	4.636 ± 0.729[b]	4.224 ± 0.537[a]
14:1	0.382 ± 0.137	0.559 ± 0.16	0.474 ± 0.113
14:2	0.164 ± 0.576	0.456 ± 0.312	0.16 ± 0.0056
15:0	0.358 ± 0.096	0.088 ± 0.038	0.142 ± 0.03
15:1	0.182 ± 0.056[a]	0.531 ± 0.118[a]	0.487 ± 0.084
15:2	0.157 ± 0.107		0.073 ± 0.021
U	1.232 ± 0.253[a,b]	1.82 ± 0.069[b]	1.712 ± 0.195[a]
16:0	27.634 ± 1.082[a]	23.71 ± 1.178	24.724 ± 0.506[a]
16:1	3.621 ± 0.871[a]	4.178 ± 0.419	5.059 ± 0.537[a]
16:2	0.058 ± 0.008		
17:1	0.174 ± 0.043	0.073 ± 0.023	0.147 ± 0.032
17:2n6	0.088 ± 0.018[a]	0.085 ± 0.083	0.158 ± 0.021[a]
U	0.917 ± 0.162	1.068 ± 0.075	0.859 ± 0.118
18:0	11.712 ± 1.296	10.214 ± 0.657	11.382 ± 0.763
18:1n9	23.027 ± 1.422	23.808 ± 2.467	25.802 ± 0.96
18:2 n6	11.122 ± 1.307[a,b]	3.821 ± 0.326[b]	2.374 ± 0.366[a]
U	0.052 ± 0.002		0.062 ± 0.012
18:3n6			0.1 ± 0.05
U			0.53 ± 0.004
18:3n3	0.327 ± 0.071[a]	0.094 ± 0.004	0.056 ± 0.005
U	0.101 ± 0.051		
20:0			0.092 ± 0.034
20:1n9	0.49 ± 0.132	0.32 ± 0.198	0.467 ± 0.116
20:3n9	0.555 ± 0.115	0.839 ± 0.064	0.75 ± 0.136
20:2n6	0.227 ± 0.042[a,b]	0.781 ± 0.088[b]	0.954 ± 0.149[a]
20:3n6	0.527 ± 0.089	0.762 ± 0.038[a]	0.453 ± 0.074[a]
20:4n6	7.724 ± 1.338	9.924 ± 0.772[a]	4.775 ± 0.7[a]
20:3n3	0.136 ± 0.056		0.101 ± 0.035
U	0.168 ± 0.091	0.396 ± 0.238	0.14 ± 0.048
20:5n3	0.428 ± 0.122[a]	0.156 ± 0.017[b]	2.615 ± 0.376[a,b]
22:0	0.31 ± 0.186		0.11 ± 0.057
U	0.181 ± 0.114		
22:1n9	0.208 ± 0.111		
U	0.324 ± 0.118	0.202 ± 0.102	0.508 ± 0.114
U		1.64 ± 0.931	0.153 ± 0.075
22:4n6	1.952 ± 0.519[a]	3.454 ± 0.222[b]	0.903 ± 0.307[a,b]
22:5n6	0.108 ± 0.028[a]	0.909 ± 0.27[a]	
22:5n3	0.592 ± 0.176[a]	0.501 ± 0.208[b]	1.89 ± 0.238[a,b]
22:6n3	1.542 ± 0.318[a]	0.48 ± 0.126[b]	3.059 ± 0.471[a,b]

Table 3.2

Percentage fatty acid content of uterine total lipid in adult rats (151-586 days old), fed on either a control pelleted diet or a semisynthetic diet supplemented with fish oil or evening primrose oil. Figures are presented as mean ± standard error. n=14 for control rats, n=4 for EPO rats,and n=21 for FO rats. Like superscript indicates a significant different of at least $P<0.05$ between diet groups. Fatty acids at less than 0.05% are not shown. U indicates an unidentified fatty acid. SEM are expressed for interest, despite non-parametric statistics being used for diet group comparisons. Results are expressed as obtained from the integrator and do not reflect sensitivity of analysis. Refer to methods section 2.16 for variance of peak size measurements from duplicate injections.

Fatty Acid	Diet Group		
	Control	Fish Oil	Evening Primrose Oil
C20:1n9	0.113 ± 0.0518	0.047 ± 0.014	0.065 ± 0.046
C20:3n9	0.244 ± 0.138	0.094 ± 0.02	0.156 ± 0.024
C20:2n6	0.064 ± 0.436	0.107 ± 0.013	0.139 ± 0.009
C20:3n6	0.127 ± 0.0626	0.049 ± 0.008	0.140 ± 0.019
C20:4n6	0.868 ± 0.162[a]	0.607 ± 0.129[b]	1.823 ± 0.289[a,b]
C20:3n3	0.093 ± 0.0712	0.004 ± 0.005	<0.001
C20:4n3	0.01 ± 0.007	0.063 ± 0.048	0.068 ± 0.042
C20:5n3	0.298 ± 0.251	0.292 ± 0.045	0.030 ± 0.012
C22:3n6	0.139 ± 0.116	0.056 ± 0.017	0.064 ± 0.029
C22:4n6	0.143 ± 0.0404[a]	0.143 ± 0.057[b]	0.656 ± 0.195[a,b]
C22:5n6	0.01 ± 0.0052	<0.001	0.180 ± 0.067
C22:3n3	<0.001	0.013 ± 0.008	0.246 ± 0.148
C22:5n3	0.197 ± 0.134	0.216 ± 0.03[a]	0.096 ± 0.042[a]
C22:6n3	0.356 ± 0.102	0.338 ± 0.045	0.089 ± 0.031

Table 3.3

The C20 and C22 fatty acid content (mg fatty acid per g wet weight uterus) of adult rats (mean age 231 days) detected by gas chromatography using C17:0 as internal standard in 14 rats fed control diet, 21 rats fed fish oil diet, and 4 rats fed evening primrose oil diet. Results are the mean mg fatty acid/g uterus ± SEM, and significantly different ($p<0.01$) results are denoted by the superscripts [a] or [b].

F.A.	Control	EPO	Hi-EPA
12:0	3.314 ± 1.041	1.224 ± 0.654	1.94 ± 0.574
13:0	0.088 ± 0.24		0.084 ± 0.034
13:1			0.077 ± 0.027
14:0	5.011 ± 0.852	3.434 ± 0.709	4.175 ± 0.668
14:1	0.992 ± 0.22	1.652 ± 0.154	0.996 ± 0.312
14:2	0.278 ± 0.143	0.362 ± 0.245	0.365 ± 0.19
15:0	0.419 ± 0.064[a]	0.126 ± 0.044[a]	0.306 ± 0.092
U	0.113 ± 0.063	0.194 ± 0.117	0.141 ± 0.063
15:1	0.787 ± 0.065	2.05 ± 0.699	0.562 ± 0.297
15:2	1.508 ± 0.122	1.953 ± 0.47	1.826 ± 0.264
16:0	24.78 ± 0.617[a]	24.2 ± 1.654[a]	23.02 ± 1.16
16:1	5.972 ± 0.401	6.786 ± 0.675	7.374 ± 0.774
17:1	0.366 ± 0.024[a]	0.423 ± 0.172	0.56 ± 0.054[a]
17:2n6	1.178 ± 0.136	1.285 ± 0.3	1.43 ± 0.156
U	0.124 ± 0.074		0.187 ± 0.088
18:0	10.85 ± 0.511	12.078 ± 1.45	11.291 ± 0.828
18:1n9	22.78 ± 0.726[a]	24.861 ± 0.81[b]	28.997 ± 1.177[a,b]
18:2n6	6.926 ± 1.085[a]	4.207 ± 1.218	2.739 ± 0.393[a]
18:3n6	0.056 ± 0.004		
18:3n3	0.195 ± 0.087	0.105 ± 0.055	<0.05
U	0.089 ± 0.025		
20:1n9	0.276 ± 0.069	0.244 ± 0.105	0.5 ± 0.247
20:3n9			0.053 ± 0.003
20:2n6	0.116 ± 0.063	0.99 ± 0.354	0.92 ± 0.368
U			0.636 ± 0.459
20:3n6	0.491 ± 0.139	0.307 ± 0.165	0.141 ± 0.063
20:4n6	8.162 ± 0.702[a]	9.538 ± 1.519[b]	3.596 ± 0.272[a,b]
U	0.091 ± 0.041		
20:5n3	0.157 ± 0.064[a]	0.132 ± 0.087[b]	2.617 ± 0.258[a,b]
22:1n9	0.089 ± 0.039	0.103 ± 0.053	0.314 ± 0.167
U		0.608 ± 0.464	0.244 ± 0.086
22:4n6	1.987 ± 0.236[a]	1.77 ± 0.398[b]	0.153 ± 0.098[a,b]
22:5n6	0.146 ± 0.0	0.453 ± 0.257	
U	0.138 ± 0.061		
22:5n3	0.097 ± 0.047		0.997 ± 0.411
22:6n3	2.312 ± 0.545	0.975 ± 0.288	3.11 ± 0.833

Table 3.4

Percentage fatty acid content of uterine phospholipid in young rats (42-49 days old), fed on either a control pelleted diet or a semisynthetic diet supplemented with fish oil or evening primrose oil. Figures are presented as mean ± standard error. n=4 for control rats, n=5 for EPO rats, and n=6 for FO rats. Like superscript indicates a significant difference of at least $P<0.05$. Fatty acids at less than 0.05% are not shown. U indicates an unidentified fatty acid.

F.A.	Control	EPO	Hi-EPA
12:0	0.966 ± 0.191^{ab}	12.135 ± 3.016^{a}	9.29 ± 2.104^{b}
U	0.154 ± 0.104	0.051 ± 0.007	
13:0	0.142 ± 0.03^{a}	0.112 ± 0.005^{a}	0.175 ± 0.01
13:1	0.098 ± 0.035	0.083 ± 0.001^{a}	0.131 ± 0.013^{a}
14:0	3.988 ± 0.289^{ab}	7.902 ± 1.653^{a}	8.715 ± 0.9^{b}
14:1	2.43 ± 0.924^{a}	1.201 ± 0.1^{b}	1.566 ± 0.094^{ab}
14:2	0.102 ± 0.052	0.059 ± 0.008^{a}	0.155 ± 0.029^{a}
15:0	0.566 ± 0.057^{a}	0.2 ± 0.008^{ab}	0.314 ± 0.033^{b}
U	0.052 ± 0.002	0.051 ± 0.001	0.054 ± 0.005
15:1	0.069 ± 0.019		0.105 ± 0.032
U			0.051 ± 0.001
16:0	33.33 ± 1.01^{ab}	28.06 ± 1.37^{a}	28.965 ± 0.795^{b}
16:1	10.05 ± 0.603^{a}	11.306 ± 1.665	14.04 ± 0.913^{a}
16:2n6			0.055 ± 0.005
17:1n9	0.486 ± 0.12^{ab}	0.142 ± 0.028^{ac}	0.229 ± 0.016^{bc}
18:0	2.484 ± 0.061	3.383 ± 0.51	2.676 ± 0.167
18:1n9	27.068 ± 1.088	28.447 ± 1.86	30.997 ± 1.734
U		0.053 ± 0.003	
18:2n6	16:82 ± 0.665^{ab}	3.438 ± 0.109^{ac}	1.261 ± 0.119^{bc}
18:3n6	0.08 ± 0.018^{a}	0.162 ± 0.016^{ac}	0.058 ± 0.008^{c}
18:3n3	0.994 ± 0.065^{a}		0.074 ± 0.017^{a}
U		0.164 ± 0.054	0.118 ± 0.033
20:0		0.050 ± 0.002	
20:1n9	0.312 ± 0.099^{a}	0.11 ± 0.04	0.067 ± 0.03^{a}
U		0.109 ± 0.033	
20:4n6	0.508 ± 0.13^{ab}	0.132 ± 0.04^{a}	0.06 ± 0.006^{b}
U		0.055 ± 0.003	0.085 ± 0.031
20:5n3	0.071 ± 0.021		0.058 ± 0.008
22:5n6		0.086 ± 0.024	0.128 ± 0.08
22:6n3	0.272 ± 0.165		

Table 3.5

Percentage fatty acid content of uterine neutral lipid in young rats (42-49 days old), fed on either a control pelleted diet or a semisynthetic diet supplemented with fish oil or evening primrose oil. Figures are presented as mean ± standard error. n=4 for control rats, n=5 for EPO rats, and n=6 for FO rats. Like superscript indicates a significant difference of at least $P<0.05$ between diet groups. Fatty acids at less than 0.05% are not shown. U indicates an unidentified fatty acid.

	Control	Fish Oil	Evening Primrose Oil
	PL	PL	PL
C20:1n9	0.028 ± 0.004	0.055 ± 0.015	0.039 ± 0.018
C20:3n9	<0.001	0.001 ± 0.003	<0.001
C20:2n6	0.015 ± 0.008[a]	0.057 ± 0.023	0.072 ± 0.025[a]
C20:3n6	0.051 ± 0.016[c,d]	0.005 ± 0.003[c]	0.022 ± 0.013[d]
C20:4n6	0.88 ± 0.139	0.299 ± 0.055[a,d]	0.699 ± 0.118[b]
C20:3n3	<0.001	<0.001	<0.001
C20:4n3	0.009 ± 0.008	<0.001	<0.001
C20:5n3	0.013 ± 0.008[a]	0.221 ± 0.042[a,b]	0.009 ± 0.008[b]
C22:3n6	<0.001	0.016 ± 0.007	0.054 ± 0.045
C22:4n6	0.215 ± 0.039[a]	0.014 ± 0.012[a,c]	0.129 ± 0.031[a,c]
C22:5n6	0.016 ± 0.009	<0.001	0.034 ± 0.020
C22:3n3	<0.001	<0.001	<0.001
C22:5n3	0.008 ± 0.007[a]	0.068 ± 0.031[a]	<0.001[a]
C22:6n3	0.248 ± 0.07	0.549 ± 0.322[c]	0.54 ± 0.026[c]
	NL	NL	NL
C20:1n9	0.037 ± 0.018	0.013 ± 0.005	0.055 ± 0.022
C20:3n9	<0.001	0.001 ± 0.003	<0.001
C20:2n6	<0.001	<0.001	<0.001
C20:3n6	<0.001	0.001	<0.001
C20:4n6	0.069 ± 0.035	0.011 ± 0.006	0.075 ± 0.025
C20:3n3	<0.001	0.001	<0.001
C20:4n3	<0.001	0.009 ± 0.005	0.023 ± 0.017
C20:5n3	0.007 ± 0.006	0.008 ± 0.008	0.001
C22:3n6	<0.001	<0.001	<0.001
C22:4n6	<0.001	<0.001	<0.001
C22:5n6	<0.001	0.011 ± 0.008	0.037 ± 0.016
C22:3n3	<0.001	<0.001	<0.001
C22:5n3	<0.001	<0.001	<0.001
C22:6n3	0.04 ± 0.035	<0.001	<0.001

Table 3.6

The C20 and C22 fatty acid content of the uteri of young 42-49d rats (mgs fatty acid per g wet weight uterus) detected by gas chromatography using C17:0 internal standard in 4 rats fed control diet, 6 rats fed fish oil diet and 5 rats fed evening primrose oil diet. Results are mean mg fatty acid/g uterus ± SEM, in neutral lipid (NL) and phospholipid (PL). Significantly different results are denoted by the superscripts [a] [b] ($P<0.01$), and [c] [d] ($P<0.05$).

group than in the FO group (both $P<0.05$) and linoleic acid, arachidonic acid and 22:4 n6 were significantly higher ($P<0.05$) in the control than the FO group. The FO group had significantly higher levels of 20:5 n3 than the control and EPO groups ($P<0.05$). In both the adult rats and the phospholipid of young rats, levels of gamma-linolenic acid were very low if detected at all and no significant differences were found between the three dietary groups. Levels of 20:3n6 were also found to be low and no significant differences between diet groups were found.

In the neutral lipid fraction of the young rats many more of the non-essential fatty acids were found to be significantly different between diet groups (table 3.5), than in the adult rats or the young rats phospholipid fraction. The control group had significantly higher levels of linoleic acid, α-linolenic acid and arachidonic acid than the EPO (all $P<0.05$) and the FO group (all $P<0.01$), and the EPO group had significantly higher proportions of gamma-linolenic acid than both the control ($P<0.05$)and FO ($P<0.001$) groups and linoleic acid than the FO group ($P<0.001$). Significantly higher proportons of n3 fatty acids in the FO group compared to the other groups were not found in the neutral lipid fatty acids.

3.3.3 The effect of dietary fatty acids on uterine tissue/diet fatty acid ratios

Table 3.7 and figure 3.1 show tissue/diet fatty acid ratios in total lipid in adult rats and table 3.8 and figure 3.2 show ratios in the phospholipid fraction of young rats. In both adult rats and phospholipid in young rats, arachidonic acid had by far the highest tissue/diet ratio in all three diet groups, with ratios of 77.24 $\pm$

F.A.	Control Ratios	EPO Ratios	FO Ratios
18:2n6	0.24 ± 0.03^{ab}	0.38 ± 0.03^{bc}	2.46 ± 0.4^{ac}
18:3n6			2.55 ± 1.3
18:3n3	0.12 ± 0.02^{ab}	1.3 ± 0.37^{b}	0.49 ± 0.05^{a}
20:4n6	77.24 ± 13.38^{c}	198.48 ± 15.45^{ab}	38.9 ± 5.34^{b}
20:5n3	0.18 ± 0.05^{a}		1.53 ± 0.21^{a}
22:4n6	18.82 ± 5.34		
22:5n6	0.36 ± 0.09		
22:5n3	1.92 ± 0.59		
22:6n3	0.38 ± 0.08^{a}		7.56 ± 1.3^{a}

Table 3.7

Adult rat (mean age 231 days) uterine total lipid essential fatty acid to dietary essential fatty acid ratios. Where a ratio is not given the fatty acid was undetected in tissue and/or diet. Figures are means ± standard errors for fourteen determinations in control rats, four determinations in EPO rats, and twenty one determinations in FO rats. A significant difference of at least $P<0.05$ between ratios of a fatty acid in two diet groups is indicated by like superscripts.

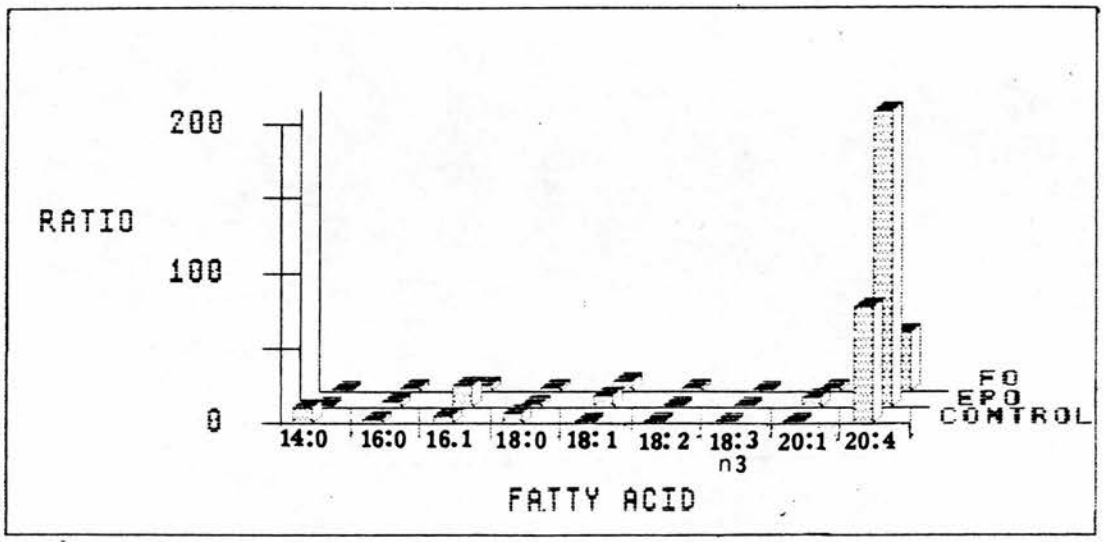


Fig 3.1
Adult rat (mean age 231 days) total lipid fatty acid to dietary fatty acid ratios. Ratios were calculated for all fatty acids detected in both diet and tissue in rats fed either a control pelleted diet (n = 14) or a semisynthetic diet supplemented with fish oil (n = 21) or evening primrose oil (n = 4).

FA.	Control Ratios	EPO Ratios	FO Ratios
18:2n6	0.15 ± 0.02[a,b]	5.01 ± 1.45[b]	3.42 ± 0.49[a]
18:3n6	0.02 ± 0.002		
18:3n3	0.07 ± 0.03	3.5 ± 1.83	
20:4n6	81.62 ± 7.03[a]	190.75 ± 30.38[a,b]	44.95 ± 3.4[b]
20:5n3	0.06 ± 0.03[a]		1.2 ± 0.12[a]
22:4n6	19.87 ± 2.36		
22:5n6	2.32 ± 1.95		
22:5n3	0.32 ± 0.16		4.75 ± 1.96
22:6n3	0.56 ± 0.13		7.81 ± 1.96

Table 3.8

Young rat (42-49 days) uterine phospholipid essential fatty acid to dietary essental fatty acid ratios. Where a ratio is not given the fatty acid was undetected in tissue and/or diet. Figures are means ± standard errors for four determinations in control rats, four determinations in EPO rats, and six determinations in FO rats. A significant difference of at least $P<0.05$ between ratios of a fatty acid in two diet groups is indicated by like superscripts.

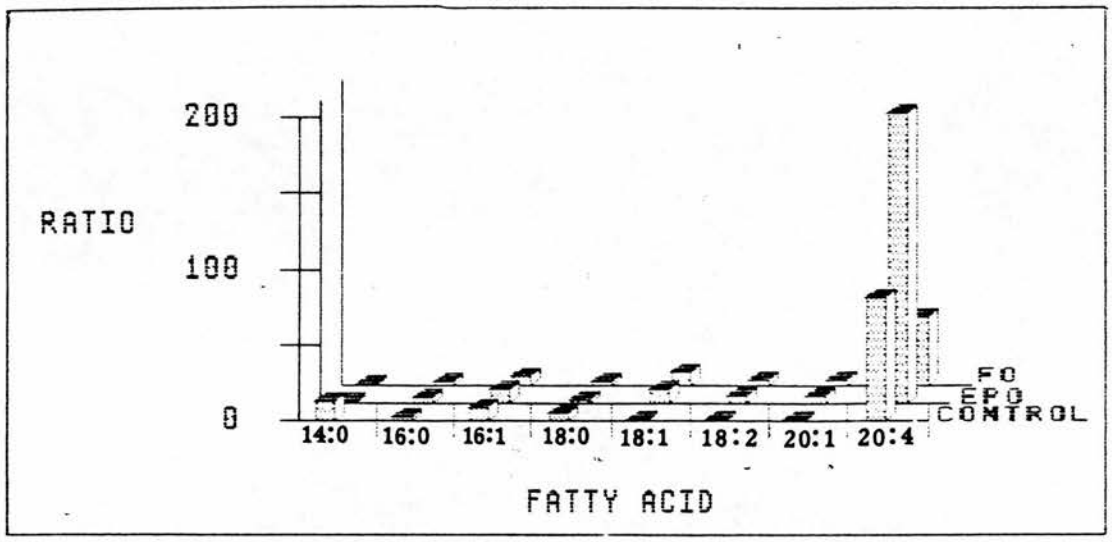


Fig 3.2
Young rat (mean age 44 days) phospholipid fatty acid to dietary fatty acid ratios. Ratios were calculated for all fatty acids that were detected in both diet and tissue in rats fed either a control pelleted diet (n = 4) or a semisynthetic diet supplemented with fish oil (n = 6) or evening primrose oil (n = 5).

13.38, 198.48 ± 15.45 and 38.9 ± 5.34 being recorded in the control, EPO and FO groups respectively, of the adult rats, and ratios of 81.62 ± 7.03, 190.75 ± 30.38 and 44.95 ± 3.4 being recorded in the same groups of the young rats. The ratios for arachidonic acid were significantly higher in the EPO group than in both the control ($P<0.01$ adult, $P<0.05$ young) and the FO groups ($P<0.001$ adult, $P<0.05$ young). Control rats also had high 22:4 n6 ratios (18.82 in adult and 19.87 in young) and fish oil rats high 22:6 n3 fatty acid ratios (7.56 in adult and 7.81 in young). In the neutral lipid fatty acids the highest ratio was found in palmitoleic acid with ratios of 14.32 ± 0.88, 37.68 ± 5.55 and 10.32 ± 0.67 being found in control, EPO and FO groups respectively, but the arachidonic acid ratio was very low (5.08 ± 1.3, 2.64 ± 0.84 and 0.74 ± 0.07 in the control, EPO and FO groups respectively) compared to the levels in the phospholipid fraction and in the adult rats (see figure 3.3).

3.3.4 The effect of dietary fatty acids on uterine n3/n6 fatty acid ratios

Diet and tissue n3/n6 ratios are illustrated in table 3.9. Adult and young rat phospholipid values were very similar to each other. The ratios in FO groups in both adult rats (0.901) and young rats phospholipid fraction (0.992) were more than 10 times that of the EPO fed rats (0.071 and 0.074 respectively). The control rats had intermediary ratios (0.147 in adult and 0.166 in young rats phospholipid fraction). The difference in ratios in the tissue between the different diet groups was, however, far less than the differences in the diet itself, where the FO diets had n3/n6 ratios 700-800 times higher than the EPO diet. The control dietary n3/n6 ratio (0.183), however, was very close to that found in the control

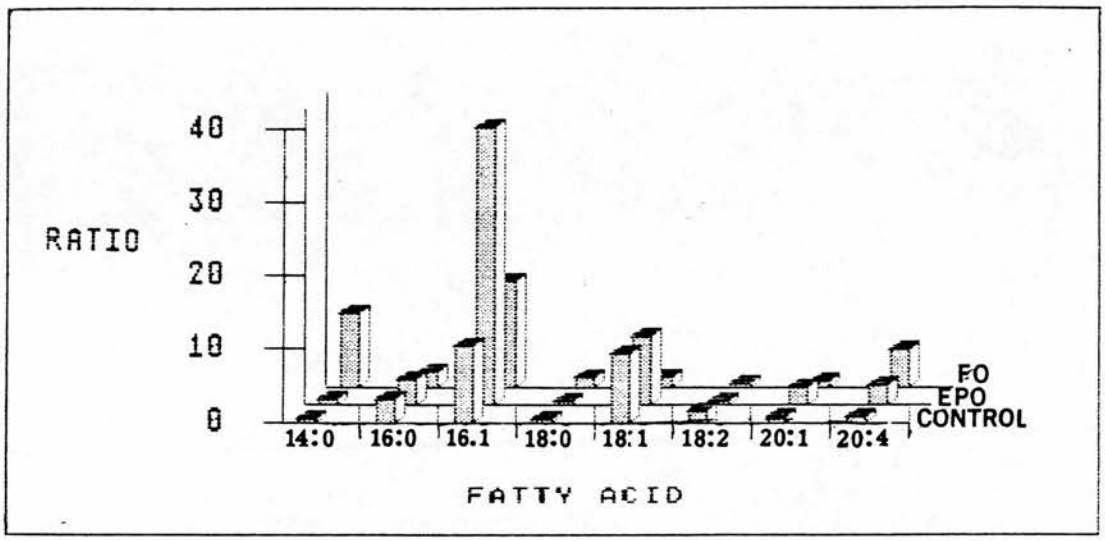


Fig 3.3
Young rat (mean age 44 days) neutral lipid fatty acid to dietary fatty acid ratios. Ratios were calculated for all fatty acids that were detected in both diet and tissue in rats fed either a control pelleted diet (n = 4) or a semisynthetic diet supplemented with fish oil (n = 6) or evening primrose oil (n = 5).

	Control (n)	EPO (n)	FO (n)	
Adult	0.147 (14)	0.071 (4)	0.901 (21)	
Young PL	0.166 (4)	0.074 (4)	0.992 (6)	
Young NL	0.08 (4)	0.028 (5)	0.171 (6)	
Diet	0.183	<0.004	2.899[a]	3.522[b]
			a-Maxepa	b-Hi-EPA

Table 3.9
n3/n6 fatty acid ratios of uterine tissue in control, EPO, and FO fed rats and diet n3/n6 ratios in control, EPO, and FO diets. Ratios for EPO and FO diets take into account fatty acid contribution from coconut oil.

rat uterus of the adult rats (0.147) and the phospholipid of the young rats (0.166). In the EPO fed rats there was a more than 10 fold increase in n3/n6 ratio over that of the EPO diet in adult rats and both phospholipid and neutral lipid in young rats, whereas the FO group exhibited at least a 4.5 fold decrease in adult rats and both phospholipid and neutral lipid in young rats compared to that of the FO diet. The young rat neutral lipid ratios showed a similar trend to the other groups, with the FO group having the highest n3/n6 ratio and the EPO group the lowest, however the differences were less pronounced. The neutral lipid ratios in the control and FO groups were both considerably lower than those in the corresponding diets, 0.08 and 0.171 compared to 0.187 and 3.522 respectively, whereas the EPO, as with the adult and phospholipid groups, was considerably higher, 0.028 in the tissue compared to <0.004 in the diet.

The proportion of n3 and n6 EFA in the uterus of each diet group was very similar in control and EPO groups of total lipid in adult rats and phospholipid and neutral lipid in young rats (see table 3.7). However, the FO fed rats had very much lower n6 and higher n3 proportions, except in the neutral lipid of the young rats where the FO diet did not have much effect on the n6-n3 proportions. In the total lipid of the adult rats and phospholipid of the young rats, the control diet groups contained the highest proportion of EFA as a percentage of total fatty acid (23% and 20.4% respectively), and the FO groups the lowest (18.2% and 14.3% respectively). The proportion of EFA compared to other fatty acids is shown in table 3.10. In neutral lipid the proportion of EFA was low in both the EPO and FO fed rats (3.69 and 1.64 % respectively), compared with the EFA in

	Adult Rats			Young Phospholipid			Young Neutral Lipid		
	Con	EPO	FO	Con	EPO	FO	Con	EPO	FO
% n6	87.2	93.4	52.6	85.8	93.1	50.2	92.6	97.3	85.4
% n3	12.8	6.6	47.4	14.2	6.9	49.8	7.4	2.7	14.6
% EFA	23	20.9	18.2	20.4	18.02	14.3	18.7	3.9	1.64

Table 3.10

Mean n6 and n3 fatty acids as percentages of total essential fatty acids and mean essential fatty acid as percentage of total fatty acid in uterine total lipid of adult rats and phospholipid of young rats. Rats were fed either a control pelleted diet or a semisynthetic diet supplemented with evening primrose oil or fish oil.

the phospholipid of young rats and total lipid of adult rats. However, the neutral lipids in the control rats had a high EFA content (18.79%), 89% of which was linoleic acid.

The 20:3n9/20:4n6 ratio was not greater than 0.2 in any of the rats (a ratio of greater than 0.4 indicates fatty acid deficiency, Holman, 1960).

3.3.5 Age-related changes in uterine fatty acid composition and essential fatty acid content

Table 3.11 shows total uterine fatty acid and <C18 and ≥C18 fatty acid content of rats in the three different age groups. The total uterine fatty acid content was significantly higher ($p<0.001$) in newly weaned rats (199.59 ± 46.63 mg fatty acid/g tissue), compared with adult rats (14.02 ± 2.47 mg fatty acid/g tissue). The proportion of short chain fatty acids (<C18) was also significantly greater ($P<0.01$) in newly weaned rats, at 52.92% ± 8.94, compared to 35.87% ± 1.39 in adult rats.

3.4 Discussion

Dietary evening primrose oil and fish oil induced significant changes in the fatty acid content of the uterus of rats in all three age groups. In adult rats and phospholipids of young rats the major differences between diet groups were found in the essential fatty acids. The proportion of tissue n6 EFA was higher in the control and EPO groups than in the FO groups. The uteri of control rats had significantly higher proportions of linoleic acid than the adult and young neutral lipid EPO groups (both $P<0.05$) and FO ($P<0.001$ and $P<0.01$ respectively) as well as the young phospholipid

Age Group	n	Total Fatty Acid mgs/g tissue	Fatty Acids <C18 %	Fatty Acids ≥C18 %
Newly Weaned	10	200 ± 44	53 ± 9	44 ± 8
Young	14	64 ± 5	38 ± 55	55 ± 1
Adult	39	14 ± 2	36 ± 1	57 ± 1

Table 3.11

Total fatty acid (mg/g wet weight tissue), <C18 fatty acid (%FA) and ≥C18 fatty acid (% FA) of rat uteri from different age groups, expressed as mean mgs fatty acid/g wet weight of uterus of n determinations ± standard error of mean. Newly weaned rats were 21-25 days old (22.6 ± 0.6), young rats were 42-49 days old (44.4 ± 0.8) and adult rats were 151-586 days old (230.6 ± 16.9).

FO group ($P<0.05$). This corresponds with the high levels of linoleic acid in the control diet. However, the uterine lipids of rats fed EPO contained the highest concentration of arachidonic acid from a diet that contained the least arachidonic acid. This suggests that large quantities of the linoleic acid in the control groups was not being converted to arachidonic acid, indicating low D6 desaturase activity in the uterus. Also, competetive inhibition of the n6 desaturase by relatively high levels of α-linolenic acid in the tissue of control rats may be inhibiting the D6 desaturase. This also suggests that the quantities of arachidonic acid found in the EPO groups were being considerably increased by the presence of high levels of gamma-linolenic acid in the EPO diet. This may be more readily converted to arachidonic acid than linoleic acid because of the omission of the D6 desaturase rate determining step which converts linoleic acid to gamma-linolenic acid. The fact that no gamma-linolenic acid was detected in the uteri of the EPO fed rats suggests that it was all converted to 20:3n6 and arachidonic acid rather than it was not incorporated into the tissue.

The FO rats had similar uterine tissue proportions of linoleic acid to the EPO rats, despite the fish oil diet containing less linoleic acid (0.98% compared to 10.19% in adult rats and 0.8% compared to 10.19% in young rats), this was probably due to inhibition of the conversion of linoleic acid to gamma-linolenic acid due to the high concentrations of n3 fatty acids in the FO diet. Arachidonic acid levels in the uterine lipid of rats fed fish oil were significantly lower than those in the EPO groups ($P<0.05$) in both adult rats and young rat phospholipids, despite the presence of higher concentrations of arachidonic acid in the FO diet. This was

probably due to increased arachidonic acid production from gamma-linolenic acid in the EPO groups, and competetive inhibition of the D5 and D6 desaturase by n3 fatty acids in the FO group (Nasser et al., 1986).

Proportions of 20:3n6 in the EPO groups of both the phospholipid fraction of young rats and the total lipid fraction of adult rats were very low compared to proportions of arachidonic acid. This, together with the low proportions of gamma-linolenic acid found in this group, despite the high dietary level, suggests very active D5 desaturase activity in the uterus and suggests that 20:3n6 will not accumulate in the uterus as has been observed in other tissues (Ayala et al., 1973; Horrobin, 1980; Lands & Samuelsson, 1968; Willis, 1981; Moore & Williams, 1966; Takayasu et al., 1970). The levels of gamma-linolenic acid in the EPO diet of the young and adult rats were only slightly higher than those in the control diet (0.84% and 1.22% compared to 0.8% respectively) but the control diet contained far greater quantities of linoleic acid than the young and adult EPO diets (45.6% compared to 7.23% and 10.19% respectively), despite this the levels of 20:3n6 in young rat phospholipids and adult rat total lipids were very similar between EPO and control groups, again suggesting low D6 desaturase activity and conversion of linoleic acid to arachidonic acid in the uterus. The fish oil groups had by far the lowest levels of 20:3n6 precursors available to them (see table 3.1), but despite this proportions of 20:3n6 in the fish oil fed rats were relatively high (tables 3.2 and 3.4). This suggested that the high levels of n3 fatty acids in the uterine lipids of rats fed fish oil inhibited the D5 desaturase. There was

also higher levels of linoleic acid in the FO fed rats than in the FO diet, suggesting there was inhibition of the D6 desaturase. Consequently, the accumulation of 20:3n6 was detected in uterine lipid of the rats fed the fish oil diet. A similar result was reported by Nasser et al. (1986) who observed increasing 20:3 n6 proportions in rat liver and plasma fatty acids with increasing proportions of fish oil in a fish oil-EPO diet mixture. There is evidence that 20:5n3 is more effective at competing with arachidonic acid for cyclooxygenase enzymes than with 20:3n6 (Boukhchache & Lagarde, 1982), thus possibly resulting in a proportional increase of PGE_1 compared to PGE_2 in the FO fed rats.

The dietary proportions of arachidonic acid were very much lower than the corresponding tissue levels in all three diet groups, this suggests that the major source of arachidonic acid in the tissue was from precursors rather than from arachidonic acid in the diet. The proportions of arachidonic acid and linoleic acid found in the uterus in this study were relatively low compared to proportions of these fatty acids found in other tissues of animals fed on diets containing similar fatty acid contents, including; rat liver (Hwang and Carrol, 1980; Hwang, 1988; Nasser, 1986), heart (Charnock, 1983), serum (Hwang, 1980), plasma (Nasser, 1986), and human platelets (Sanders et al., 1981). Due to the profound effects of 2-series prostaglandins, and possibly of free arachidonic acid itself, on the uterus, as seen at parturition, it may be beneficial for the uterus to maintain low levels of arachidonic acid when prostaglandins are not required for parturition. Arachidonic acid incorporation in human amnion has been found to increase during labour (Schwartz et al., 1977) and arachidonic acid composition of

cholesterol esters in amniotic fluid have been found to increase with gestational age (Das et al., 1975), so a possiblity exists of extra incorporation of arachidonic acid into uterine tissues during gestation. Specific tissues thought to be involved in arachidonic acid release and prostaglandin production at parturition, such as human uterine decidua and foetal membranes have also been shown to contain high arachidonic acid concentrations (Schwarz et al., 1975).

The fish oil diet groups in total lipid in adult rats and phospholipid in young rats had significantly greater quantities of eicosapentaenoic acid than both control ($P<0.001$ in adult and $P<0.05$ in young) and EPO groups ($P<0.05$ in both adult and young). This was despite the fact that the control diet had a higher percentage 20:5n3 content than the FO diet. This suggests that the uterus will only incorporate large quantities of n3 fatty acids when sufficient quantities of n6 fatty acids are not available, as is the case with the FO diet but not the pelleted diet. Similar results have been observed in rat plasma and liver (Huang et al., 1987). The 22 carbon EFA show a similar trend to that of the 20C EFA with control and EPO groups having significantly higher levels of the n6 FA and significantly lower of the n3 FA. In both the phospholipids of young rats and the total lipid of adult rats there was relatively low tissue proportions of adrenic acid and 22:5n6 compared to arachidonic acid, despite levels of all three free fatty acids in the diet being very low. In the FO groups the relative quantitites of adrenic acid and 22:5n6 compared to arachidonic acid were lower than those in the control and EPO groups, this was probably due to inhibition of the D4 desaturase by high levels of 20:5n3, 22:5n3 and

22:6n3 in the FO fed rats. The n3 22C fatty acids, however, were present in higher levels than 20:5n3, especially in the EPO and control groups. This suggests that either D4 desaturase has greater specificity for the n3 fatty acids than the n6 fatty acids or that the 22C n3 fatty acids are preferentially incorporated compared to the 20C n3 fatty acids. Preferential incorporation in platelets after fish oil feeding has been reported (Simonsen et al., 1987).

The results suggest that the uterus may compensate for deficiencies of arachidonic acid by incorporating available n3 C20 and C22 EFA. Figures 3.4 and 3.5 show the combined arachidonic acid, 20:5n3 and 22:6n3 contents of the 3 diet groups of adult rats and young rat phospholipids respectively. This illustrates clearly how the combined arachidonic acid, 20:5n3 and 22:6n3 levels are very close in the three diet groups despite widely differing individual proportions of the three fatty acids. However, this compensation was structural rather than functional, as a number of studies have illustrated the difficulties encountered at parturition in animals receiving a diet with a high n3 fatty acid content. The tissue/diet ratios shown in tables 3.7 and 3.8 and figures 3.1 and 3.2 illustrate the importance of high arachidonic acid content in the uterus. Even in the FO groups where only low levels of arachidonic acid precursors were available arachidonic acid still had by far the highest ratio. In the control groups, adrenic acid (22:4n6) also had a relatively high ratio, suggesting that it may also play a role in uterine functions (22:4n6 was not detected in the EPO or the FO diet and so ratios could not be calculated). Little is known about the synthesis and biological activity of the 22C n6 prostaglandins.

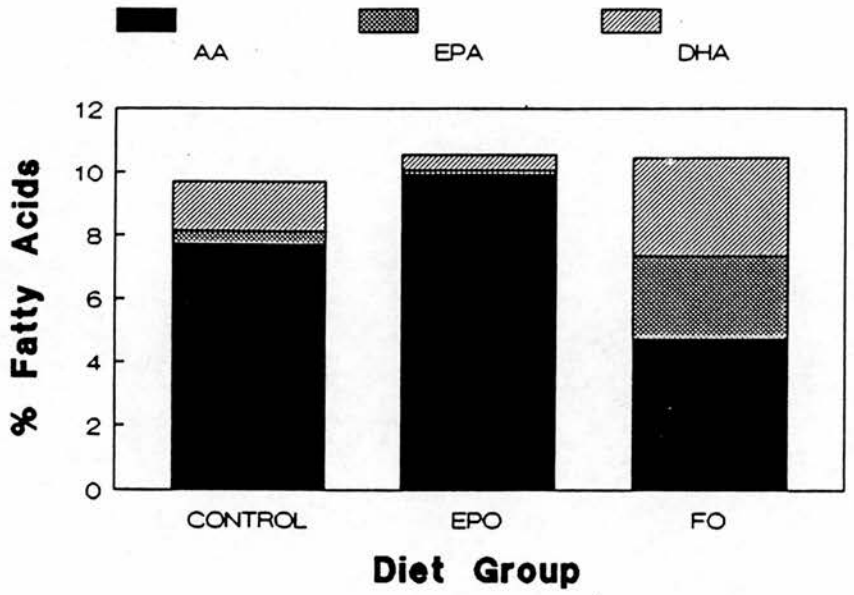


Figure 3.4
Proportions of arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid in total lipid of adult rats (mean age 231 days) fed control pelleted diet (n = 14), semi-synthetic diet with evening primrose oil (EPO, n = 4), and semi-synthetic diet with fish oil (FO, n = 21). Proportions are expressed as percentages of total detected fatty acid.

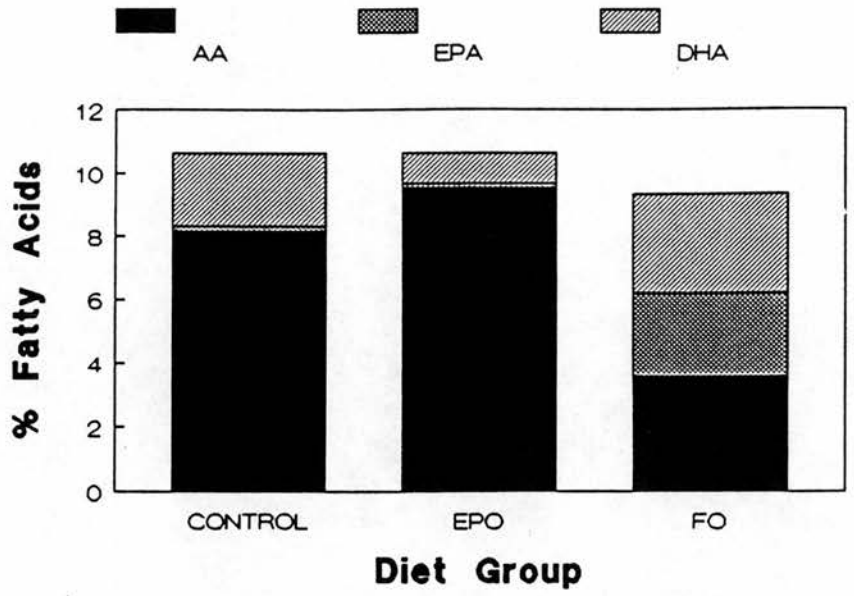


Figure 3.5
Proportions of arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid in phospholipids of young rats (mean age 44 days) fed control pelleted diet (n = 4), semi-synthetic diet with evening primrose oil (EPO, n = 5), and semi-synthetic diet with fish oil (FO, n = 6). Proportions are expressed as percentages of total fatty acid detected

The n3/n6 ratios of the total lipid in the adult rats and the phospholipid in the young rats showed considerable differences from the n3/n6 ratios detected in the corresponding diets. The control diet groups had n3/n6 ratios similar to the control diet ratio, however, the EPO rats had much higher ratios than the EPO diet and the FO rats had much lower ratios than the FO diet. This suggests that some compensation for the high n6 content in the EPO diet and high n3 content in the FO diet was taking place within the rats, and suggests a requirement for the presence of both n3 and n6 fatty acids in the uterus. However, despite any compensation taking place within the uterus the FO diet resulted in a far higher n3/n6 ratio than either the EPO or control diets. Since little difference in n3/n6 fatty acid ratio was seen between the control diet and the control rats this may suggest that the n3 and n6 fatty acid content of this diet was close to the balance required in the uterus.

The FO diet resulted in a decrease of the proportion of n6 EFA by approximately 30%, from around 80% in the control groups to nearer 50% in the FO groups in both total lipid of adult rats and phospholipid of young rats (table 3.10). These changes, however, were quantitatively smaller than the observed changes in the efficacy of parturition in fish oil fed rats, which has been shown to decrease by 80%, or the uterine content of prostaglandin E_2 which decreases by over 80% in uterine wall and foetal membranes of rats fed FO diets compared to those on control diets (Leaver et al., 1986). However, the fatty acid that increased the most in this study in relation to levels in the control rats was 20:5n3, which increased by 610% in adult rats and by 1666% in the phospholipid of young rats. These very large increases could clearly have a marked

effect on prostaglandin production from arachidonic acid. Differences in n3 and n6 content of different lipid pools could also have to be considered in relation to localisation, metabolic turnover, and their physiological role in parturition (see chapter 4).

The phospholipid fatty acids in the young rats illustrated the same trends as the total fatty acid in the adult rats. Only 3 weeks were therefore necessary for significant dietary induced changes to result. The fatty acid content of the neutral lipid in the three diet groups also demonstrated a number of significant differences, however, in the neutral lipid only a very few of the differences were seen in the essential fatty acids. In the tissue/diet ratios of the neutral lipid pool the highest ratios were found in palmitoleic and oleic acids, as compared to arachidonic acid in the phospholipid. This suggests the neutral lipid pool is a quantitatively minor source of the eicosanoid precursor fatty acids. The levels of all the EFA, except linoleic acid and gamma-linolenic acid, were very much lower in the neutral lipid than the phospholipid fraction. There was little evidence of active elongation or desaturation of EFA in the neutral lipids as the relatively high levels of linoleic acid and gamma-linolenic acid corresponded to low levels of arachidonic acid and 22:4 n6. In the FO group the quantities of n3 EFA were very much lower than those in the phospholipid fraction, with the exception of α-linolenic acid. These results suggest that the major part of the neutral lipid pool in the uterus does not store or metabolise EFA to any extent. However, the metabolism of these EFA in quantitatively

minor neutral lipid constituents such as diacylglycerol and monoacylglycerol would require further analysis (this has been investigated in chapter 4).

Significant differences were detected in the fatty acid composition of the uteri of newly weaned rats compared to adult rats. The newly weaned rats had a significantly higher total fatty acid content ($P<0.001$), and a significantly higher proportion of short chain fatty acids of less than 18C ($P<0.01$), but a significantly lower proportion of fatty acids of 18 carbons or more ($P<0.01$). The increased proportion of short chain fatty acids in the newly weaned rats may be a result of high quantities of these fatty acids in the mothers' milk (Ross et al., 1985). However, it seems unlikely that adult rats would incorporate this level of short chain fatty acids even if the diet contained large quantities of these fatty acids. This is supported by a comparison of the adult rats fed the pelleted diet to those fed the semi-synthetic diet as the semi-synthetic diets contained approximately four times the quantity of short chain fatty acids of the pelleted diet, however, there was little difference between uterine proportions of short chain fatty acids of rats fed pelleted diet or semi-synthetic diets. It therefore seems likely that this difference was due to age rather than diet.

In conclusion, we demonstrated both a diet induced and an age related change in the fatty acid composition of the rat uterus. The diet induced change took place after only three weeks of feeding and little further changes were observed when feeding was continued for up to 18 months. Conservation of arachidonic acid by the uterus was also seen, even in rats on the FO diet. The levels of EFA in the

uterus suggest low D6 desaturase and high D5 desaturase activity. There was also evidence for structural replacement of n6 EFA by n3 EFA in the FO fed rats and to some extent in the control rats. Differences in EFA content of neutral lipid and phospholipid fractions were also observed, suggesting that phospholipids were the major source of EFA in the uterus.

Chapter 4

The Effects of Dietary n3 and n6 Fatty Acids on the Fatty Acid Composition of the Major Lipid Classes of the Rat Uterus

4.1 Introduction

The release of fatty acid prostaglandin precursors from specific lipid species has been observed in a number of tissues, including the uterus and in intrauterine tissues thought to be involved in prostaglandin production during parturition such as the foetal membranes. In the guinea pig uterus, Leaver and Poyser (1981) observed release of arachidonic acid from PC and triglyceride and Ning et al. (1983) observed specific release of tritiated arachidonic acid from PC, PE and triglyceride from guinea pig endometrium in culture. In human foetal membranes, specific release of arachidonic acid from PE and PI was detected during early labour (Okita et al, 1982). Phospholipase A_2 with a preference for PE with arachidonic acid in the sn-2 position, and phospholipase C specific for PI have been identified in human foetal membranes (Okazaki et al., 1978; Di Renzo et al., 1981).

The essential fatty acids may also be incorporated preferentially into specific lipid species. In the guinea pig uterus, 93% of total arachidonic acid was esterified to phospholipids of which 80% was esterified to PE and PC (Leaver and Poyser, 1981) and in the guinea-pig endometrium exogenous tritiated arachidonic acid was incorporated predominantly into PE, PC and triglyceride (Ning et al, 1983). Human amnion at term, also incorporated exogenous arachidonic acid primarily into PC and PE and a high rate of turnover of arachidonic acid relative to palmitic acid was detected

in PC (Schwartz et al., 1977).

The effects of diets with different n3 fatty acid contents on n6 fatty acid incorporation into specific lipid groups was investigated in rat myocardium, and a greater displacement of arachidonic acid by n3 fatty acids was detected in PE compared with other phospholipids (Abeywardena et al., 1987). Huang et al., (1987) also reported preferential incorporation of n6 above n3 fatty acids into liver and plasma phospholipids and cholesterol esters compared to triglyceride. However, no investigation of this sort has yet been conducted in uterine tissue. Knowledge of the relative distribution of the n3 fatty acids and n6 fatty acids in the lipid pools involved in the release of eicosanoid precursor fatty acids is important if a better understanding of the inhibitory effect of n3 fatty acids on parturition is to be gained. We therefore studied the effect of three different diets, containing different levels of n3 and n6 fatty acids, on the incorporation and metabolism of fatty acids in the monoglyceride, diglyceride, triglyceride, cholesterol ester, free fatty acid, PI, PE and PC lipid fractions in the rat uterus.

In chapter 3, evidence for significant changes in uterine fatty acid composition after only three weeks of feeding diets containing different proportions of n3 and n6 fatty acids was presented. In the following experiments, purified n3 and n6 fatty acid ethyl esters, (linoleic acid ethyl ester and α-linolenic acid ethyl ester, both >99% pure) were used as the main source of EFA for rats on a semisynthetic diet and the incorporation of EFA into the major lipid pools in the rat uterus compared with rats fed a control pelleted

diet. The purified ethyl esters were used in preference to the EPO and FO used in chapter 3 to minimise the quantity of n6 EFA present in the n3 diet and n3 EFA present in the n6 diet. This also clarified identification of the metabolism of the dietary EFA in the uterus as the only source of EFA was either linoleic acid or α-linolenic acid, whereas the FO and EPO provided a range of different n3 and n6 EFA. The 2- and 3- series PGE and PGF released by these uteri were analysed using mass spectroscopy (see chapter 5).

4.2 Methods

Nine newly weaned female Sprague-Dawley rats were randomly divided into three groups and fed either a control pelleted diet or a diet supplemented with linoleic acid or α-linolenic acid ethyl esters (both >99% pure). Section 2.1 describes the fatty acid content of the control and semi-synthetic diets. Rats were killed after three weeks on the diets and uteri removed as described in section 2.2. Uterine lipid extraction, separation, methylation and analysis were as described in sections 2.9, 2.11, 2.12, 2.13, 2.14, 2.15, and 2.16. Due to the small number of rats in each group (n=3), it was not possible to show statistically significant differences between diet groups.

4.3 Results

The body weights and uterine weights at time of sacrifice were not significantly different between diet groups. Fur and skin condition indicated no signs of EFA deficiency and 20:3n9/20:4n6 ratio was never greater than 0.2 in any of the lipid fractions from any of the diet groups (a ratio of greater than 0.4 indicates fatty acid deficiency; Holman, 1960). Tables 4.1-4.7 show the mean percentage of each fatty acid detected in the three diet groups in the seven

different lipid fractions. In the major saturated fatty acids (16:0 and 18:0) there was no clear trends in dietary effects on the proportions of these fatty acids common to all the lipid groups, and in most of the lipid groups the different diets had little effect on the proportions of these fatty acids, however, some differences could be seen. Proportions of palmitic acid in the triglyceride - cholesterol ester (table 4.7) and PC (table 4.2) fractions were higher in linoleic and linolenic diet groups than in the control diet group (30.02% ± 1.62 and 27.71% ± 2.68 compared to 21.19% ± 2 respectively in the triglyceride-cholesterol ester fraction, and 33.61% ± 5.45 and 31.93% ± 4.48 compared to 16.68% ± 4.82 respectively in the PC fraction), and in the PE fraction (table 4.3) levels in the linolenic group were higher than in the other two groups (40.27% ± 1.56 compared to 33.92% ± 2.71 in control and 28.68% ± 3.43 in linoleic groups). Proportions of stearic acid in PI (table 4.1) were higher in the linoleic and linolenic groups than in the control group (14.78% ± 1.35 and 17.86% ± 4.35 compared to 3.33% ± 2.23 respectively) and in the free fatty acid fraction stearic acid proportions were higher in the linoleic group than in the other two groups (table 4.4) (19.88% ± 2.96 compared to 10.28% ± 3.82 in the control group and 12.61% ± 1.26 in the linolenic group). There was little difference in proportions of palmitic acid between the different lipid fractions, however, stearic acid was present in considerably lower proportions in the triglyceride - cholesterol ester lipid fraction (table 4.7) than in all the other lipid fractions.

The monounsaturated fatty acids present in the highest proportions

were 16:1 and 18:1. Palmitoleic acid (16:1) proportions in the linoleic and linolenic groups in the triglyceride - cholesterol lipid fraction (table 4.7) were slightly higher than in the control group (12.01% ± 0.4 and 10.25% ± 0.41 compared to 6.46% ± 0.57 respectively) and proportions of this fatty acid were also higher in the linolenic acid fed rats in the diglyceride lipid fraction than in the rats fed the control or linoleic acid supplemented diets (7.85% ± 0.46 compared to 3.48% ± 1.75 and 4.83% ± 0.29 respectively). Apart from this there was little evidence of dietary effect on the proportions of palmitoleic acid. However, a dietary effect was detected in the proportion of oleic acid which was higher in the linolenic acid diet groups of the diglyceride, monoglyceride, free fatty acid, PC and PI lipid fractions than in both the control or the linoleic acid diet groups (tables 4.2, 4.3, 4.4, 4.6 and 4.7). Both palmitoleic and oleic acid were incorporated to a greater extent into the triglyceride - cholesterol ester fraction than the other lipid fractions (tables 4.1 - 4.7).

Arachidonic acid proportions were higher in linoleic and control rats than in linolenic fed rats in all lipid fractions except PC and PI, where proportions were similar in all three diet groups (tables 4.1 - 4.7). The free fatty acid fraction contained the highest proportion of arachidonic acid of all the lipid fractions in the control and linolenic diet groups, with mean percentages of 8.47% ± 2.58 and 6.78% ± 2.63 respectively, being recorded. The proportion of arachidonic acid in the linoleic diet group, however, was highest in the PE lipid fraction with a level of 11.35% ± 2.24 compared to 10.57 ± 0.42 in the free fatty acid fraction. Proportions of arachidonic acid were lowest in the triglyceride - cholesterol ester

F.A.	Control PI	Linoleic PI	Linolenic PI
12:0	1.03 ± 0.47	<0.1	<0.1
12:1	2.07 ± 1.97	<0.1	<0.1
13:0	2.92 ± 1.94	2.75 ± 1.32	1.081 ± 0.98
13:1	0.18 ± 0.08	0.41 ± 0.31	<0.1
14:0	2.42 ± 1.58	2.95 ± 1.18	<0.1
14:1	0.41 ± 0.31	<0.1	<0.1
14:2	<0.1	<0.1	<0.1
15:0	0.41 ± 0.2	0.48 ± 0.22	2.63 ± 2.53
15:1	4.22 ± 2.12	3.69 ± 1.53	1.28 ± 1.69
15:2	0.2 ± 0.1	0.49 ± 0.39	<0.1
16:0	16.79 ± 5.03	16.74 ± 3.46	17.92 ± 3.96
16:1	0.75 ± 0.65	3.22 ± 1.89	1.45 ± 1.36
16:2	<0.1	<0.1	<0.1
17:1	<0.1	<0.1	<0.1
17:2	<0.1	5.22 ± 2.22	3.8 ± 0.3
18:0	3.33 ± 2.23	14.78 ± 1.35	17.86 ± 4.35
18:1n9	10.41 ± 5.98	9.52 ± 1.46	11.8 ± 1.94
18:2n6	2.03 ± 1.22	2.56 ± 0.85	2.1 ± 0.66
18:3n6	<0.1	<0.1	<0.1
U	1.58 ± 0.86	1.84 ± 0.94	2.67 ± 1.32
18:3n3	0.19 ± 0.09	0.73 ± 0.37	0.64 ± 0.54
18:4n3	<0.1	<0.1	<0.1
20:0	<0.1	0.52 ± 0.42	0.34 ± 0.17
20:1n9	0.24 ± 0.1	2.41 ± 1.5	0.15 ± 0.05
U	<0.1	<0.1	<0.1
20:3n9	<0.1	0.71 ± 0.61	<0.1
20:2n6	7.05 ± 1.51	9.47 ± 2.41	10.74 ± 2.6
20:3n6	<0.1	0.16 ± 0.06	0.51 ± 0.29
20:4n6	4.25 ± 3.3	7.29 ± 2.02	6.78 ± 2.49
20:3n3	1.04 ± 0.94	0.44 ± 0.34	0.67 ± 0.31
20:5n3	0.28 ± 0.18	0.41 ± 0.22	2.09 ± 0.66
U	<0.1	<0.1	<0.1
22:4n6	0.74 ± 0.64	2.41 ± 1.8	<0.1
22:5n6	0.75 ± 0.41	<0.1	<0.1
22:5n3	<0.1	<0.1	0.4 ± 0.24
22:6n3	2.55 ± 1.54	3.56 ± 2.85	0.46 ± 0.2

Table 4.1

Fatty acid content of uterine phosphatidylinositol in rats fed for three weeks on either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid ethyl ester or α-linolenic acid ethyl ester. Fatty acids are shown as a mean percentage of total fatty acid present in PI ± standard error (n = 3 in each diet group). U indicates an unidentified fatty acid.

F.A.	Control PC	Linoleic PC	Linolenic PC
12:0	<0.1	2.2 ± 1.48	0.31 ± 0.12
12:1	<0.1	2.07 ± 1.97	<0.1
13:0	<0.1	1.64 ± 1.56	0.17 ± 0.07
13:1	<0.1	1.05 ± 0.95	<0.1
14:0	0.44 ± 0.34	2.69 ± 0.04	1.72 ± 0.82
14:1	<0.1	<0.1	<0.1
14:2	<0.1	<0.1	0.66 ± 0.58
15:0	<0.1	0.32 ± 0.13	0.36 ± 0.13
15:1	1.86 ± 1.44	<0.1	0.68 ± 0.3
15:2	1.85 ± 1.43	<0.1	1.02 ± 0.44
16:0	16.68 ± 4.82	33.11 ± 5.45	31.93 ± 4.48
16:1	8.33 ± 5.11	5.82 ± 0.67	9.14 ± 4.62
16:2	2.05 ± 1.59	<0.1	<0.1
17:1	<0.1	<0.1	0.2 ± 0.1
17:2	2.24 ± 0.2	3.54 ± 2.12	1.24 ± 0.72
18:0	9.77 ± 0.51	11.06 ± 2.03	9.41 ± 1.41
18:1n9	15.33 ± 5.62	14.84 ± 1.86	20.96 ± 2.98
18:2n6	6.08 ± 2.22	2.44 ± 0.37	1.52 ± 0.77
18:3n6	<0.1	<0.1	<0.1
U	<0.1	<0.1	<0.1
18:3n3	<0.1	<0.1	0.14 ± 0.04
18:4n3	<0.1	<0.1	<0.1
20:0	<0.1	0.4 ± 0.16	0.14 ± 0.04
20:1n9	4.05 ± 3.23	<0.1	2.39 ± 2.04
U	<0.1	<0.1	<0.1
20:3n9	<0.1	<0.1	1.26 ± 1.48
20:2n6	<0.1	0.92 ± 0.41	1.29 ± 0.83
20:3n6	<0.1	0.19 ± 0.09	0.22 ± 0.12
20:4n6	5.3 ± 1.72	4.94 ± 0.12	6.2 ± 1.16
20:3n3	<0.1	<0.1	<0.1
20:5n3	<0.1	0.46 ± 0.36	1.05 ± 0.51
U	<0.1	<0.1	<0.1
22:4n6	7.44 ± 2.81	3.62 ± 2.32	1.0 ± 0.82
22:5n6	2.24 ± 1.74	<0.1	<0.1
22:5n3	2.05 ± 1.59	0.78 ± 0.35	0.41 ± 0.21
22:6n3	2.8 ± 1.31	<0.1	1.3 ± 0.68

Table 4.2

Fatty acid content of uterine phosphatidylcholine in rats fed for three weeks on either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid ethyl ester or α-linolenic acid ethyl ester. Fatty acids are shown as a mean percentage of total fatty acid present in PC ± standard error (n = 3 in each diet group). U indicates an unidentified fatty acid.

F.A.	Control PE	Linoleic PE	Linolenic PE
12:0	2.63 ± 1.36	6.64 ± 3.4	1.92 ± 0.37
12:1	0.77 ± 0.41	0.49 ± 0.39	<0.1
13:0	<0.1	0.29 ± 0.19	<0.1
13:1	0.91 ± 0.44	1.86 ± 0.9	<0.1
14:0	5.03 ± 1.2	5.7 ± 0.76	5.19 ± 0.44
14:1	0.2 ± 0.15	0.2 ± 0.1	<0.1
14:2	0.15 ± 0.1	0.14 ± 0.04	<0.1
15:0	0.16 ± 0.06	0.64 ± 0.27	0.66 ± 0.31
15:1	0.53 ± 0.3	0.65 ± 0.48	0.81 ± 0.41
15:2	0.68 ± 0.58	0.95 ± 0.85	<0.1
16:0	33.92 ± 2.71	28.68 ± 3.43	40.27 ± 1.56
16:1	3.31 ± 1.67	4.03 ± 1.04	3.66 ± 0.41
16:2	<0.1	<0.1	<0.1
17:1	0.28 ± 0.09	0.43 ± 0.16	0.61 ± 0.08
17:2	0.5 ± 0.22	1.24 ± 0.32	0.92 ± 0.19
18:0	11.13 ± 4.06	11.49 ± 1.94	15.2 ± 0.88
18:1n9	15.59 ± 0.93	12.7 ± 0.9	15.36 ± 2.5
18:2n6	7.68 ± 2.57	3.48 ± 0.5	2.95 ± 0.12
18:3n6	0.1 ± 0.001	<0.1	0.14 ± 0.04
U	<0.1	0.13 ± 0.3	0.13 ± 0.03
18:3n3	0.34 ± 0.14	0.15 ± 0.05	0.38 ± 0.07
18:4n3	0.18 ± 0.08	<0.1	<0.1
20:0	0.13 ± 0.03	<0.1	0.18 ± 0.06
20:1n9	0.19 ± 0.05	0.18 ± 0.05	0.2 ± 0.1
U	0.23 ± 0.13	<0.1	<0.1
20:3n9	<0.1	<0.1	<0.1
20:2n6	1.1 ± 0.6	0.45 ± 0.07	0.74 ± 0.22
20:3n6	0.58 ± 0.3	1.36 ± 0.7	0.53 ± 0.09
20:4n6	6.3 ± 1.93	11.35 ± 2.24	4.15 ± 0.4
20:3n3	0.25 ± 0.15	0.47 ± 0.34	<0.1
20:5n3	<0.1	<0.1	1.98 ± 0.49
U	<0.1	<0.1	<0.1
22:4n6	0.94 ± 0.34	1.49 ± 0.54	0.28 ± 0.07
22:5n6	0.36 ± 0.2	0.71 ± 0.07	0.13 ± 0.03
22:5n3	0.25 ± 0.13	0.18 ± 0.05	0.83 ± 0.15
22:6n3	0.65 ± 0.19	0.52 ± 0.16	0.98 ± 0.36

Table 4.3

Fatty acid content of uterine phosphatidylethanolamine in rats fed for three weeks on either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid ethyl ester or α-linolenic acid ethyl ester. Fatty acids are shown as a mean percentage of total fatty acid present in PE ± standard error (n = 3 in each diet group). U indicates an unidentified fatty acid.

F.A.	Control FFA	Linoleic FFA	Linolenic FFA
12:0	2.36 ± 1.74	1.29 ± 0.32	0.3 ± 0.25
12:1	0.08 ± 0.03	<0.1	<0.1
13:0	0.99 ± 0.94	0.72 ± 0.37	3.62 ± 1.32
13:1	1.18 ± 0.54	1.15 ± 0.67	0.2 ± 0.15
14:0	1.1 ± 0.95	1.11 ± 0.7	0.96 ± 0.47
14:1	0.13 ± 0.08	<0.1	0.11 ± 0.06
14:2	0.08 ± 0.03	<0.1	0.15 ± 0.1
15:0	0.9 ± 0.81	3 ± 2.84	0.17 ± 0.12
15:1	0.45 ± 0.4	0.18 ± 0.13	<0.1
15:2	0.2 ± 0.1	0.33 ± 0.28	<0.1
16:0	23.32 ± 11.69	25.02 ± 3.94	21.6 ± 2.44
16:1	1.78 ± 0.89	1.13 ± 0.1	1.07 ± 0.54
16:2	3.24 ± 1.63	0.72 ± 0.39	1.23 ± 1.03
17:1	0.15 ± 0.1	0.5 ± 0.41	<0.1
17:2	1.56 ± 1.46	0.15 ± 0.1	1.58 ± 0.91
18:0	10.28 ± 3.82	19.88 ± 2.96	12.61 ± 1.26
18:1n9	10.58 ± 3.56	13.28 ± 1.17	17.06 ± 1.49
18:2n6	4.94 ± 1.23	2.24 ± 0.03	2.75 ± 0.66
18:3n6	0.15 ± 0.06	0.5 ± 0.43	<0.1
U	0.26 ± 0.16	0.92 ± 0.45	0.45 ± 0.2
18:3n3	<0.1	<0.1	<0.1
18:4n3	<0.1	<0.1	<0.1
20:0	0.27 ± 0.13	0.17 ± 0.06	<0.1
20:1n9	0.35 ± 0.06	0.52 ± 0.05	0.32 ± 0.17
U	0.36 ± 0.31	0.52 ± 0.19	1.45 ± 0.45
20:3n9	<0.1	0.87 ± 0.6	1.74 ± 0.96
20:2n6	1.72 ± 0.66	2.79 ± 1.14	2.72 ± 1.57
20:3n6	0.77 ± 0.22	0.73 ± 0.11	0.76 ± 0.38
20:4n6	8.47 ± 2.58	10.57 ± 0.42	6.98 ± 2.63
20:3n3	<0.1	<0.1	0.43 ± 0.38
20:5n3	0.74 ± 0.18	0.64 ± 0.24	1.24 ± 0.64
U	1.55 ± 1.42	2.9 ± 1.47	5.1 ± 2.58
22:4n6	1.04 ± 1.01	2.74 ± 0.44	1.07 ± 0.53
22:5n6	0.85 ± 0.81	0.51 ± 0.14	0.46 ± 0.21
22:5n3	0.19 ± 0.07	0.18 ± 0.11	1.04 ± 0.5
22:6n3	0.56 ± 0.24	0.29 ± 0.12	0.56 ± 0.28

Table 4.4

Free fatty acid content of the uteri of rats fed for three weeks on either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid ethyl ester or α-linolenic acid ethyl ester. Fatty acids are shown as a mean percentage of total uterine free fatty acid ± standard error (n = 3 in each diet group). U indicates an unidentified fatty acid.

F.A.	Control MG	Linoleic MG	Linolenic MG
12:0	1.11 ± 0.25	<0.1	4.34 ± 3.03
12:1	0.49 ± 0.44	<0.1	<0.1
13:0	0.71 ± 0.34	0.41 ± 0.36	0.55 ± 0.49
13:1	0.64 ± 0.3	0.41 ± 0.36	<0.1
14:0	3.86 ± 1	0.82 ± 0.77	5.02 ± 1.96
14:1	0.61 ± 0.28	<0.1	<0.1
14:2	1.38 ± 1.09	<0.1	<0.1
15:0	0.39 ± 0.34	0.8 ± 0.75	<0.1
15:1	1.59 ± 0.86	0.59 ± 0.54	1.38 ± 1.02
15:2	0.65 ± 0.6	3.52 ± 3.47	<0.1
16:0	23.22 ± 5.52	22.08 ± 5.31	36.92 ± 11.11
16:1	3.89 ± 0.9	1.37 ± 1.32	3.72 ± 2.72
16:2	<0.1	1.98 ± 1.94	0.51 ± 0.46
17:1	0.48 ± 0.44	1.73 ± 1.37	<0.1
17:2	0.9 ± 0.44	5.37 ± 3.29	1.92 ± 0.94
18:0	11.96 ± 2.08	15.66 ± 2.4	14.31 ± 3.14
18:1n9	11.6 ± 2.06	12.13 ± 2.02	14.47 ± 4.42
18:2n6	4.2 ± 0.85	2.75 ± 0.36	2.71 ± 1.05
18:3n6	<0.1	<0.1	<0.1
U	<0.1	0.3 ± 0.25	<0.1
18:3n3	<0.1	0.47 ± 0.42	0.13 ± 0.08
18:4n3	<0.1	<0.1	<0.1
20:0	<0.1	0.47 ± 0.42	<0.1
20:1n9	0.19 ± 0.14	0.77 ± 0.72	<0.1
U	<0.1	<0.1	<0.1
20:3n9	0.34 ± 0.3	0.29 ± 0.24	<0.1
20:2n6	2.34 ± 2.08	7.78 ± 5.36	0.38 ± 0.26
20:3n6	<0.1	<0.1	<0.1
20:4n6	7.64 ± 4.81	3.09 ± 1.11	2.53 ± 0.41
20:3n3	<0.1	0.4 ± 0.35	<0.1
20:5n3	<0.1	<0.1	0.84 ± 0.79
U	<0.1	<0.1	<0.1
22:4n6	1.57 ± 0.24	2.02 ± 1.44	0.64 ± 0.22
22:5n6	0.46 ± 0.3	<0.1	0.23 ± 0.18
22:5n3	0.36 ± 0.31	0.63 ± 0.58	1.54 ± 0.98
22:6n3	1.68 ± 0.24	0.51 ± 0.43	1.53 ± 0.79

Table 4.5

Fatty acid content of uterine monoglyceride in rats fed for three weeks on either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid ethyl ester or α-linolenic acid ethyl ester. Fatty acids are shown as a mean percentage of total fatty acid present in uterine monoglyceride ± standard error (n = 3 in each diet group). U indicates an unidentified fatty acid.

F.A.	Control DG	Linoleic DG	Linolenic DG
12:0	4.2 ± 2.55	2.56 ± 1.27	4.23 ± 3.46
12:1	0.25 ± 0.2	<0.1	0.26 ± 0.21
13:0	2.19 ± 0.83	0.85 ± 0.4	0.04 ± 0.004
13:1	0.79 ± 0.62	2.93 ± 0.27	0.36 ± 0.3
14:0	3.98 ± 0.3	4.32 ± 0.05	4.21 ± 2.27
14:1	0.75 ± 0.26	<0.1	0.36 ± 0.19
14:2	0.14 ± 0.08	<0.1	0.14 ± 0.09
15:0	1.21 ± 0.38	<0.1	0.16 ± 0.11
15:1	1.58 ± 0.42	4.1 ± 1.16	0.48 ± 0.39
15:2	1.63 ± 0.74	<0.1	0.16 ± 0.11
16:0	23.38 ± 1.59	23.29 ± 0.38	26.2 ± 2.6
16:1	3.46 ± 1.75	4.83 ± 0.29	7.85 ± 0.46
16:2	1.7 ± 1.65	6.04 ± 3.25	0.25 ± 0.2
17:1	0.87 ± 0.69	<0.1	0.12 ± 0.08
17:2	3.64 ± 2.41	6.69 ± 1.74	2.72 ± 2.67
18:0	8.02 ± 0.71	10.58 ± 0.77	6.78 ± 2.26
18:1n9	13.84 ± 1.59	11.52 ± 0.55	21.04 ± 0.99
18:2n6	5.68 ± 0.5	2.64 ± 0.15	1.92 ± 0.3
18:3n6	0.24 ± 0.01	0.24 ± 0.19	<0.1
U	1.65 ± 1.12	<0.1	<0.1
18:3n3	0.3 ± 0.12	<0.1	2.0 ± 0.1
18:4n3	0.14 ± 0.09	<0.1	0.6 ± 0.52
20:0	0.6 ± 0.35	<0.1	<0.1
20:1n9	0.81 ± 0.76	<0.1	<0.1
U	0.14 ± 0.09	<0.1	0.66 ± 0.6
20:3n9	<0.1	1.07 ± 0.52	<0.1
20:2n6	2.71 ± 0.32	<0.1	1.1 ± 0.68
20:3n6	0.14 ± 0.09	<0.1	0.49 ± 0.34
20:4n6	4.09 ± 1.16	4.61 ± 2.02	2.28 ± 1.29
20:3n3	0.23 ± 0.09	0.35 ± 0.3	<0.1
20:5n3	0.58 ± 0.27	<0.1	0.28 ± 0.23
U	<0.1	<0.1	<0.1
22:4n6	1.23 ± 0.64	<0.1	0.09 ± 0.04
22:5n6	0.41 ± 0.36	<0.1	0.13 ± 0.01
22:5n3	0.46 ± 0.12	<0.1	1.11 ± 0.86
22:6n3	1.43 ± 0.74	<0.1	2.63 ± 2.35

Table 4.6

Fatty acid content of uterine diglyceride in rats fed for three weeks on either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid ethyl ester or α-linolenic acid ethyl ester. Fatty acids are shown as a mean percentage of total fatty acid present in uterine diglyceride ± standard error (n = 3 in each diet group). U indicates an unidentified fatty acid.

F.A.	Con TG, Chol	Linoleic TG, Chol	Linolenic TG, Chol
12:0	1.744 ± 0.716	3.08 ± 3.01	13.28 ± 2.32
12:1	0.485 ± 0.187	0.64 ± 0.4	0.63 ± 0.11
13:0	0.624 ± 0.187	0.39 ± 0.19	0.06 ± 0.01
13:1	0.213 ± 0.048	0.3 ± 0.17	0.09 ± 0.04
14:0	3.296 ± 0.554	10.04 ± 2.12	10.03 ± 0.94
14:1	0.56 ± 0.358	0.87 ± 0.18	0.99 ± 0.03
14:2	0.22 ± 0.06	0.13 ± 0.08	<0.1
15:0	0.71 ± 0.28	0.34 ± 0.15	0.19 ± 0.08
15:1	0.3 ± 0.1	0.21 ± 0.16	0.06 ± 0.01
15:2	0.34 ± 0.26	<0.1	<0.1
16:0	21.19 ± 2	30.02 ± 1.62	27.71 ± 2.68
16:1	6.46 ± 0.57	12.01 ± 0.4	10.24 ± 0.41
16:2	0.33 ± 0.16	0.39 ± 0.34	<0.1
17:1	0.451 ± 0.05	0.24 ± 0.04	0.09 ± 0.04
17:2	0.45 ± 0.23	0.78 ± 0.34	0.25 ± 0.2
18:0	2.74 ± 1.18	3.74 ± 0.35	2.77 ± 0.18
18:1n9	22.85 ± 3.9	25.76 ± 0.94	22.64 ± 0.46
18:2n6	15.0 ± 2.96	4.3 ± 0.83	1.17 ± 0.19
18:3n6	0.265 ± 0.21	0.15 ± 0.1	<0.1
U	<0.1	<0.1	<0.1
18:3n3	0.88 ± 0.21	0.27 ± 0.19	1.77 ± 1
18:4n3	<0.1	0.15 ± 0.02	0.08 ± 0.04
20:0	0.88 ± 0.21	0.32 ± 0.28	<0.1
20:1n9	0.27 ± 0.12	0.63 ± 0.55	0.13 ± 0.08
U	<0.1	<0.1	0.12 ± 0.07
20:3n9	0.06 ± 0.01	0.17 ± 0.03	0.15 ± 0.07
20:2n6	0.45 ± 0.13	0.21 ± 0.06	0.16 ± 0.11
20:3n6	0.1 ± 0.05	0.12 ± 0.04	<0.1
20:4n6	2.2 ± 0.6	1.98 ± 0.4	0.64 ± 0.39
20:3n3	<0.1	<0.1	<0.1
20:5n3	0.12 ± 0.05	<0.1	0.33 ± 0.06
U	<0.1	<0.1	<0.1
22:4n6	0.6 ± 0.12	1.09 ± 0.5	0.08 ± 0.02
22:5n6	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.03
22:5n3	0.07 ± 0.02	0.61 ± 0.55	0.33 ± 0.12
22:6n3	0.7 ± 0.18	0.21 ± 0.09	1.2 ± 1.0

Table 4.7

Fatty acid content of uterine triglycerides and cholesterol esters in rats fed for three weeks on either a control pelleted diet or semisynthetic diet supplemented with linoleic acid ethyl ester or α-linolenic acid ethyl ester. Fatty acids are shown as a mean percentage of total fatty acid present in uterine triglycerides and cholesterol esters ± standard error (n = 3 in each diet group). U indicates an unidentified fatty acid.

lipid fraction with mean levels of 2.22%, 1.98% and 0.64% in the control, linoleic and linolenic diet groups respectively. The proportions of arachidonic acid in the other neutral lipid fractions (diglyceride and monoglyceride - tables 4.4 and 4.3) were also lower than those in the free fatty acid and phospholipid fractions (tables 4.4 - 4.7).

Proportions of eicosapentaenoic acid (20:5n3) were highest in the linolenic acid diet group in all the lipid fractions except diglyceride where it was highest in the control group (tables 4.1 - 4.7). In the control and linoleic diet groups, the free fatty acid lipid fraction contained the highest proportion of 20:5n3 (0.74% $\pm$ 0.18 and 0.64% $\pm$ 0.24 respectively). However, in the linolenic group, the highest proportions were found in the PI and PE at 2.09% $\pm$ 0.66 and 1.98% $\pm$ 0.49 respectively, compared to 1.24% $\pm$ 0.64 in the free fatty acids. There was no clear dietary effect on levels of dihomo-gamma-linolenic acid (20:3n6) in any of the lipid fractions. The free fatty acid and PE fractions contained the highest proportions of this fatty acid, with percentages of 0.77 $\pm$ 0.22, 0.73 $\pm$ 0.11 and 0.76 $\pm$ 0.38 in the control, linoleic and liolenic groups respectively in the free fatty acid fraction, and proportions 0.58% $\pm$ 0.3, 1.36% $\pm$ 0.7 and 0.53% $\pm$ 0.09 in the control, linoleic and linolenic groups respectively, in the PE fraction.

Control proportions of linoleic acid were considerably higher than those of linoleic and linolenic acid ethyl ester fed rats in all lipid fractions except PI where proportions were similar in all three diet groups (tables 4.1 - 4.7). The proportions of linoleic

acid in the linoleic acid fed rats were slightly higher than those of the linolenic acid fed rats in all except the free fatty acid fraction where the linolenic acid fed rats had a higher proportion. There was a higher proportion of linoleic acid in the triglyceride - cholesterol ester lipid fraction in both control and linoleic groups (15% ± 2.96 and 4.3% ± 0.83 respectively) than in any of the other lipid fractions in the different diet groups. However, in the linolenic group there was little difference between lipid fractions, with proportions of around 2% being found in all lipid frations. α-linolenic acid proportions were highest in the diglyceride and triglyceride - cholesterol ester lipid fractions of the linolenic acid diet group, with proportions of 2.0% ± 0.1 and 1.77% ± 1 respectively, being observed. These were also the only lipid groups where a clear dietary effect on α-linolenic acid levels was observed, with the linolenic acid fed rats having the highest percentages and the linoleic the lowest.

The n6 22 carbon fatty acids (22:4n6 and 22:5n6) were present in considerably lower proportions than arachidonic acid in each of the diet groups in all of the lipid fractions, with the exception of the control PC. The 22:4n6 was also generally present in higher proportions than 22:5n6. This was in contrast to the n3 22 carbon fatty acids (22:5n3 and 22:6n3) which were present in equal or greater proportions than 20:5n3 throughout most of the lipid fractions (with the exception of the free fatty acids). 22:6n3 was also usually present in higher proportions than 22:5n3. Figures 4.1 - 4.14 illustrate these results. Proportions of 22:4n6 were higher in linoleic and control rats than in linolenic rats, whereas 22:5n3 and 22:6n3 were higher in the linolenic and control groups than in

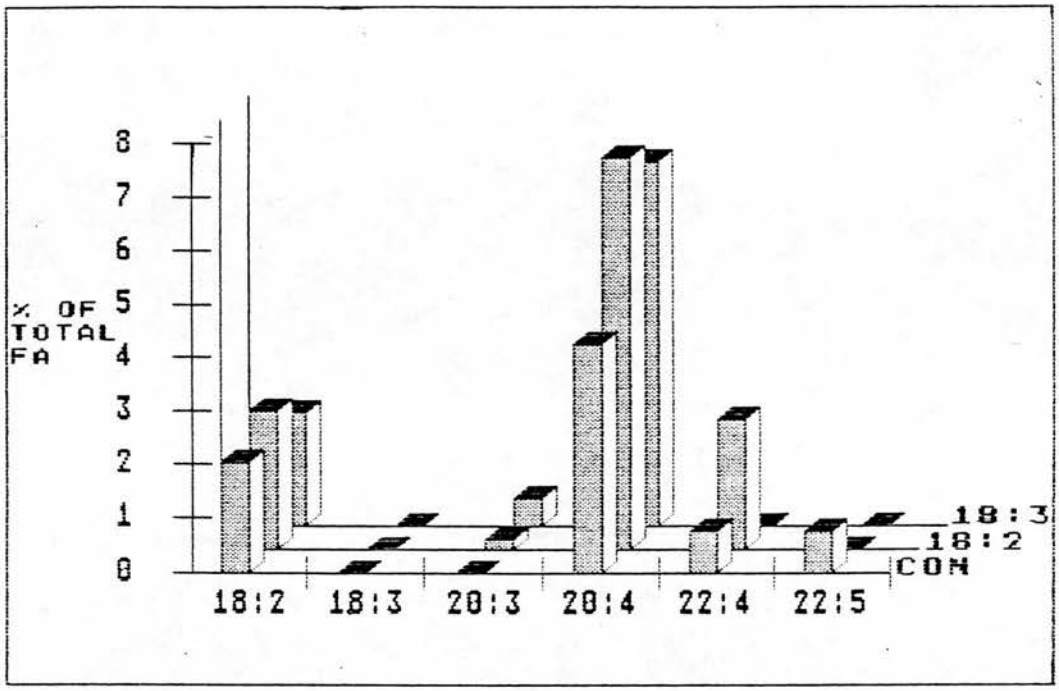


Figure 4.1
Proportions of n6 essential fatty acids in the phosphatidylinositol lipid fraction of uterine tissue from rats fed for three weeks on either a control pelleted diet (83.6% n6 and 17.4% n3 EFA), a semisynthetic diet supplemented with linoleic acid (99.9% n6 EFA) or a semisynthetic diet supplemented with α-linolenic acid (97.6% n3 EFA). n = 3 for each diet group. Proportions are expressed as a percentage of the total fatty acid detected.

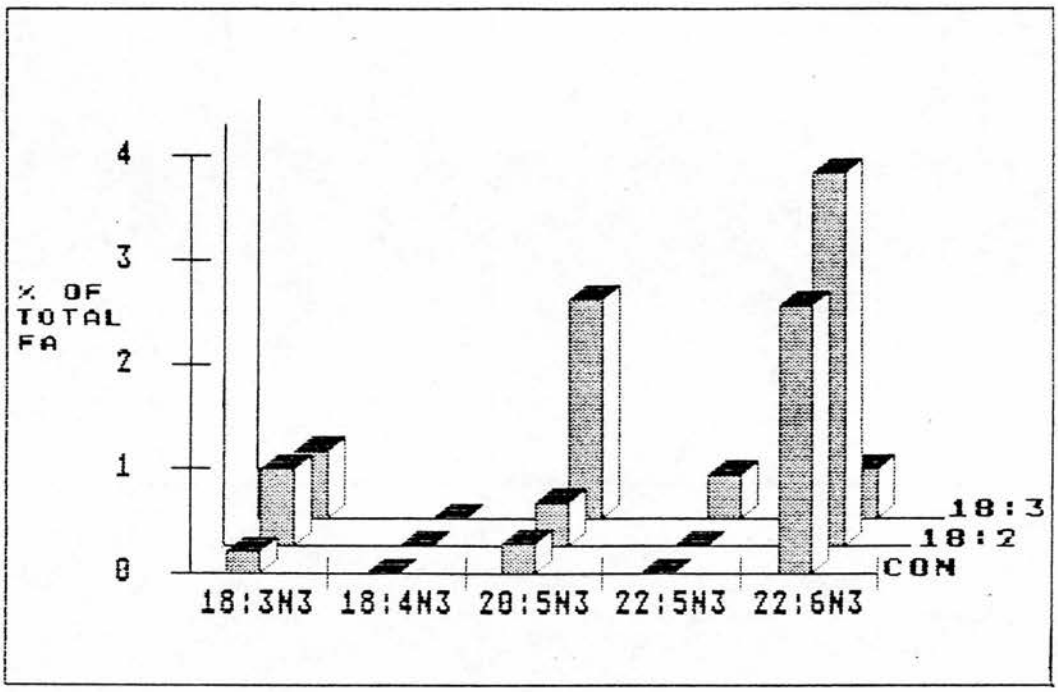


Figure 4.2
Proportions of n3 essential fatty acids in the phosphatidylinositol lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

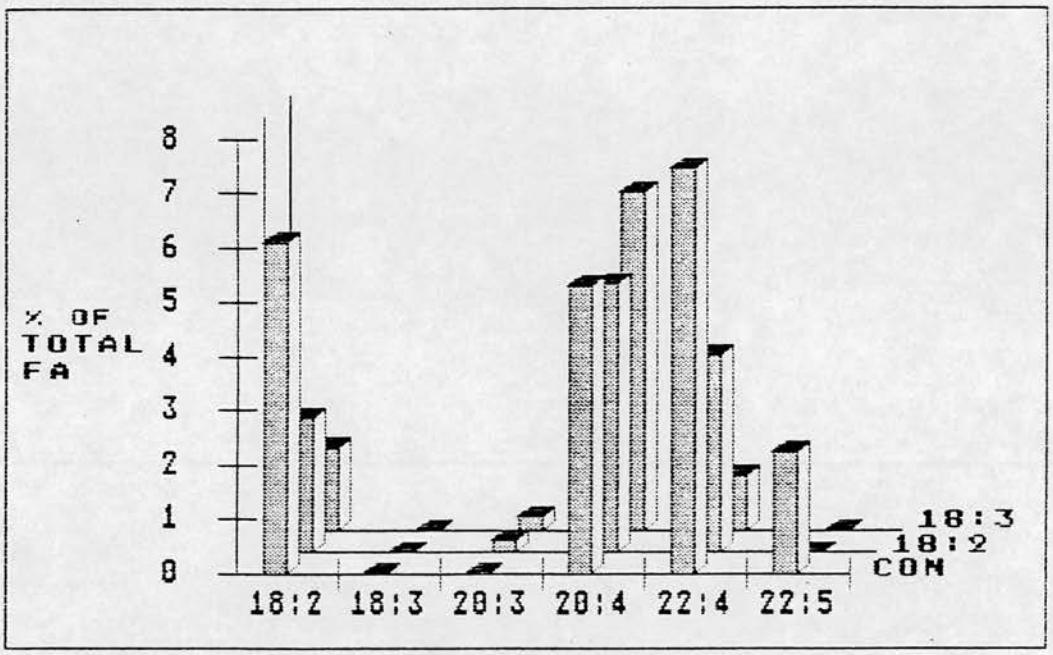


Figure 4.3
Proportions of n6 essential fatty acids in the phophatidylcholine lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

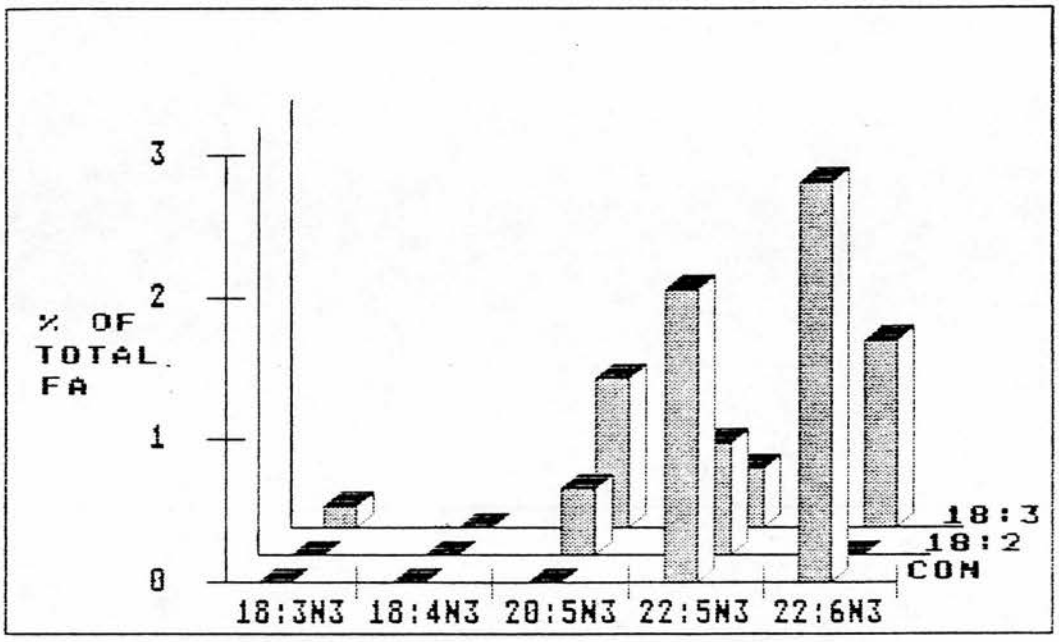


Figure 4.4
Proportions of n3 essential fatty acids in the phosphatidylcholine lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

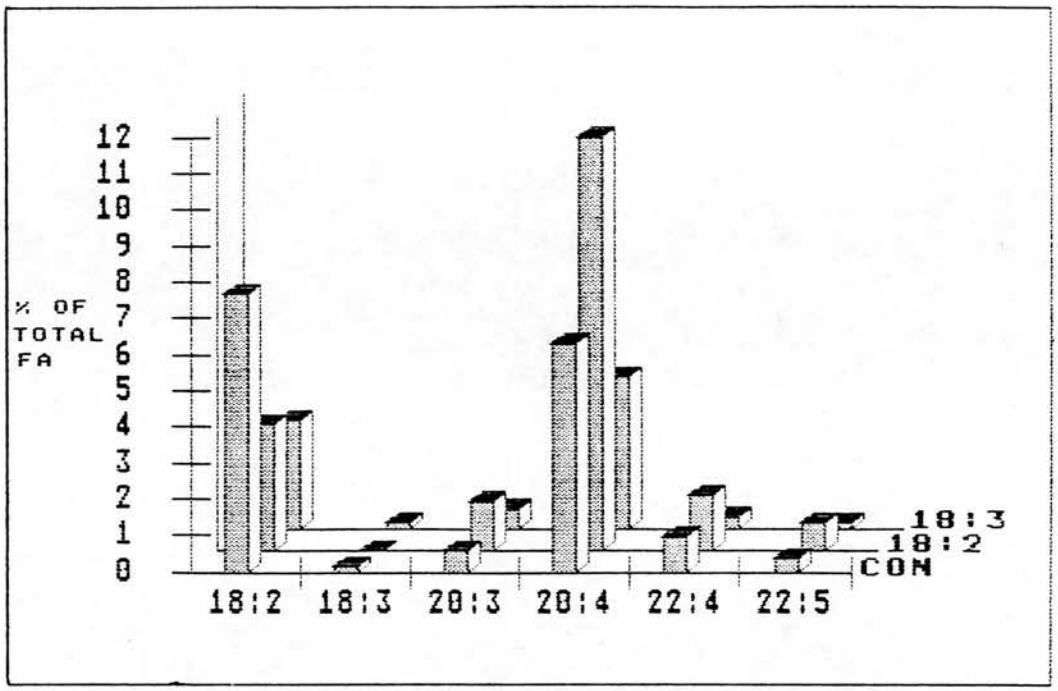


Figure 4.5
Proportions of n6 essential fatty acids in the phosphatidylethanolamine lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

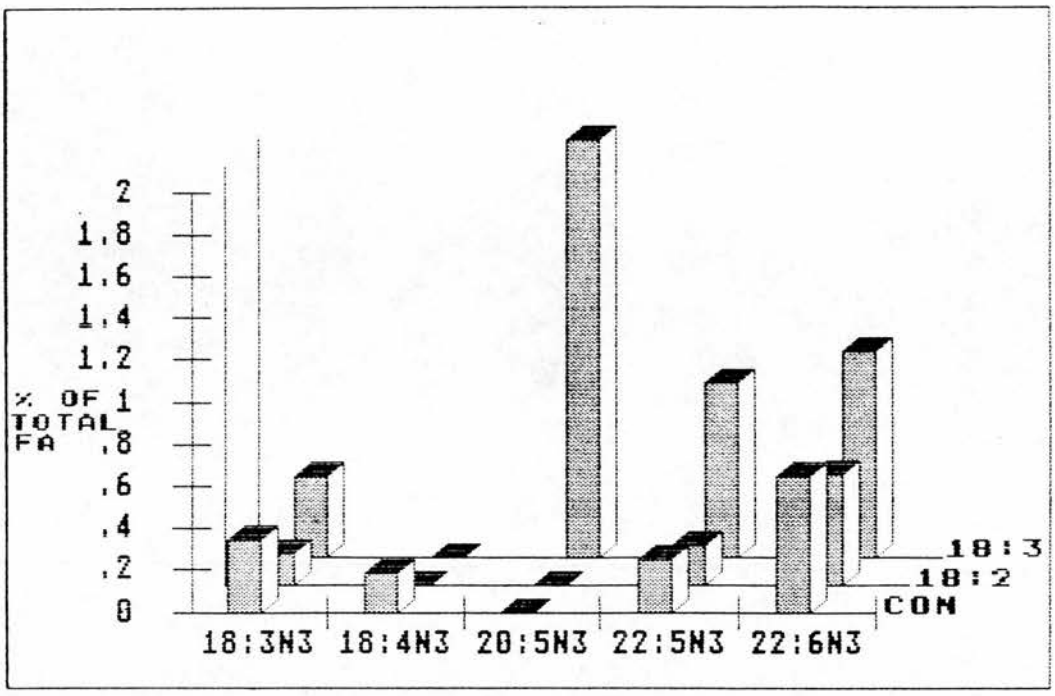


Figure 4.6
Proportions of n3 essential fatty acids in the phosphatidylethanolamine lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

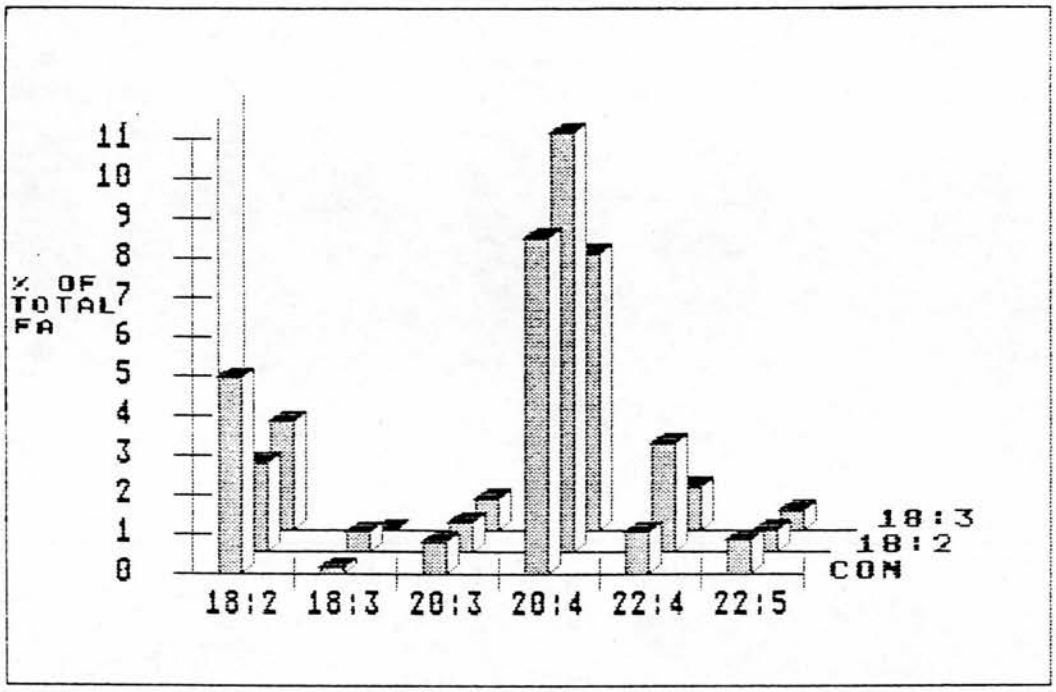


Figure 4.7
Proportions of n6 essential fatty acids in the free fatty acid lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

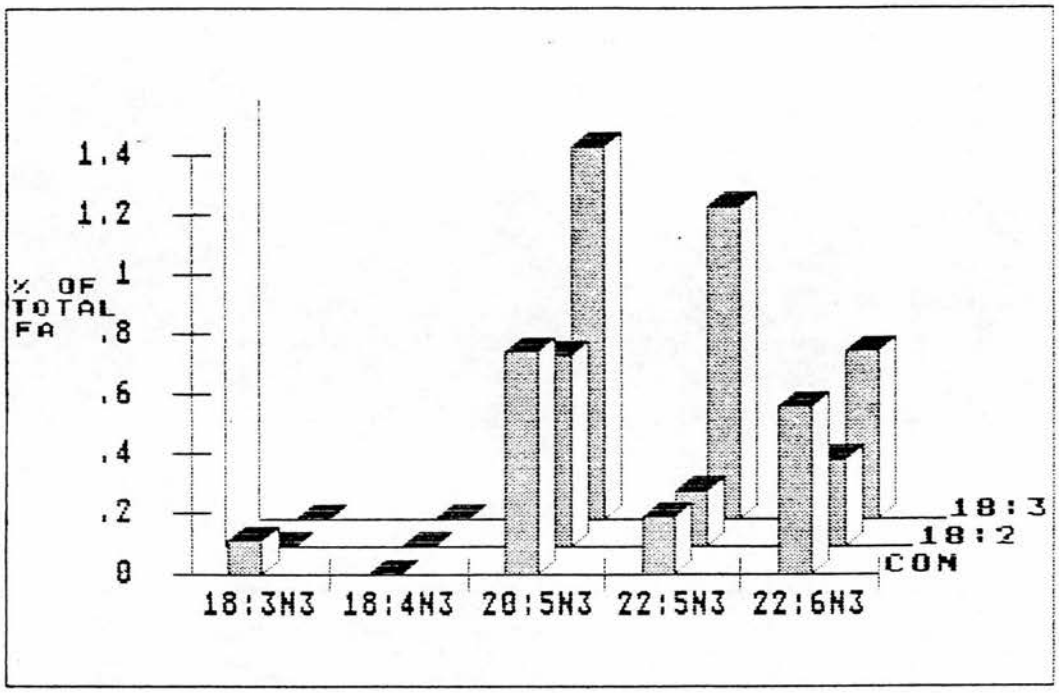


Figure 4.8
Proportions of n3 essential fatty acids in the free fatty acid lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

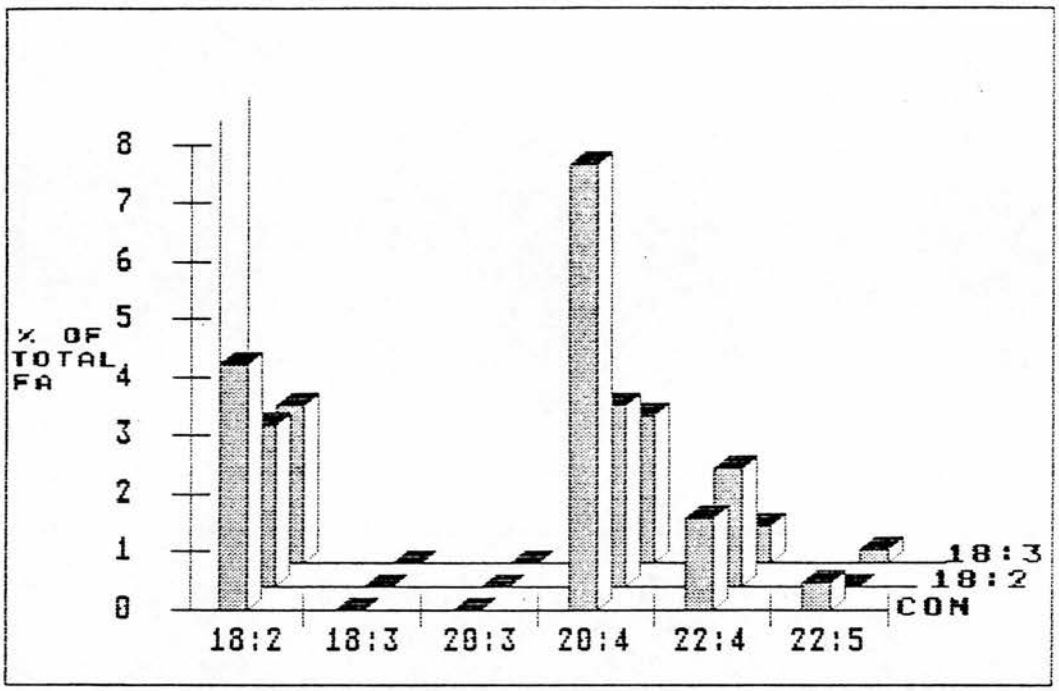


Figure 4.9
Proportions of n6 essential fatty acids in the monoglyceride lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

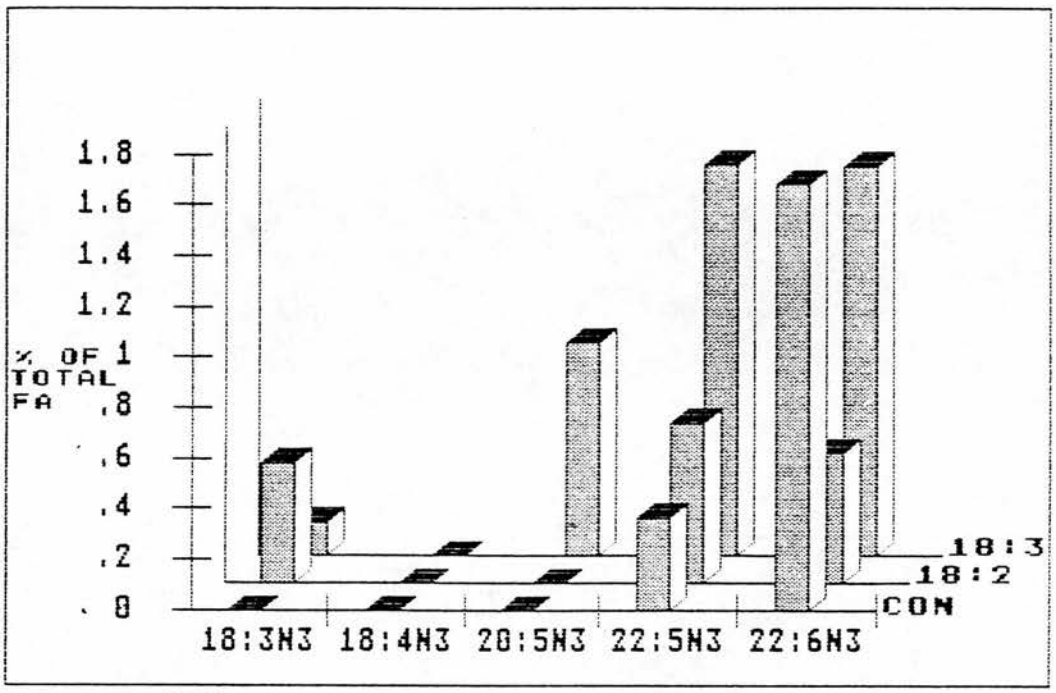


Figure 4.10
Proportions of n3 essential fatty acids in the monoglyceride lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

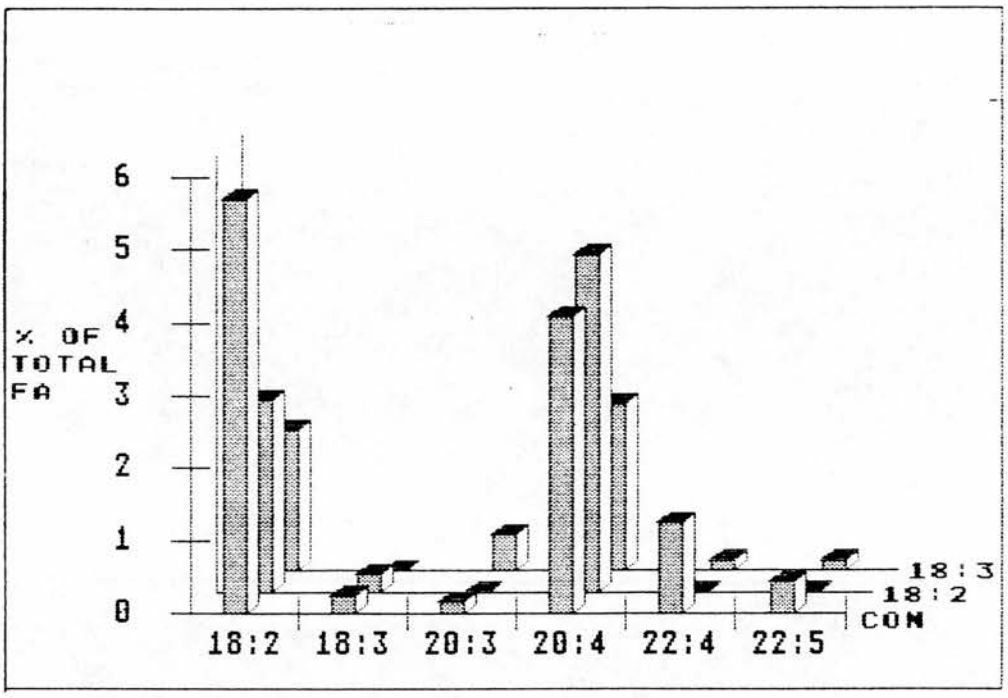


Figure 4.11
Proportions of n6 essential fatty acids in the diglyceride lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

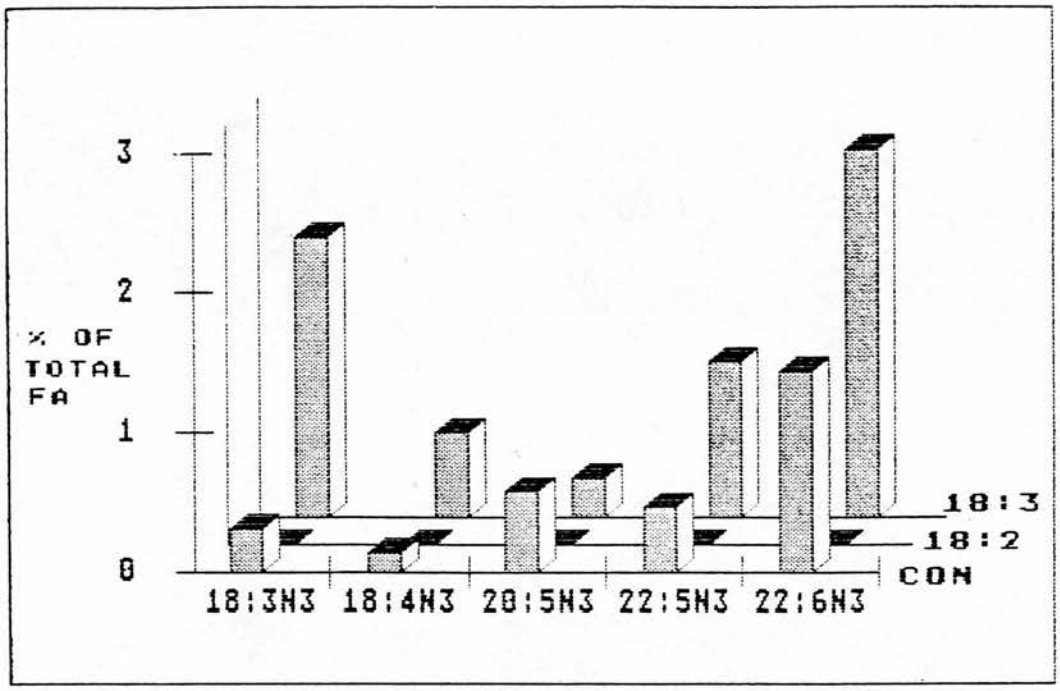


Figure 4.12
Proportions of n3 essential fatty acids in the diglyceride lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

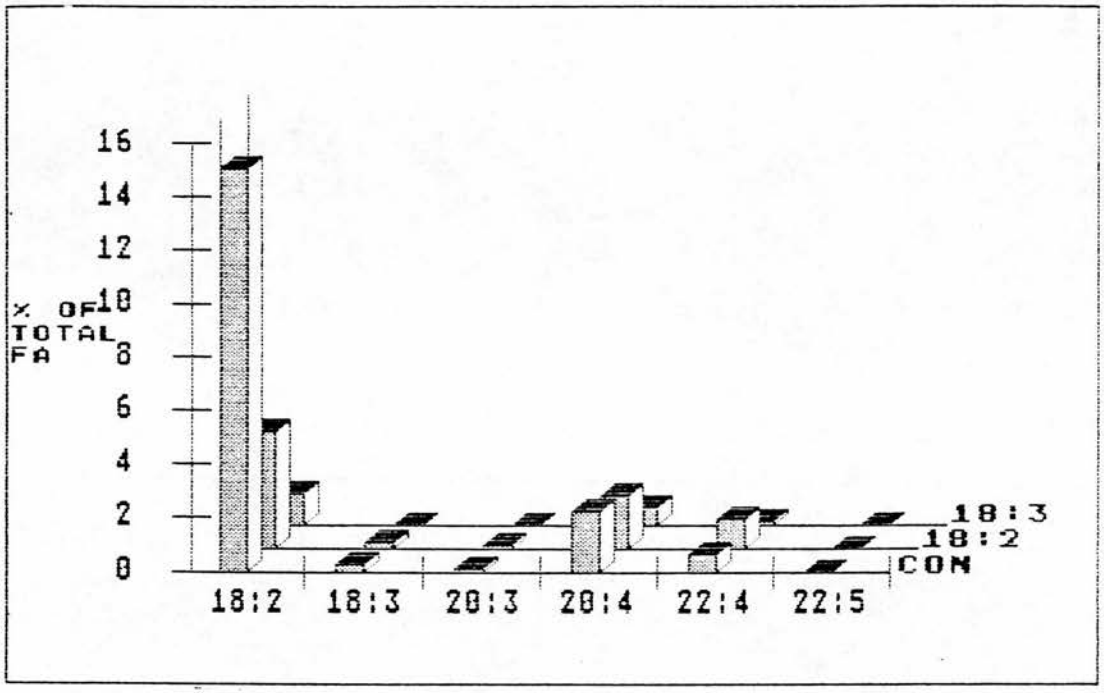


Figure 4.13
Proportions of n6 essential fatty acids in the triglyceride and cholesterol ester lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

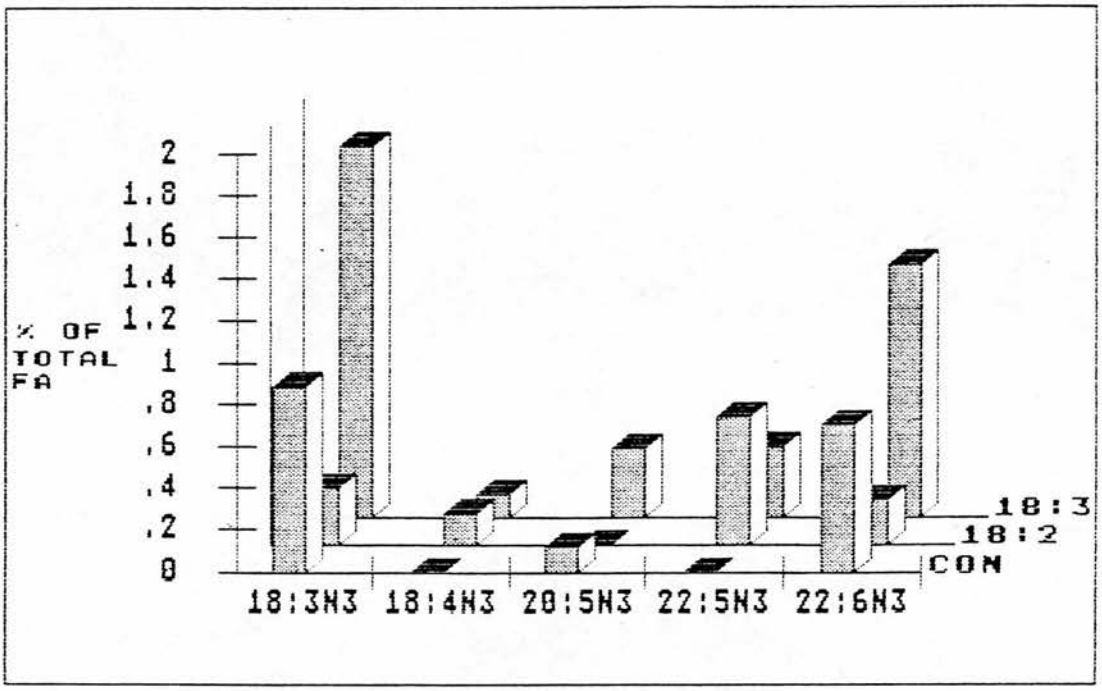


Figure 4.14
Proportions of n3 essential fatty acids in the triglyceride and cholesterol ester lipid fraction of uterine tissue from rats fed for three weeks on diets as described in figure 4.1.

the linoleic group.

Figure 4.15 shows the n3/n6 ratios of the different diet groups in each lipid fraction. The ratios were highest in the linolenic fed rats in the triglyceride - cholesterol ester and diglyceride lipid fractions (ratios of 1.39 and 1.33 respectively) and lowest in the linoleic fed rats in the free fatty acid and PE fractions (both ratios were 0.08). In each lipid fraction the linolenic group had the highest n3/n6 ratio and the linoleic the lowest, with the exception of the triglyceride - cholesterol ester fraction where the control ratio was slightly lower than that of the linoleic. The PI and free fatty acid lipid fractions were least affected by the linolenic acid diet with ratios of 0.23 and 0.26 respectively. PE was the phospholipid group most affected by the linolenic acid diet with a ratio of 0.47. The dietary n3/n6 ratio of control, linoleic and linolenic acid diets were 0.18, 0.002 and 17.43 respectively. The value for the linoleic acid diet was far lower than any of the tissue values found, whereas the ratio for the linolenic acid diet was far greater than any of the tissue ratios. This suggests conservation of both n3 and n6 fatty acids in the uterus and is in accordance with the results presented in chapter 3, where rats were fed evening primrose oil and fish oil diets high in n6 and n3 fatty acids respectively.

4.4 Discussion

The results of this chapter confirm the results described in chapter 3 which indicated that three weeks of feeding diets with high n3 or n6 fatty acid contents induced significant changes in the fatty acid composition of uterine tissue of the rat.

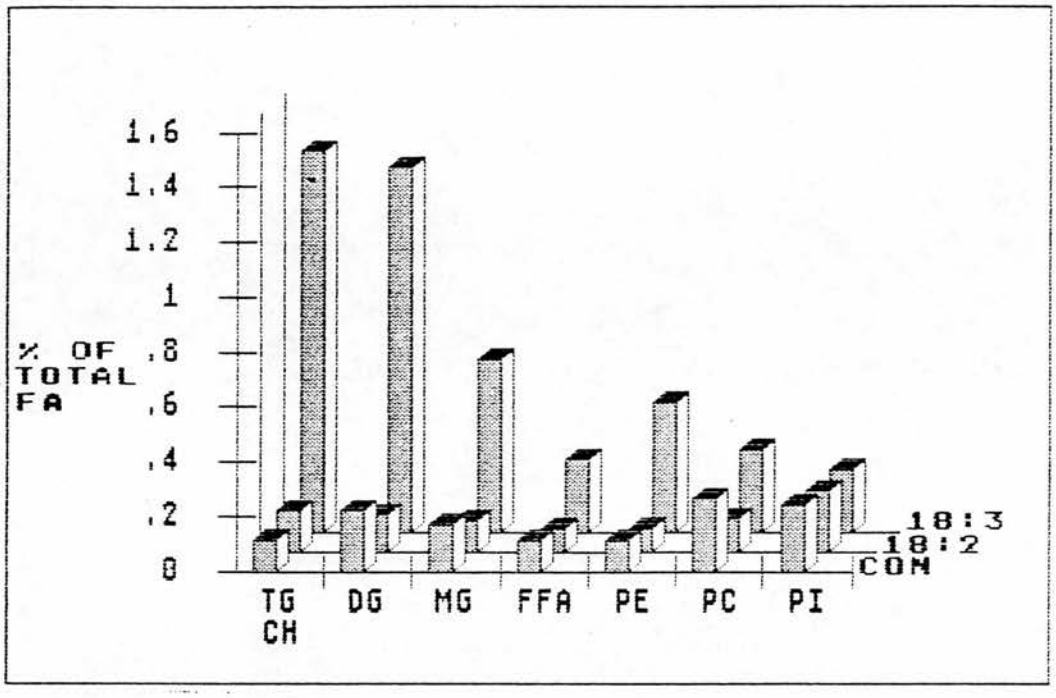


Figure 4.15
n3/n6 EFA ratios in triglyceride-cholesterol ester, diglyceride, monoglyceride, free fatty acid, PE, PC and PI lipid fractions from uterine tissue of three groups of three rats fed for three weeks on either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid (99.9% n6 EFA) or a semisynthetic diet supplemented with α-linolenic acid (97.6% n3 EFA).

In the phospholipids, the fatty acid content of PE was more susceptible to changes in dietary n3 and n6 fatty acid than either PC or PI (Figure 4.15). In rats fed α-linolenic acid the n3/n6 ratio was higher in PE than in either PC or PI (0.47 compared to 0.3 and 0.23 respectively). The PE 20C essential fatty acids were also more susceptible to dietary change than those of PC and PI with high proportions of 20:5n3 (1.98% ± 0.49) but low arachidonic acid (4.15% ± 0.4) in the α-linolenic acid fed rats (figures 4.5 and 4.6). Abeywardena et al. (1987) also reported the largest decrease in arachidonic acid to occur in PE on feeding a diet high in n3 fatty acids in rat cardiac tissue. In the linoleic acid fed rats the opposite was true (figures 4.5 and 4.6) with low 20:5n3 (<0.1%) and high arachidonic acid proportions (11.35% ± 2.24). In both PC and PI 20:5n3 levels were higher in the linolenic group than in the other diet groups, however the difference was less than in the PE fraction (figures 4.2 and 4.4). The α-linolenic acid diet had no effect on the arachidonic acid proportion in PI and PC (figures 4.1 and 4.3 respectively). In the PC and PI fractions there were low levels of 22:4n6 in the linolenic group compared to the control and linoleic groups. Inhibition of conversion of arachidonic acid to adrenic acid may be one way in which the uterus conserves arachidonic acid.

There was a marked difference in the effect of the n3 and n6 diets on the essential fatty acid composition of individual lipid groups. Neutral lipids (with the exception of free fatty acids) and in particular the triglyceride - cholesterol ester and diglyceride fractions exhibited the largest increase in n3/n6 fatty acid ratio

in response to the α-linolenic acid diet (figure 4.15). The essential fatty acid content of the triglyceride - cholesterol ester fraction consisted mainly of 18C fatty acids and only contained low proportions of the eicosanoid precursors (table 4.7). In chapter 3 a relatively high accumulation of linoleic and α-linolenic acids in comparison to the longer chain essential fatty acids was observed in the neutral lipids. This accumulation of the 18C n3 and n6 fatty acids was not observed in diglyceride, monoglyceride or free fatty acid lipid fractions. The arachidonic acid and eicosapentaenoic acid composition of the diacylglycerol, monacylglycerol and free fatty acids also differed from that of the triacylglcerol - cholesterol ester pool, in containing higher proportions of these fatty acids (tables 4.4-4.6). This suggests the possible actions of phospholipase C, diacylglycerol lipase and monoacylglycerol lipase in releasing free fatty acids in uterine tissues. These enzymes have been identified in human uterine decidua and foetal membranes (Di Renzo et al., 1981; Okazaki et al., 1981)

In the free fatty acid fraction, arachidonic acid and eicosapentaenoic acid comprised a large proportion of the essential fatty acids (figures 4.7 and 4.8), suggesting these fatty acids may be selectively released. The dietary effect on the proportions of free fatty acids was not as pronounced as that seen in the PE, monoglyceride or diglyceride fractions, suggesting that the major sources of free fatty acid in the non-pregnant rat uterus may be from PI and PC. Leaver and Poyser (1981) suggested that PC may form the major source of arachidonic acid for prostaglandin synthesis in the guinea pig uterus, and further studies using tritiated arachidonic acid indicated release of arachidonic acid in vitro from

PC, PE and possibly triglyceride (Ning et al., 1983). In human foetal membranes during labour, PI and diacyl PE have been suggested to be the sources of free arachidonic acid (Okita et al., 1982).

Despite the high dietary levels of linoleic or α-linolenic acids received by the rats on the semi-synthetic diet (greater than 95% of total unsaturated fatty acid in both cases), this did not increase the tissue levels of these fatty acids compared to those from rats on the control diet (which contained only 45.6% linoleic acid and less than 2.8% α-linolenic acid). In most of the lipid fractions, rats from the control group contained higher tissue levels of linoleic acid than those from the linoleic group. The high levels of linoleic acid in the control rats may have resulted from impaired desaturation and elongation by n3 fatty acids also present in the control diet. However, there was little overall difference between arachidonic acid levels in the control and linoleic acid fed rats. The control levels may, however, have been increased by small quantities of gamma-linolenic acid and arachidonic acid in the control diet. One other possibility for the lower levels of linoleic acid in the linoleic acid fed rats is that the ethyl ester form of the fatty acid may not have been well absorbed by the rats. It has been suggested that the ethyl ester forms of fatty acids are not as well absorbed as the free acid, or other forms such as triacylglycerols, in humans (El Boustani et al., 1987; Lawson and Hughes, 1988) and rats (Lawson et al., 1985).

In all of the lipid fractions and dietary groups there were high proportions of linoleic acid compared to gamma-linolenic and dihomo-

gamma-linolenic acids. There was no accummulation of either gamma- or dihomo-gamma-linolenic acids in any of the lipid groups. This suggests low D6-desaturase activity but high elongase and D5 desaturase activity. The same results were found in chapter 3. The high proportions of n3 22C fatty acids compared to n3 20C fatty acids and the low proportions of n6 22C compared to n6 20C fatty acids also correlated with results presented in chapter 3. The rats fed the linoleic and linolenic acid diets in this study received no detectable quantities of 22C fatty acids in the diet, suggesting that the tissue levels of these fatty acids were a result of synthesis from precursors. The accummulation of 22C n3 fatty acids relative to 20C n3 fatty acids has been reported in other studies (Abeywardena et al., 1987). The considerably greater accummulation of 22C n3 fatty acids compared to 22C n6 fatty acids suggested that 20:5n3 was a better substrate than 20:4n6 for the elongase enzymes and that 22:5n3 was a much better substrate than 22:4n6 for D4 desaturase.

In conclusion, the dietary n3 and n6 fatty acids were incorporated differently in individual lipid fractions of the rat uterus. The phospholipid fractions of PC and PI conserved arachidonic acid when rats were fed a diet high in α-linolenic acid and low in n6 fatty acids. PE was more susceptible to dietary change than phosphatidylinositol or phosphatidylcholine. Evidence was found for the selective release of the prostaglandin precursor essential fatty acids, with the free fatty acid fraction containing high levels of these fatty acids compared to 18C and 22C essential fatty acids. Low levels of the eicosanoid precursor fatty acids were detected in

the triglyceride and the cholesterol ester pools.

Chapter 5

The Biosynthesis of the 2- and 3-Series Prostaglandins in Rat Uterus After Linoleic and α-Linolenic Acid Feeding: Mass Spectroscopy of Prostaglandin E and F Produced by Rat Uteri in Tissue Culture

5.1 Introduction

The uterine tissues of the human responsible for prostaglandin synthesis are characterised by a high arachidonic acid content and by increasing arachidonic acid incorporation with gestational age (Schwarz et al., 1975; Schwartz et al., 1977). Large quantities of prostaglandins are released at parturition (Leaver et al., 1989; Leaver et al., 1987; Leaver & Seawright, 1982; Leaver & Richmond, 1984; Roberts et al., 1975) and the selective esterification of arachidonic acid by uterine phospholipids has been described (Leaver & Ning, 1981; Ning et al., 1983; Leaver & Peatty, 1985). Both arachidonic acid esterification and prostaglandin release are influenced by endocrine control (Leaver et al., 1989; Leaver & Poyser, 1981; Leaver & Ning, 1981; Olund & Lunell, 1980; Ning et al., 1983; Leaver & Seawright, 1982; Leaver & Richmond, 1984; Roberts et al., 1985; Leaver & Peatty, 1985). The inhibitory effect of the n3 essential fatty acids on uterine activity at parturition was identified in rats fed a diet containing α-linolenic acid as the major essential fatty acid (Quackenbush et al., 1942; Leat & Northrop, 1979; Leat & Northrop, 1981). These rats showed defects in initiating and sustaining labour. As a result of the protracted labour, fetal mortality was high and it was found that if Caesarian section was carried out early in labour, live animals were delivered. Similar effects on the initiation of parturition were

observed when fish oil was given as the major dietary essential fatty acid (Leaver et al., 1986). In these experiments, an inhibition of the uterine synthesis of the 2-series prostaglandin, PGE_2, was detected in fish oil-fed rats.

The composition of uterine lipid may influence the release and the activity of a range of stimuli at parturition. These stimuli include the prostaglandins, platelet-activating factor, cytokines and other mediators (Leaver et al., 1989; Roberts et al., 1985; Billah & Johnston, 1983; Romero et al., 1988). The best characterised of these stimuli are $PGF_{2\alpha}$ and PGE_2. The involvement of PGE_2 and $PGF_{2\alpha}$ in the excitatory phases of uterine activity has been established (Wickland et al., 1984), although the control of their release remains poorly defined. It has been proposed that a major regulator of cyclooxygenase activity is substrate availability. The dietary n3 fatty acids are rapidly incorporated into uterine lipid (chapters 3 and 4), and selective turnover of the n6 fatty acids in the various lipid pools of the uterus has been detected (Leaver & Poyser, 1981; Leaver & Ning, 1981; Olund & Lunell, 1980; Okita et al., 1982; Ning et al., 1983; Okazaki et al., 1978; Leaver & Peatty, 1985). There is also evidence of selective release of n6 fatty acids during parturition (Ogburn et al., 1980; Olund & Lunell, 1980; Okita et al., 1982; Okazaki et al., 1981). These studies of fatty acid uptake and release indicate that the enzymes of uterine phospholipid metabolism regulate the release of n6 fatty acids at parturition. It has been proposed that the n3 effect on parturition may be explained principally in terms of the competetive inhibition of n6 specific cyclooxygenase metabolism (Leaver & Poyser, 1981; Leaver & Ning, 1981; Olund & lunell, 1980;

Ning et al., 1983; Leaver et al., 1986). However, little is known about the capacity of the cyclooxygenase enzyme to metabolise the n3 fatty acids in the uterus (Leaver et al., 1989). We therefore investigated the molecular species of PGF and PGE synthesised by the uteri of rats after short-term feeding with diets containing either predominantly n3 fatty acids, predominantly n6 fatty acids or a normal pelleted diet. The GC-MS fragmentation of a variety of derivatives of $PGF_{2}\alpha$, $PGF_{3}\alpha$, PGE_{2}, PGE_{3}, PGB_{2} and PGB_{3} were investigated and compared with the spectra of PGF, PGE and PGB synthesised by the uteri of rats fed the n3, n6 and control diets. The uterine prostaglandin synthesis was studied under two conditions, using either unstimulated uterine explants in tissue culture, or tissue whose phospholipase A_{2} had been maximally stimulated using the calcium ionophore A23187. The aim of this study was to determine the capacity of the unstimulated and the stimulated uterus to release the 3-series prostaglandins and to define the influence of dietary EFA on uterine prostaglandin release.

5.2 Materials and Methods

5.2.1 Rats and Diet

Twenty four newly weaned 21 day old female Sprague Dawley rats were randomly divided into three groups and maintained on different diets of either a control pelleted diet or a semisynthetic diet supplemented with linoleic acid or α-linolenic acid (see section 2.1 for diet compositions). After 21 days on the diet, rats were killed and the uteri removed as described in section 2.3. Rats used in these experiments were the same as those in chapter 4.

5.2.2 GC-MS of Prostaglandins

A number of different methods of purification and derivatisation of prostaglandins were tested to identify the best method of analysis. These will be described in the order in which they were carried out.

(a) Analysis of PGF (Me,TMS) and PGE (Me,BuO,TMS) Derivatives.

2 uteri from each diet group were cultured in medium 199 containing 5µg/ml A23187. After culture, the medium from the two uteri of each diet group was pooled and the prostaglandins extracted into ethyl acetate (section 2.4). The samples were then purified by passing them through a Sep-Pak and each sample split into two halfs (section 2.4). PGF (Me,TMS) and PGE (Me,BuO,TMS) derivatives were then prepared from one half of each sample (section 2.7a). These samples were analysed by total ion monitoring (TIM, see section 2.8a) and single ion monitoring (SIM, see section 2.8b) for the PGF 423 ion. The other half of the samples were further purified by t.l.c. (section 2.4) and were analysed using TIM and SIM for the 510 and 295 ions in the E samples and the 423 ion in the F samples. Standards of PGE_2, PGE_3, $PGF_{2\alpha}$ and $PGF_{3\alpha}$ were run prior to samples in each case to obtain elution times for the prostaglandins.

(b) Analysis of PGB (Me,TBDMS) Derivatives.

Six uteri from each diet group were divided into two groups of three, and one group of three from each diet group was cultured in medium 199 with 5 ug/ml A23187 and the other group of three cultured in medium 199 without A23187. Prostaglandins from each group were extracted from the medium and pooled (section 2.4). They were then purified by passing through a Sep-Pak, followed by t.l.c.. PGE was converted to PGB (section 2.5) and further purified by HPLC (see

section 2.6). Me,TBDMS derivatives were prepared from purified samples (section 2.7b), and standards were injected into the GC to obtain column retention times and ratios for multiple ion detection. Samples were then analysed using SIM for the 405 ion and multiple ion detection (MID, see section 2.8c) for the 403 (PGB_3) and 405 (PGB_2) ions.

(c) Analysis of PGF (Me,TBDMS) Derivatives.

Samples were prepared as in (b) up to t.l.c., where E and F prostaglandins were separated. F samples were not purified by HPLC. Standard PGF (Me,TBDMS) derivatives were used to obtain GC column retention times and ratios for the MID. Standards were injected at regular intervals during analysis to monitor retention times. These remained constant provided the column was maintained at injection temperature between injections. Repeated injections had a range of ± 0.05 minutes from the mean retention time. Samples were analysed by SIM for the 653 ion and MID for the 653 and 651 ions. Deuterated prostaglandin internal standards were not available for improved quantitation and identification of prostaglandins in samples

5.3 Results

5.3.1 TIM and SIM of PGE (Me,BuO,TMS) Derivatives

PGE (Me,BuO,TMS) has syn and anti butoxime isomers that had different GC column retention times. Standards of PGE_2 and PGE_3 were found to elute at 19.2 - 19.3 minutes for the first isomer and 20.2 - 20.3 minutes for the second isomer. The column would not separate 2 and 3 series prostaglandins. TIM analysis of PGE samples only purified by Sep-Paks did not produce any clear results with the chromatograms obtained having many unidentified peaks that hid any peaks appearing from PGE. Spectra taken at the correct time exhibited a large number of non-prostanoid ions that prevented identification of PGE.

To try and remove some of the contaminating material it was therefore decided to purify the sample by t.l.c. after purification

with Sep-Paks. It was also decided to use SIM instead of TIM to increase the sensitivity of the mass spectrometer for PGE specific ions. SIM was first used for the PGE_3 510 ion, this is only found in PGE_3 and so a peak appearing on the chromatogram at a time corresponding to that of the PGE standards would be evidence for the presence of PGE_3 in the samples. However, this ion is present in the greatest quantity in the first E isomer which is the minor isomer and so reduces the chance of detection against the background noise and ions produced from non-prostanoid substances. SIM for the 510 ion did not produce a peak on the chromatogram at the correct time in any of the samples, suggesting levels of PGE_3 were below the level of detection for this method.

The 295 ion is the major ion of the second isomer of both PGE_2 and E_3 and so is the ion that would give the largest signal and have the best chance of detection. However, since it is also present in PGE_2, a peak at the correct time on the chromatogram would only be evidence for the presence of PGE. Identification of PGE_3 would have to rely on spectra of the samples. Using SIM for the 295 ion clear peaks were identified in all three samples at the correct time on the chromatograms, however, interference from other substances on the spectra again prevented identification of PGE_2 or PGE_3.

5.3.2 SIM of PGB (Me,TBDMS) Derivatives

Due to the problems of contaminating substances masking the detection of the PGE, a number of measures were taken to further

purify and concentrate the samples and improve the sensitivity of detection on the mass spectrometer. To concentrate the samples it was decided to use three uteri instead of two for prostaglandin production and to convert PGE into PGB, this eliminates the problem of the two oxime isomers, meaning that all the E can be detected in one peak instead of two. To further purify the samples it was decided to use uteri that had not been stimulated with A23187 in addition to uteri that had been stimulated with A23187, since the A23187 may have been causing indiscriminate production of non-prostanoid substances as well as prostaglandins. It was also decided to purify the PGB by HPLC, this cannot be done conveniently with PGE as it does not have an ultraviolet chromophore for ease of defining retention times. Multiple ion detection was also used for analysis, this is a more sensitive and accurate method of detection of selected ions than SIM. One disadvantage of the MID is that spectra cannot be taken when the mass spectrometer is in this mode of operation, therefore SIM was also used to obtain spectra from the samples.

PGB (Me,TBDMS) standards had a GC column retention time of 15 minutes. SIM for the 405 ion was used. This ion is specific for PGB_2, however, the spectrometer was set to cover a range of $\pm$ 3.5 mass units from the mid-setting so it would also monitor the 403 PGB_3 ion. The 405 ion was chosen because it is the largest PGB_2 ion and is also a heavy ion and so is likely to avoid the majority of the interfering ions. This is also an advantage of the TBDMS derivative over the TMS derivative. Standard PGB_2 and PGB_3 (Me,TBDMS) spectra are shown in figures 5.1 and 5.2. The chromatograms produced using SIM for the 405 ion in the samples not stimulated with A23187 exhibited very few interfering peaks and a large peak was produced in each sample at the time corresponding

exactly to that of the PGB_2 and PGB_3 (Me,TBDMS) standards. Spectra taken also contained few interfering ions and clear identification of PGB_2 could be made in all samples with characteristic ions being easily identifiable. In the control sample PGB_2 ions were identified at 462, 405, 391, 363, 300, 248 and 216. In the linoleic sample ions were identified at 462, 405, 391, 373, 363, 300, 248 and 216, and in the linolenic sample, 462, 405, 391, 373, 363 and 216 ions could be seen. The ions were all present in approximately the correct proportions for PGB_2, except for the 391 ion in the linolenic sample which was higher than in B_2, since this is the major ion in PGB_3 and has a far higher abundance than in PGB_2, this may suggest the presence of some B_3. However, no exclusive PGB_3 ions could be observed in the spectra.

In the samples stimulated with A23187, the chromatograms from the linoleic and linolenic samples produced clear peaks at the correct time of 15 mins. Spectra taken had a large nunber of interfering ions present, however, identification of PGB_2 could be made with the 462, 448, 405, 391, 363 and 300 ions being visible in both samples. The chromatogram from the control sample, however, had many contaminating substances present which prevented identification of PGB ions.

Estimates of the quantity of PGB in the samples were made by comparison of heights of peaks produced on the chromatograms from known quantities of standards with those produced from the samples. These estimates are shown in table 5.1.

5.3.3 MID of PGB (Me,TBDMS) Derivatives

In MID measurements, peak heights were measured from the mid-point

	PGB ug/g Tissue			PGF ug/g Tissue		
	Control	Linoleic	Linolenic	Control	Linoleic	Linolenic
Normal tissue	3	8	3	~0.1	~0.1	~0.1
A23187 induced tissue	1	13	12	1	1	~0.3

Table 5.1

Estimated uterine production of prostaglandins E and F in rats fed either a control pelleted diet or a semisynthetic diet supplemented with linoleic or α-linolenic acid ethyl esters (n = 3 for each diet group in normal and A23187 stimulated tissue). Incubating medium was replaced after 30 minutes, 3 hours and 15 hours and the prostaglandins immediately extracted from the medium after each incubation. Prostaglandins were identified by gas chromatography. Results are expressed as µg prostaglandin produced per g wet weight uterus from three uteri in each group. Quantitation of PGF in unstimulated uteri was innacurate due to small peak sizes produced in the chromatograms. Results are estimates based on one chromatographic peak.

of noise at the bottom of the peak to the mid-point of noise at the top of the peak. Cross channel interference was expressed as a percentage of the peak height in the blank channel compared to the peak height in the sample channel, where the peak height in the sample channel was equal to 100%. Noise was expressed as a ratio of the noise height to the peak height for each ion in each sample.

The 405 PGB_2 and 403 PGB_3 ions were monitored. Standard PGB_2 (Me,TBDMS) was used to obtain a ratio of the height of the peak produced in the channel monitoring the 405 ion to that produced in the channel monitoring the 403 ion. Any reduction in this ratio in the samples would suggest the presence of the 403 ion and therefore the presence of PGB_3. The results are shown in table 5.2. In the unstimulated uteri there was no evidence of PGB_3 production in either control or linoleic acid fed rats. In the linolenic acid fed rats a slight increase in the 403 to 405 ion ratio of 3.3 in the PGB_2 standard to 3.5 in the linolenic acid fed rats was detected. The difference, however, could have arisen from background noise. In the A23187 stimulated uteri, increased ratios above that of the PGB_2 standard were observed in all three diet groups, indicating PGB_3 production in each case. The linolenic acid fed rats gave the highest ratio of 21.3, compared to 9.7 in the control rats.

5.3.4 TIM and SIM of PGF (Me,TMS) Derivatives

PGF (Me,TMS) standards eluted at 16.3 - 16.4 minutes and spectra were taken. These are illustrated in figures 5.3 and 5.4. Samples before t.l.c. analysed by TIM and SIM for the 423 ion exhibited a large number of unidentified peaks that masked the identification of PGF on the chromatograms. TIM after t.l.c. of the control sample

	Proportion of 403 to 405			
	Standard B_2	Control	Linoleic	Linolenic
Normal tissue	3.3% (127,28)	2.3% (96,7.3)	2.7% (222,20)	3.5%(131,11.5)
A23187 stimulated tissue	3.3% (127, 28)	9.7%(12.4,24)	4.8(49.5,6.8)	21.3%(23,10.9)

Table 5.2

MID analysis of PGB (Me,TBDMS) derivatives from standard PGB_2 and PGB produced by uteri from eighteen rats (n = 3 for each diet group for normal and A23187 stimulated tissue) fed either a control pelleted diet or a semisynthetic diet supplemented with either linoleic or α-linolenic acid ethyl esters. Proportions are expressed as percentages of the 403 ion to 405 ion taking the 405 ion as 100%. Signal to noise ratios are expressed for each ion in brackets after each measurement with the first figure representing the 405 ion and the second the 403 ion.

gave a clear peak at the correct time on the chromatogram, however, the spectrum taken still contained a high level of contaminating material and only the large PGF 191 ion was clearly visible. SIM for the 423 ion in the linoleic and linolenic samples was used. This gave very clear results on the chromatograms with clear peaks appearing at the time corresponding to that of the PGF standard. The spectrum from the linoleic sample contained a large number of interfering ions present which prevented identification of PGF ions. In the linolenic sample, however, $PGF_{2\alpha}$ was clearly identified by the presence of the 191, 397, 423, 494 and 513 ions. These were also in the correct ratio for $PGF_{2\alpha}$. A 492 ion was identified in the spectrum, this is the largest of the $PGF_{3\alpha}$ exclusive ions and so could indicate the presence of $PGF_{3\alpha}$.

5.3.5 SIM of PGF (Me,TBDMS) Derivatives

The TBDMS derivatives produced from F prostaglandins have a very high molecular weight due to the presence of three hydroxyl groups in the F molecule. This increases the chance of identification of PGF due to the formation of high molecular weight ions that can be identified above the majority of the lower molecular weight interfering ions.

Standard PGF (Me,TBDMS) had a retention time of 22.5 minutes. Spectra taken of $PGF_{2\alpha}$ (Me,TBDMS) and $PGF_{3\alpha}$ (Me,TBDMS) are illustrated in figures 5.5 and 5.6. SIM for the 653 ion was used in both standards and samples. Samples from uteri not stimulated with A23187 only produced very small peaks of PGF on the chromatograms. These, unfortunately, were not identified at the time of elution and so no spectra were taken. The peaks in the chromatograms from the

Figures 5.1-5.6

Fragmentation patterns of B and F_{α} Me,TBDMS and Me,TMS derivatives. A VG Analytical Micromass 7070F mass spectrometer was used. Ion source temperature was 250°C and ionisation energy was 25ev (electron impact).

Figure 5.1
Fragmentation pattern of PGB_2 (Me,TBDMS).

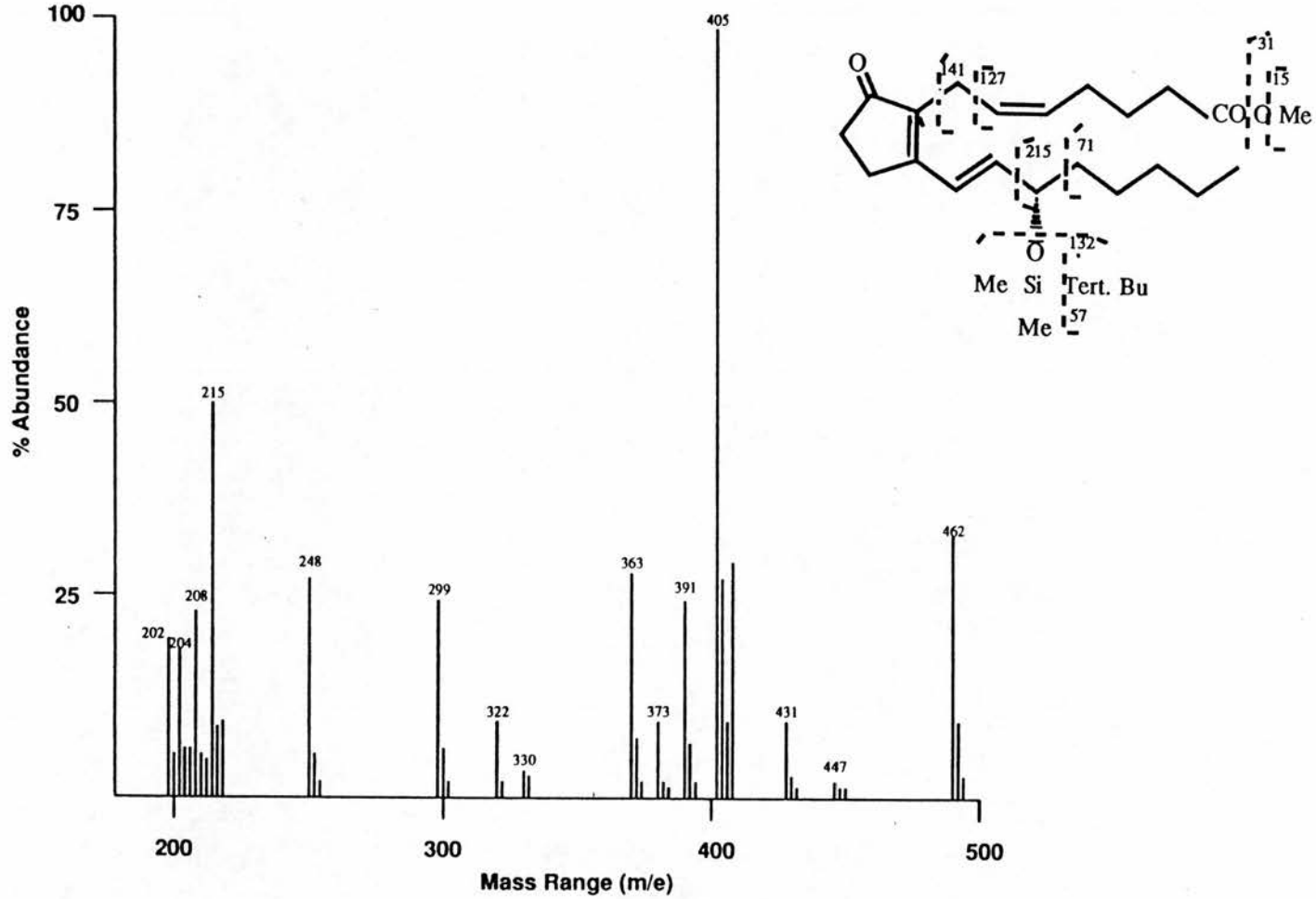


Figure 5.2
Fragmentation pattern of PGB_3 (Me,TBDMS).

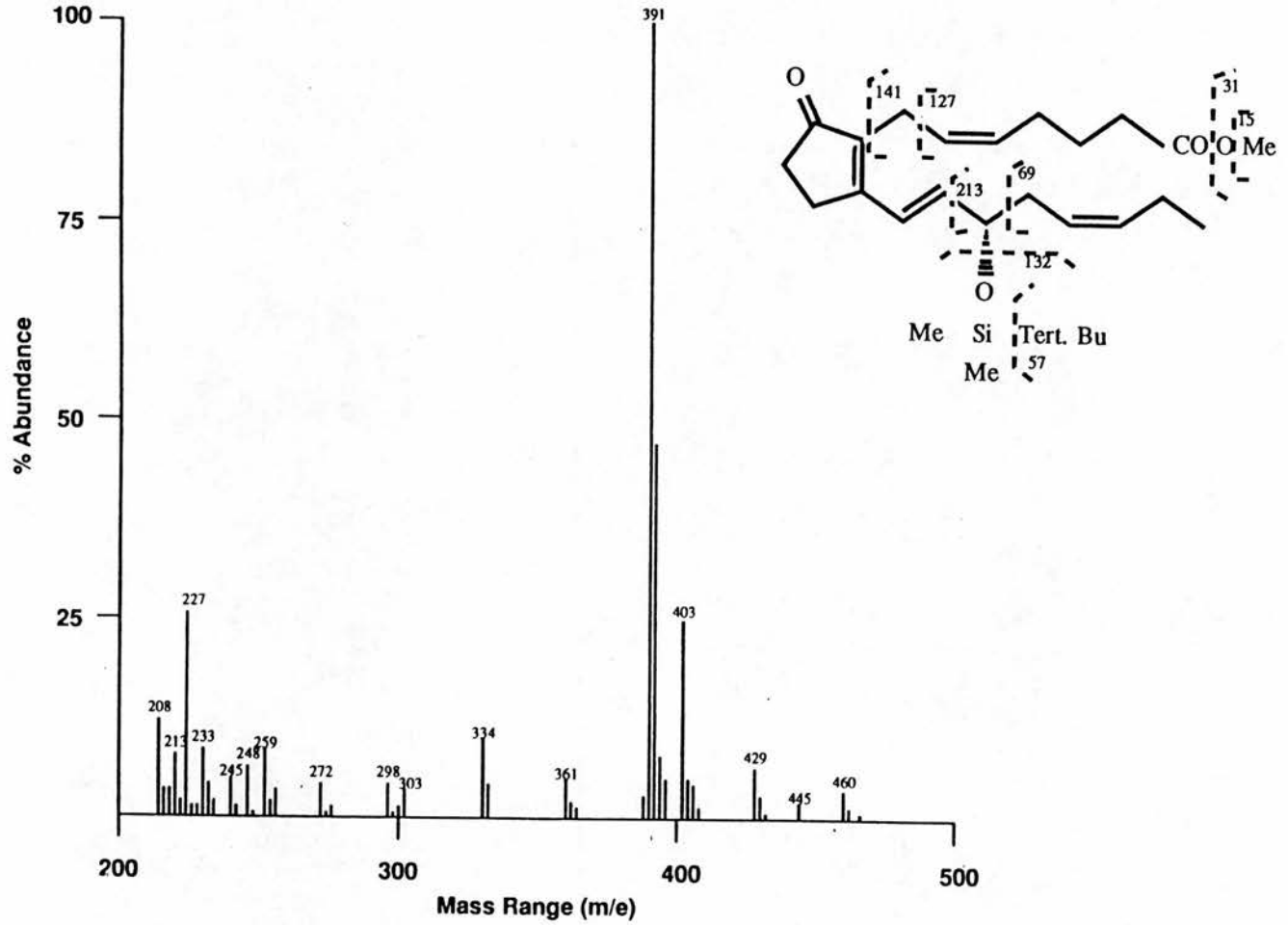


Figure 5.3
Fragmentation pattern of $PGF_{2\alpha}$ (Me,TMS).

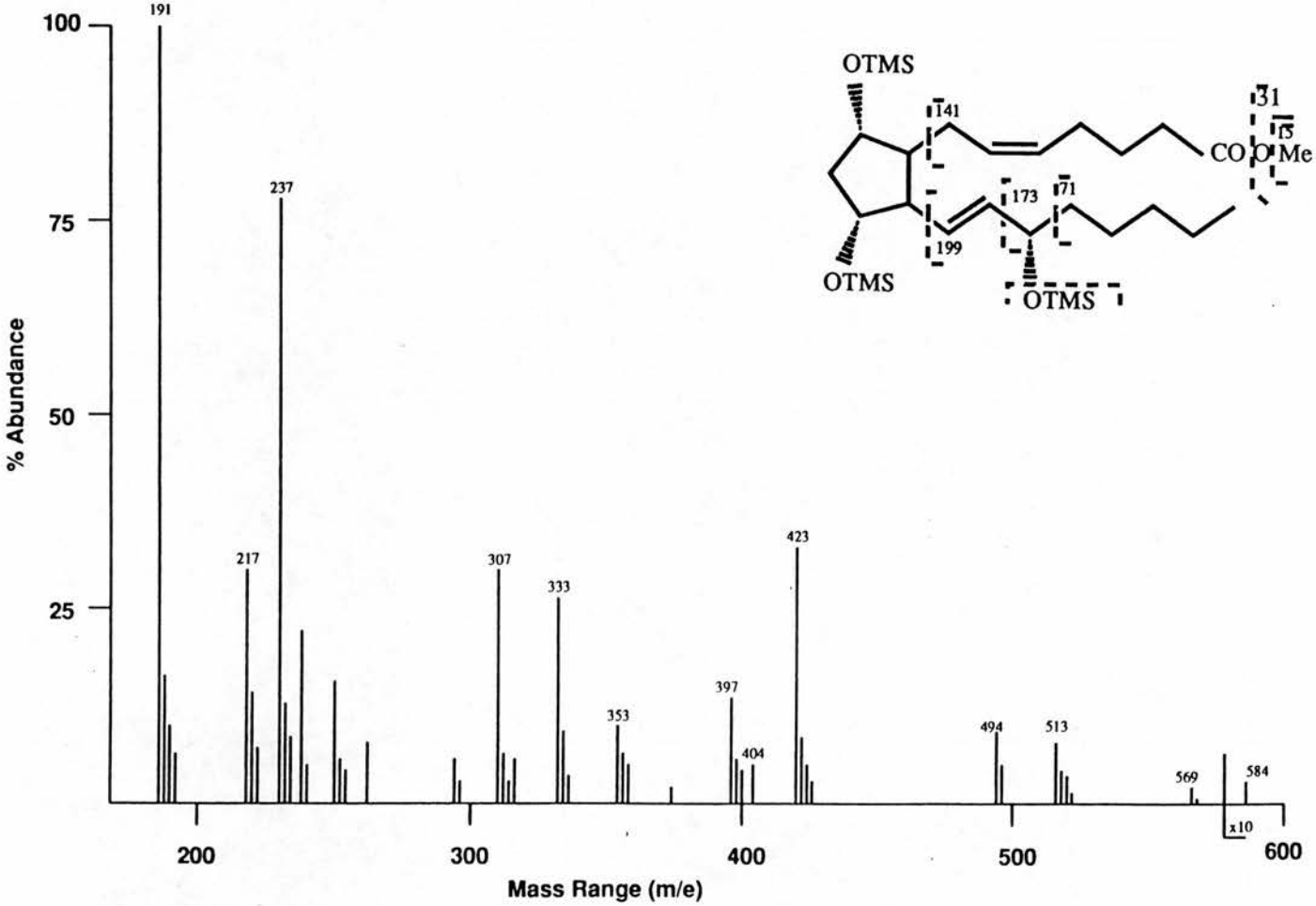


Figure 5.4
Fragmentation pattern of $PGF_{3\alpha}$ (Me,TMS).

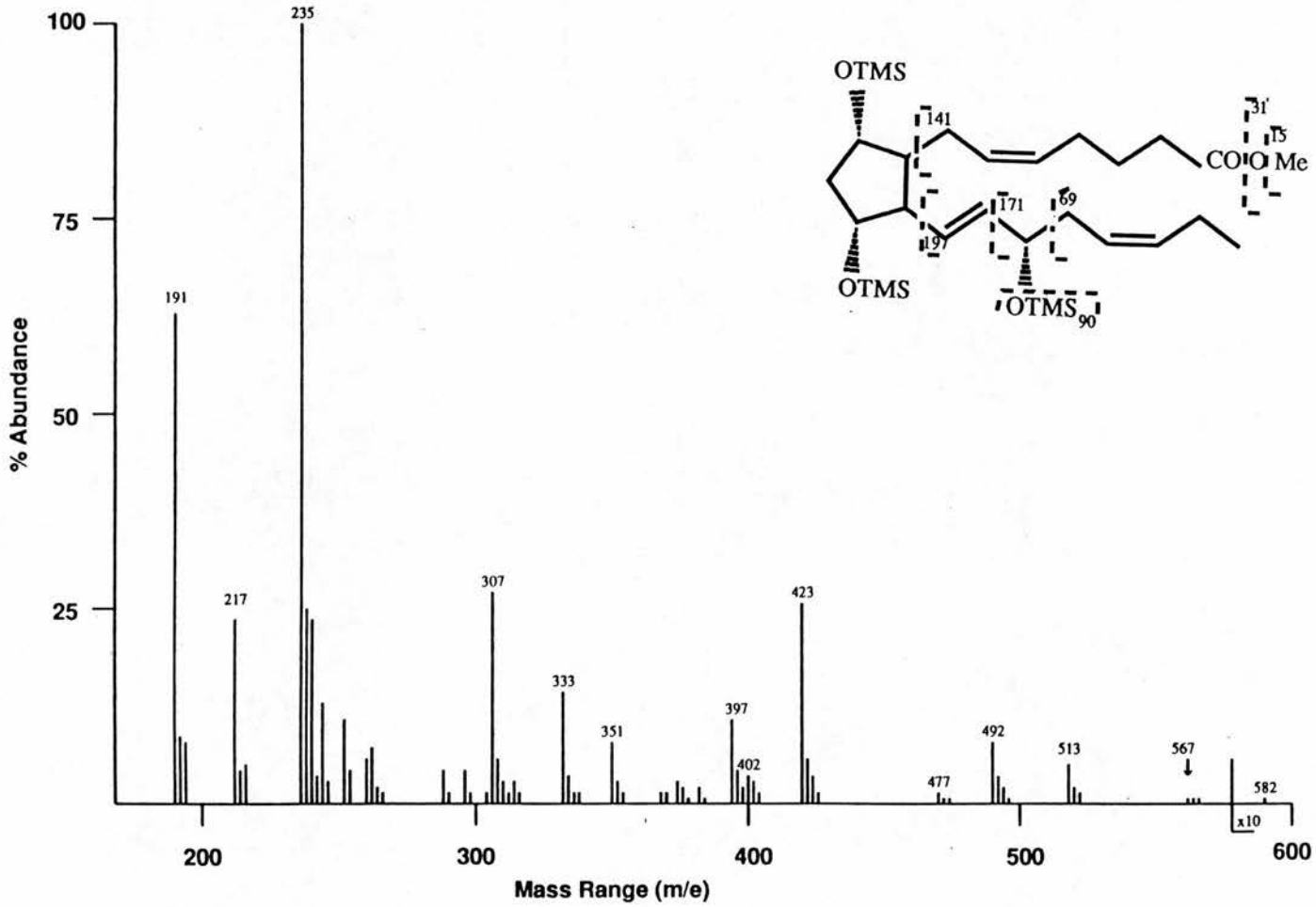


Figure 5.5
Fragmentation pattern of $PGF_{2\alpha}$ (Me,TBDMS).

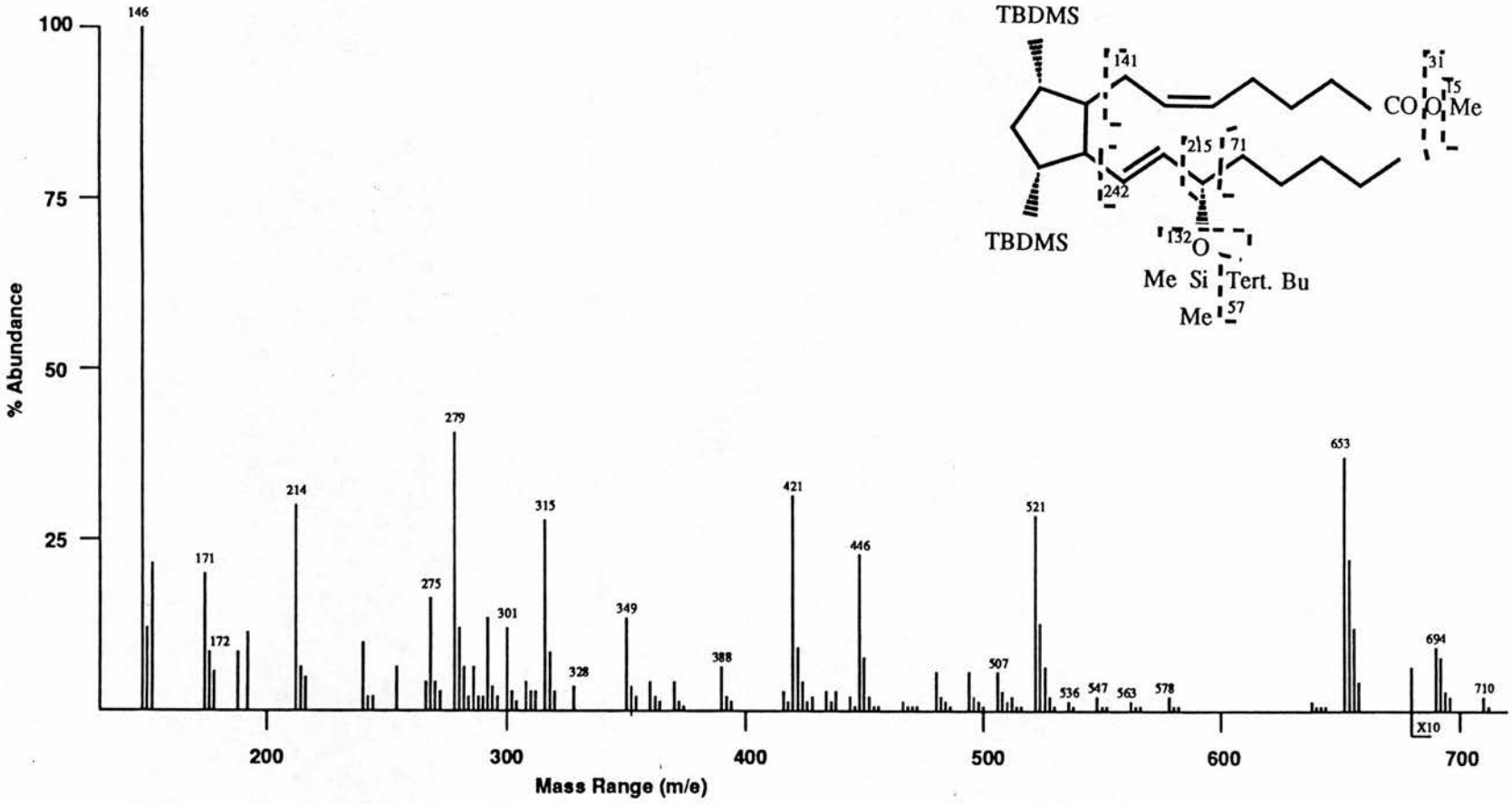
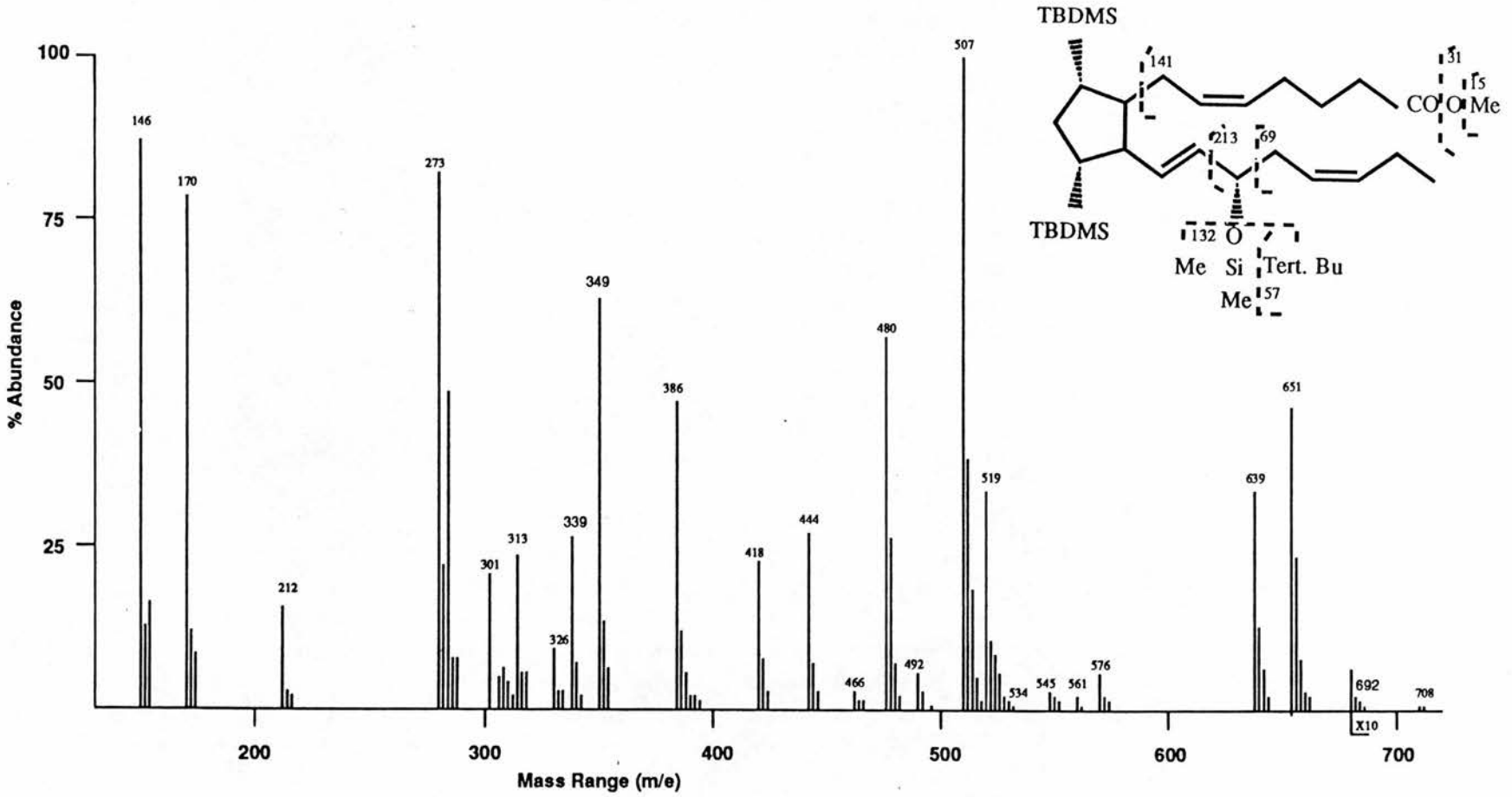


Figure 5.6
Fagmentation pattern of $PGF_{3\alpha}$ (Me,TBDMS).



A23187 - induced samples were also very small but spectra were taken at the time of sample elution. However, the spectra contained a number of interfering ions and only the $PGF_{2\alpha}$ 653 ion was identified. In the linolenic sample the 651 $PGF_{3\alpha}$ ion was also identified.

5.3.6 MID of PGF (Me,TBDMS) Derivatives

The 653 and 651 ions were monitored. The results are shown in table 5.3. There was no evidence of $PGF_{3\alpha}$ production in the unstimulated uteri of either the control or linoleic acid fed rats, however, the linolenic acid fed rats exhibited an increased 653 to 651 ion ratio of 19.2 compared to 12.9 in the standard $PGF_{2\alpha}$. In the A23187 stimulated uteri there was evidence for substancial $PGF_{3\alpha}$ production in both control and linolenic acid fed rats with proportions of 18.2 and 90.9 being recorded respectively, compared to 1.9 in the standard $PGF_{2\alpha}$.

Discussion

The prostaglandin-producing tissues of the uterus play a major role in regulating cyclical endometrial receptivity, the prolonged suppression of uterine activity during pregnancy, and perhaps most importantly, in the rapid and coordinated myometrial excitation of parturition. A functional requirement for the n6 essential fatty acids during normal parturition was established in a series of dietary studies in which n3 EFA were substituted for the n6 EFA (Quackenbush et al., 1942; Leaver et al., 1989; Leat & Northrop, 1979; Leat & Northrop, 1981; Leaver et al., 1986). It has been postulated that the "n3" effect inhibited prostaglandin synthesis

	Proportion of 653 to 651			
	Standard $F_{2\alpha}$	Control	Linoleic	Linolenic
Normal tissue	12.9%(99,13.5)	13.2%(78,14.7)	12.8%(7,2.6)	19.2%(33,15.8)
Tissue + A23187	1.9% (228,9.2	18.2% (14.7,5)	5%(14.7,1.4)	90.9% (9,10.5)

Table 5.3

MID analysis of PGF (Me,TBDMS) derivatives from standard $PGF_{2\alpha}$ and PGF produced from uteri of eighteen rats (n = 3 for each group for normal and A23187 stimulated tissue) fed either a control pelleted diet or a semisynthetic diet supplemented with linoleic or α-linolenic acid ethyl esters. Proportions are expressed as percentages of the 651 ion to the 653 ion taking the 653 ion as 100%. Signal to noise ratios are expressed in brackets after each measurement, with the first figure representing the 653 ion and the second the 651 ion. The two different proportions given for the $F_{2\alpha}$ standard are due to the $PGF_{2\alpha}$ 653 ion being used for the lock channel in the normal tissue analysis, whereas for the A23187 treated sample the 614 peak of perfluorotributylamine (Heptacosa-Aldrich Chemical Co., Poole, England) was used for the lock channel.

(Leaver et al., 1989; Leat & Northrop, 1979; Leat & Northrop, 1981; Leaver et al., 1986). However, little information about the metabolism of the n3 essential fatty acids by uterine tissue is available to indicate whether the postulated competitive inhibition of n6 metabolism occurs at the level of incorporation into uterine lipid precursor pools, release from such pools, or at the level of the cyclooxygenase enzymes. In terms of EFA uptake into the uterus, it is known that the essential fatty acid composition of the uterus responds rapidly to changes in dietary essential fatty acid intake (chapters 3 and 4). However, at the same time, 40-50% of the uterine arachidonic acid pool is conserved in rats fed 80% n3 EFA diets, indicating selectivity at the level of n3 EFA incorporation into uterine PC and PI (chapter 4). The release of EFA at parturition may be triggered by progesterone withdrawal, oestradiol release, and oxytocin in species-dependent patterns, in a sequence leading to the activation of phospholipases A_2 and C, and the release of large µg/ml quantities of arachidonic acid and prostaglandins (Ogburn et al., 1980; Olund & Lunel, 1979; Okita et al., 1982; Okazaki et al., 1978; Okazaki et al., 1981; Leaver et al., 1987). However, the EFA specificity of the uterine phospholipases involved has not been reported. Similarly, little is known about the selectivity of uterine cyclooxygenase, although there is some evidence that dietary n3 EFA inhibit the uterine synthesis of PGE_2 (Leaver et al., 1986).

In this study, the activity of uterine cyclooxygenase was studied under two conditions. Firstly in the absence of stimuli thought to release prostaglandins, and secondly in the presence of high (1.8 mM) calcium concentrations which activate tissue phospholipases A_2

and C activity. The synthesis of the 2- and 3-series PGE and PGF_{α} was analysed using total ion monitoring and multiple ion detection of a variety of derivatives of PGF and PGE. The Me,TMS and Me,TBDMS derivatives of PGF, the Me,BuO,TMS derivative of PGE, and the Me,TBDMS derivative of PGB were fragmented under high electron impact to yield a series of high molecular weight ions, which could contain the n3 double bond, thereby allowing the 2-series prostaglandins to be distinguished from the 3-series.

TIM and SIM of PGE (Me,BuO,TMS) derivatives provided no evidence for the presence of 3-series prostaglandins, although the chromatograms did show the presence of E prostaglandins in all the samples. The spectra taken provided little evidence of the presence of B_3 in the samples with only the linolenic sample from the uteri not treated with A23187 exhibiting a large 391 peak indicating the presence of PGB_3. Estimates made of PGB produced shown in table 5.1 clearly indicate the stimulatory effect of the A23187 on prostaglandin production, however, the low level found in the control A23187 sample is inconsistent with the linoleic and linolenic samples. The fact that this sample had a far greater quantity of contaminating peaks present than either the linoleic or linolenic samples suggests something may have gone wrong with the processing of this sample. In both the A23187 stimulated uteri and the unstimulated uteri the linoleic group produced greater quantities of PGE (as PGB) than the linolenic group, suggesting inhibition of PGE_2 synthesis by the high level of dietary n3 fatty acids in the linolenic acid fed rats.

The MID results shown in table 5.2 suggest there may be a low level of synthesis of PGE_3 in the linolenic acid fed rats, indicated by

the increased proportion of 403 ion from 3.3% in the standard PGB_2 to 3.5% in the linolenic sample, however, this increase may have been a result of background noise. There was no evidence of PGE_3 synthesis in either the control or linoleic acid fed rats. In the A23187 stimulated uteri, however, there was some suggestion of PGE_3 synthesis in all three diet groups, with a low level of synthesis in the linoleic acid group, indicated by only a slight increase in the proportion of the 403 ion from 3.3% in the PGB_2 standard to 4.8% in samples, but a high level of synthesis in the uteri of the linolenic acid fed rats, indicated by the high 403 ion proportion of 21.3%.

TIM and SIM of the Me,TMS PGF derivatives provided little evidence of the presence of $PGF_{3\alpha}$ with only the spectra of the linolenic sample exhibiting the major 492 ion of $PGF_{3\alpha}$. The chromatograms produced, however, gave clear evidence of the presence of quantities of PGF in all the samples. The spectra produced from the PGF (Me,TBDMS) derivatives from A23187 stimulated uteri contained a number of interfering ions and only the heavy 653 ion could be identified in all the samples, in the linolenic acid group a clear 691 ion could also be seen, indicating the presence of $PGF_{3\alpha}$. Quantitation of PGF produced in unstimulated uteri was inaccurate due to the small peak sizes produced in the chromatograms and levels of production could not be distinguished between the three diet groups. In the A23187 stimulated uteri, prostaglandin production was considerably higher than in the unstimulated uteri. In the A23187 treated uteri evidence could also be seen for the inhibition of PGF synthesis by the n3 fatty acids in the linolenic acid fed rats, with this group producing only 0.3 µg PGF/g tissue compared to

1.16 µg/g and 1.02 µg/g in the control and linoleic groups respectively. The MID analysis of unstimulated uteri provided some evidence for $PGF_{3\alpha}$ production in the linolenic acid fed rats with an increased 651 ion proportion from 13% in the $PGF_{2\alpha}$ standard to 19% in the linolenic sample. There was no evidence of $PGF_{3\alpha}$ production in the linoleic acid fed rats. As was found with the PGE_2, the A23187 induced considerable production of three series prostaglandins. By far the greatest quantity was produced by the linolenic acid rats with a very large increase in the proportion of the 651 ($PGF_{3\alpha}$) to the 653 ($PGF_{2\alpha}$) ion from 2% in the $PGF_{2\alpha}$ standard to 91% in the linolenic sample. This result supports the identification of the large 651 ion identified on the spectrum produced from this sample. The control rats also synthesised considerable quantities of $PGF_{3\alpha}$ with a 651 proportion of 18%. The linoleic acid fed rats produced the lowest level with a proportion of 5% being observed.

The possibility of 3-series prostaglandin production in A23187 stimulated uteri compared to unstimulated uteri suggests that either two different groups of cells in the uterus are responsible for prostaglandin synthesis in the unstimulated and stimulated state or that two different pools of fatty acids and/or phospholipases are involved. In the uterus, tissue heterogeneity is a possibility for the difference in prostaglandin production. Leaver et al. (1981) presented evidence for varying levels of prostaglandin precursors in the endometrium and myometrium of guinea pig uterus. In the rabbit kidney evidence has been presented for the existence of two pools of fatty acids for

prostaglandin synthesis (Schwartzman and Raz, 1979; Schwartzman and Raz, 1981), one being hormone sensitive and the other hormone insensitive. Needleman et al. (1979) have also presented evidence suggesting that arachidonic acid from varying sources may be converted to different prostaglandins in the rabbit kidney.

Evidence for the existence of more than one phospholipase pool that may be independently activated within one particular cell type also exists (Hong and Levine, 1976; Hsueh et al, 1981). If different phospholipase enzymes are involved then they may release fatty acids from phospholipids with varying n6 - n3 contents (see chapter 4), with the phospholipase active in the stimulated uterus acting on a phospholipid group with a higher n3 content than the phospholipid substrate of the phospholipase active in the unstimulated uterus. In chapter 4 PI and PC phospholipids were found to be less susceptible to dietary change than PE, and decreases in 20:4n6 and increases in 20:5n3 in PI and PC were less than those in PE when a diet high in α-linolenic acid was fed to rats. It is possible that in the unstimulated uterus PI and/or PC may form the major source of free fatty acid for prostaglandin synthesis, whereas on stimulation by A23187, PE may become the major source, resulting in a higher level of 20:5n3 being released and converted to 3-series prostaglandins. Hong and Deykin (1979) found that in mouse fibroblasts, bradykinin, thrombin and serum caused specific release of arachidonic and eicosatrienoic acids, however, A23187 stimulation also caused release of stearic, oleic and linoleic acids. They suggested that A23187 activated a less specific pool of phospholipases. This is a possible explanation for the results we

have observed. The increase in total output of prostaglandins in response to A23187 reported here has also been observed in other studies (Leaver et al., 1989; Leaver and Seawright, 1982; Leaver and Richmond, 1984; Brown et al., 1987).

The increase in 3-series prostaglandin production suggested by the results obtained after A23187 induced tissue activation indicated that the uterine cyclooxygenase was capable of producing both PGE_3 and $PGF_{3\alpha}$, and that only a limited degree of substrate specificity lay at the level of uterine cyclooxygenase. The major control of uterine PGE_3 and $PGF_{3\alpha}$ synthesis appeared to lie at the level of EFA release. A similar finding has been reported in endothelial cells, in which the liberation of eicosapentaenoic acid and arachidonic acid resulted in the formation of substantial amounts of PGI_3, PGE_3 and $PGF_{3\alpha}$ (Bordet et al., 1986). The increase in 3-series prostaglandin production observed on A23187 stimulation may explain why only parturition and not implantation or gestation are affected by diets containing a high proportion of n3 fatty acids if the tissue activation at parturition results in similar increases in 3-series prostaglandin production as those resulting from A23187 stimulation.

In conclusion, the results presented indicate the possible production of 3-series E and F_{α} prostaglandins in the rat uterus. In unstimulated uteri production of 3-series prostaglandins was only suggested in rats fed a diet containing α-linolenic acid as the main dietary EFA source. Upon stimulation with A23187, there was some suggestion of 3-series prostaglandin production in rats fed a control pelleted diet and a diet containing linoleic acid as the major EFA source. This suggested that A23187 stimulation caused a less specific

release of fatty acids from the uterus, with higher proportions of n3 fatty acids being released than in the unstimulated uterus. It is therefore important to analyse the release of the 3-series prostaglandins during the changes in phospholipase activity which occur during tissue stimulation. Our results suggested that in A23187 stimulated uteri α-linolenic acid fed rats may synthesise substantially higher proportions of 3-series compared to 2-series prostaglandins than control or linoleic acid fed rats. Linoleic acid fed rats also synthesised greater total prostaglandin levels than α-linolenic acid fed rats, indicating that the diet high in n3 fatty acids suppressed 2-series prostaglandin production. The low total and high 3-series prostaglandin production may cause the inhibition of parturition associated with high n3 fatty acid intake.

Chapter 6

Changes in Prostaglandin E_2, Oestradiol 17β and Progesterone During Late Gestation in Catheterised Sheep Experimentally Infected with an Ovine Abortion Strain of Chlamydia Psittaci

6.1 Introduction

Premature labour and abortion are major complications associated with Chlamydia psittaci infection of sheep (Studdert & McKercher, 1968; Novilla & Jensen, 1970). Ovine abortion strains of C. psittaci have been demonstrated to infect women and are associated with abortion in these subjects (Roberts et al., 1967; Beer et al., 1982; Johnson et al., 1985). As with the n3 fatty acids, infection by C. psittaci may affect parturition through alterations in prostaglandin production, or possibly through effects on the steroid hormones controlling uterine prostaglandin synthesis. In both sheep and humans, the perinatal complications caused by C. psittaci appear to be more severe than those associated with Chlamydia trachomatis but the pathophysiology of both infections is similar with respect to the site and timing of infectivity and the effect of initiating premature labour (Sweet et al., 1987; Studdert, 1968). The widespread distribution of chlamydial infection in sheep has been known for many years (McEwen et al., 1951; Studdert & McKercher, 1968). However, recent applications of monoclonal antibodies and gene probe techniques have indicated a much wider distribution of chlamydial infection than had been previously suspected in the human population (Sweet et al., 1987).

The primary focus of chlamydial infection during pregnancy is the

placenta. Three placental products are important in the control of parturition; progesterone, oestradiol 17β and PGE_2. Progesterone is important in the maintenance of pregnancy (Bedford et al., 1972; Taylor et al., 1982), and progesterone synthesis during late pregnancy in both sheep and women occurs in the placenta (Linzell & Heap, 1968). The placenta is also a major source of oestrogens in these species. The secretion and distribution of oestradiol 17β during gestation and its effect on prostaglandin synthesis, have been characterised (Allen, 1975; Liggins et al., 1972). Plasma oestradiol 17β concentrations are indicators of the placental production and secretion of this steroid. The concentrations of oestradiol 17β in the amniotic fluid and the utero-ovarian vein reflect the intrauterine production and diffusion of oestradiol 17β.

Changes in the concentrations of oestradiol 17β and progesterone influence the release of PGE_2 at parturition (Thorburn & Challis, 1979; Taylor et al., 1982; Olson et al., 1984). The primary site of placental infection of C. psittaci is the chorionic membrane, which is also a major site of placental PGE_2 synthesis. PGE_2 is also released in significant quantities by leucocytes, particularly by inflammatory macrophages (Lewis, 1983). The prostaglandins exert a range of effects within the uterus at parturition. Prostaglandins act on the myometrium, where they stimulate and co-ordinate myometrial contraction (Wickland et al., 1984). In the cervix, prostaglandins induce ultrastructural changes resulting in an increase in cervical patency (Keirse et al., 1983). Stimulation of the chorionic membrane causes release of arachidonic acid and prostaglandin E_2 from the foetal membranes (Grieves & Liggins, 1976). A role for prostaglandin E_2 in initiating labour has been

proposed (Bleasdale & Johnston, 1984).

The synthesis of PGE_2 increases during late gestation in both sheep and women (Mitchell & Flint, 1977; Olson et al., 1984; Dray & Frydman, 1976). The inhibition of prostaglandin synthesis delays premature delivery in sheep (Mitchell & Flint, 1978), and infusion of PGE_2 and $PGF_{2\alpha}$ into the aorta of pregnant sheep stimulates uterine contractions similar to those detected at term (Thorburn & Challis, 1979). An increase in uterine PGE_2 concentration in infectious abortion associated with bacterial endotoxin has been reported in women (Lamont et al., 1985; Romero et al., 1988), and there has been a recent report of elevated $PGF_{2\alpha}$ metabolite concentrations in the plasma of four sheep infected with C. psittaci (Fredriksson et al., 1988).

The predominant prostaglandin produced by sheep and human chorion is PGE_2 (Mitchell & Flint, 1977; Mitchell et al., 1977; Olson et al., 1984; Casey et al., 1983). In contrast to $PGF_{2\alpha}$, the concentration of PGE_2 increases during late gestation and early labour, and there is evidence that PGE_2 has a role in the initiation of labour, while $PGF_{2\alpha}$ is involved in the progression and the coordination of labour after it has been initiated (Bleasdale & Johnston, 1984). The PGE_2 synthesised by the fetal membranes is converted to $PGF_{2\alpha}$, and both PGE_2 and $PGF_{2\alpha}$ are metabolised, at a variable rate, to a range of inactive oxygenated metabolites. The variability of the activities of the enzymes of prostaglandin metabolism, together with the contribution of blood and vascular cells to the circulating prostaglandin pool, combine to make the measurement of peripheral prostaglandin an indirect indicator of

uterine prostaglandin synthesis (Schweer et al., 1986). In contrast, the intrauterine concentrations of PGE_2 indicate the local concentrations of biologically active PGE_2.

The timing of C. psittaci infection has led to investigation of endocrine factors which might play a role during this period of gestation. The infection of the placenta by the Chlamydiae does not begin until a relatively advanced stage of gestation. In the case of C. psittaci infection of sheep, placental infiltration is detected between days 100 to 120 of gestation, commencing in the chorioallantoic membrane, and proceeding to infiltrate the caruncular region of the placenta (Studdert, 1968; Aitken, 1986). It has been reported that oestradiol 17ß acts as a "growth factor" for the Chlamydiae in several species (Rank et al., 1982; Bose & Goswami, 1986; Sugarman & Agbor, 1986). However, there has been little investigation of the effects of Chlamydiae on the factors which play a role in controlling parturition (Martel et al., 1983; Rank et al., 1982; Fredriksson et al., 1988). This is surprising, since pathological studies indicate that the infectivity of Chlamydiae is closely related to the stage of pregnancy, and disorders in the initiation of parturition result from this infection.

The endocrinology of infectious abortion has been the subject of intermittent attention (Smith & Hughes, 1974; Roberts et al., 1975; Minkoff, 1983; Lamont et al., 1985; Helm et al., 1987). We have used a catheterised sheep model to study changes in uterine and peripheral plasma concentrations of PGE_2, progesterone and oestradiol 17ß during pregnancy in response to infection by an

ovine abortion strain of Chlamydia psittaci.

6.2 Methods

Materials and methods used in the experiments discussed in this chapter are described in sections 2.18-2.26 of chapter 2.

Results were expressed as mean ± standard error of mean for n determinations. Statistical significance of differences between paired and unpaired data was analysed using the paired and unpaired Students' t-tests (Cohen and Holliday, 1984). Snedecors' variance ratio test was used to analyse variances in samples for the paired t-test. The unpaired t-test incorporated the Behrens Fisher statistic and, therefore, did not assume that the variances of the population groups being compared were the same.

6.3 RESULTS

6.3.1 Prostaglandin E_2 in Amniotic Fluid during Chlamydial Infection

The concentration of PGE_2 in the amniotic fluid of six catheterised control sheep is shown in figure 6.1A. Amniotic fluid PGE_2 increased during late gestation in the control group. The concentration of PGE_2 between 122 to 135 days of gestation in control animals (2.72 ± 0.27 ng/ml, n=29 samples) increased significantly from day 136 of gestation to the day of parturition (7.23 ± 0.72 ng/ml, n=41 samples), in samples taken from the same animals, compared using the unpaired Students' t-test for sample groups with different variances ($P<0.01$, n=67) (see table 6.1).

In the six sheep infected with C. psittaci, the concentration of PGE_2 in the amniotic fluid increased earlier in gestation (figure

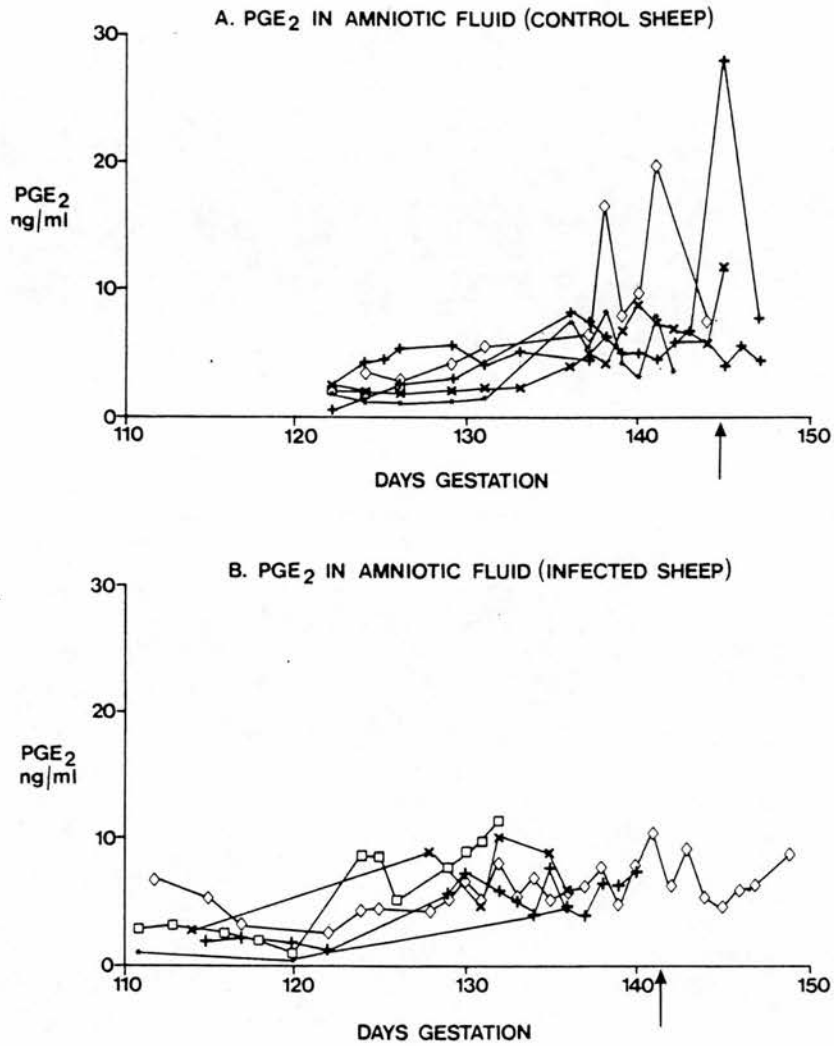


Fig. 6.1

Prostaglandin E_2 in amniotic fluid of control and *Chlamydia*-infected sheep. Amniotic fluid was withdrawn by catheter from A. control or B. sheep infected with an ovine abortion strain of *C. psittaci* (4.5×10^9 ELD_{50}) on day 115 of parturition. The arrow indicates the mean date of parturition. The PGE_2 concentrations are the mean of triplicate determinations carried out on amniotic fluid of each individual sheep. Serial results from each individual sheep are joined.

Gestation (days)	111-121	122-135	136-parturition
Control	-	2.72±0.27(29)*	7.23±0.72(41)
Infected	2.55±0.45(14)	6.42±0.44(21)*	6.48±0.42(21)

Table 6.1

Mean PGE_2 concentration in amniotic fluid of six control and six Chlamydia-infected sheep during late gestation. The mean PGE_2 concentrations in sheep whose individual PGE_2 profiles were shown in figure 6.1, in ng/ml of amniotic fluid ± SEM for n samples of amniotic fluid, withdrawn from indwelling catheters in control sheep or sheep infected with 4.5×10^9 ELD_{50} of an ovine abortion strain of C. psittaci on the stated days of gestation.

*The concentration of PGE_2 in the amniotic fluid of Chlamydia-infected sheep, between days 122 to 135 of gestation, was signficantly greater than that of control sheep, using the unpaired Students' t-test for sample groups with different variances ($P<0.01$, $n=58$).

6.1B). A significant increase in PGE_2 concentration was observed after day 122 of gestation in the Chlamydia-infected group, when samples from the same sheep were compared using the unpaired Students' t test ($P<0.001$, $n=43$). The mean concentration of PGE_2 was 2.55 ± 0.45 ng ml^{-1} ($n=14$) on days 111 to 121 of gestation, compared with 6.42 ± 0.44 ng ml^{-1} ($n=21$) in the same sheep on days 122 to 135 of gestation (see table 6.1). There was no significant increase in the concentration of PGE_2 in the amniotic fluid between days 122 to 135 of gestation, compared with day 136 of gestation onwards, in the Chlamydia-infected group.

6.3.2 Prostaglandin E_2 in Utero-ovarian Vein during Chlamydial Infection

The concentration of PGE_2 in the utero-ovarian vein of six control sheep showed a pulsatile release of PGE_2, increasing in amplitude from day 126 of gestation (figure 6.2A). The mean concentration of PGE_2 in utero-ovarian plasma of control sheep between days 122 to 125 of gestation was 1.34 ± 0.205 ng ml^{-1} ($n=12$), and this increased to a concentration of 2.38 ± 0.241 ng ml^{-1} ($n=28$) in the same sheep between days 126 to 136 of gestation. This increase in PGE_2 concentrations was significant using the unpaired t-test ($P<0.01$, $n=36$). The PGE_2 concentrations in the utero-ovarian venous plasma showed a similar pattern of release in Chlamydia-infected sheep (Fig. 6.2B). The greatest increase in PGE_2 was observed after day 122 of gestation. The concentration of PGE_2 in utero-ovarian plasma of infected sheep was 0.922 ± 0.123 ng ml^{-1} ($n=28$) between days 110 to 121 of gestation, increasing to 1.52 ± 0.153 ng ml^{-1} ($n=53$) in the same ewes between days 122 to 135 of gestation. This increase in

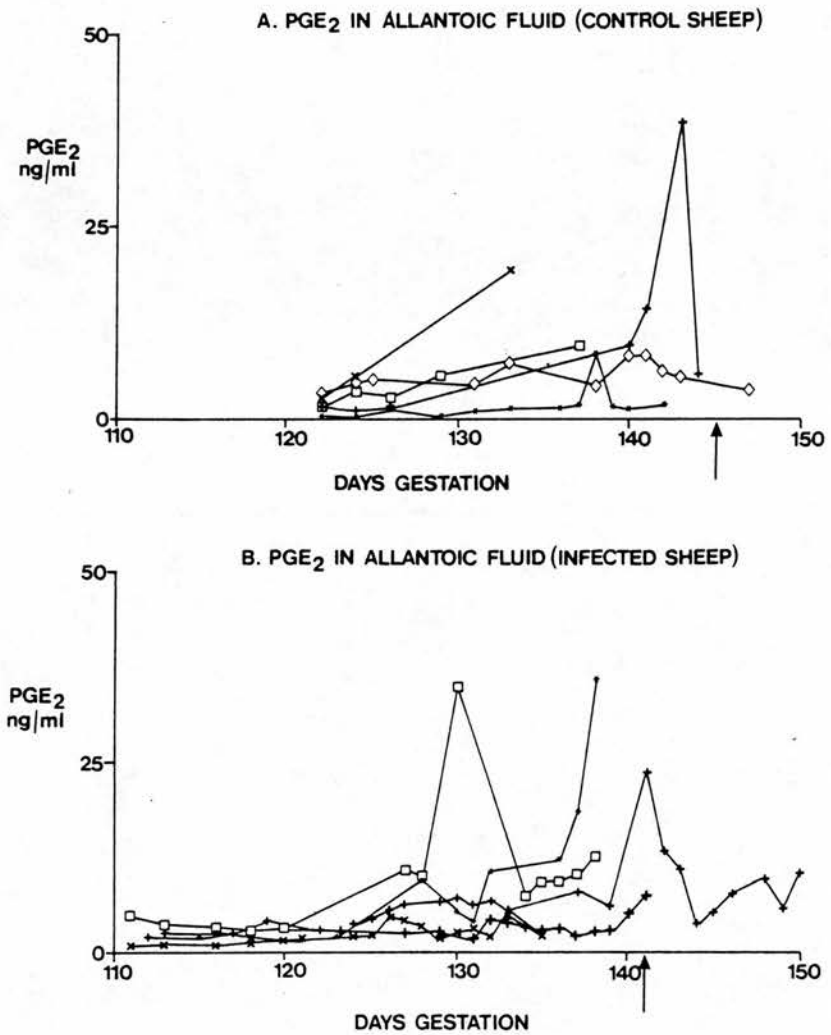


Fig. 6.2.
Prostaglandin E_2 in utero-ovarian vein of control and Chlamydia-infected sheep. Utero-ovarian plasma was withdrawn by catheter from A. control sheep or B. sheep infected with an ovine abortion strain of C. psittaci (4.5×10^8 ELD_{50}) on day 115 of parturition. The arrow indicates the mean date of parturition. The PGE_2 concentrations are the mean of triplicate determinations, carried out on utero-ovarian plasma of six individual sheep. Serial results from individual sheep are joined.

PGE_2 was significant using the unpaired t-test for sample groups with different variances ($P<0.01$, $n=81$). The increase in PGE_2 concentrations in the utero-ovarian vein of infected sheep (from 1.08 ± 0.149 ng ml^{-1} on days 122 to 125, $n=14$; to 1.55 ± 0.140 ng ml^{-1} on days 126 to 136, $n=43$), was not significant. There was a further increase in PGE_2 concentrations in utero-ovarian vein of Chlamydia-infected sheep late in gestation. The concentration of PGE_2 from day 137 of gestation in the Chlamydia-infected sheep increased to 1.96 ± 0.228 ng ml^{-1} ($n=31$) of utero-ovarian venous plasma. This was significantly greater than the PGE_2 concentration detected in the same ewes between days 122 to 125 of gestation, using the unpaired t-test for sample groups with different variances ($P<0.01$, $n=32$).

The increase in utero-ovarian venous PGE_2 concentrations in infected sheep occurred more gradually, over a longer time span, compared with the increase in utero-ovarian venous plasma PGE_2 in control sheep (see table 6.2). In the utero-ovarian vein, the concentration of PGE_2 increased sharply after day 126 of gestation. The mean PGE_2 concentration in the utero-ovarian venous plasma of control sheep increased by 78%, if samples collected in the period between days 126 to 136 of gestation were compared with samples collected between days 122 to 125. In contrast, the increase in mean PGE_2 over the same period in the infected group (44%) was proportionately lower than the increase in the control group.

The mean concentrations of PGE_2 in the utero-ovarian vein of infected sheep were compared with the mean PGE_2 concentrations detected in control sheep on the same day of gestation throughout

Gestation (days)	110–121	122–125	126–136	137–parturition
Control	–	1.34±0.205(12)	2.38±0.241(28)	2.51±0.19(53)
Infected	0.922±0.123(28)	1.08±0.149(14)	1.55±0.140(43)	1.96±0.23(31)

Table 6.2

Mean PGE_2 concentration in utero-ovarian venous plasma of six Chlamydia-infected and six control sheep during late gestation. The mean PGE_2 concentration in sheep whose individual PGE_2 profiles were shown in figure 6.2, in ng ml^{-1} of utero-ovarian venous plasma ± SEM for n samples of utero-ovarian venous blood, withdrawn from indwelling catheters in control sheep or sheep infected with 4.5 x 10^5 ELD_{50} of an ovine abortion strain of C. psittaci on the stated period of gestation.

the period when infected and control sampling overlapped (between days 122 to 141 of gestation). Over this period, the mean PGE_2 concentration in the utero-ovarian vein of infected sheep was 68.8 ± 5.2% the mean PGE_2 concentration of control sheep (range 52.6% to 96.8% for 12 data pairs). The mean PGE_2 concentrations in the utero-ovarian venous plasma of infected sheep were significantly lower than the mean PGE_2 concentrations detected in control sheep, using the paired t-test for samples with different variances ($P<0.01$, $n=12$). This suggested that the transfer of PGE_2 into the utero-ovarian vein was compromised by chlamydial infection.

6.3.3 Prostaglandin E_2 in Allantoic Fluid during Chlamydial Infection

The sampling of allantoic fuid was infrequent after day 130 of gestation, due to catheter blockage caused by particulate matter, and to the increase in allantoic fluid viscosity which occurs during this period. This problem was most acute during the five days before parturition, when the allantoic fluid was decreasing in volume (Mellor, 1980). The concentration of PGE_2 in the allantoic fluid of the six control sheep increased during late gestation (figure 6.3A). The concentration of PGE_2 in allantoic fluid between days 122 to 135 of gestation was 3.66 ± 0.922 ng ml^{-1} ($n=21$), increasing to 8.13 ± 2.15 ng ml^{-1} ($n=17$) in the same sheep between day 136 and the day of parturition. This increase was significant, when analysed using the unpaired t-test ($P<0.05$, $n=38$) (see table 6.3). The concentration of PGE_2 in the allantoic fluid of six sheep infected with *C. psittaci* is shown in figure 6.3B. The increase in allantoic fluid PGE_2 occurred earlier in infected sheep than in

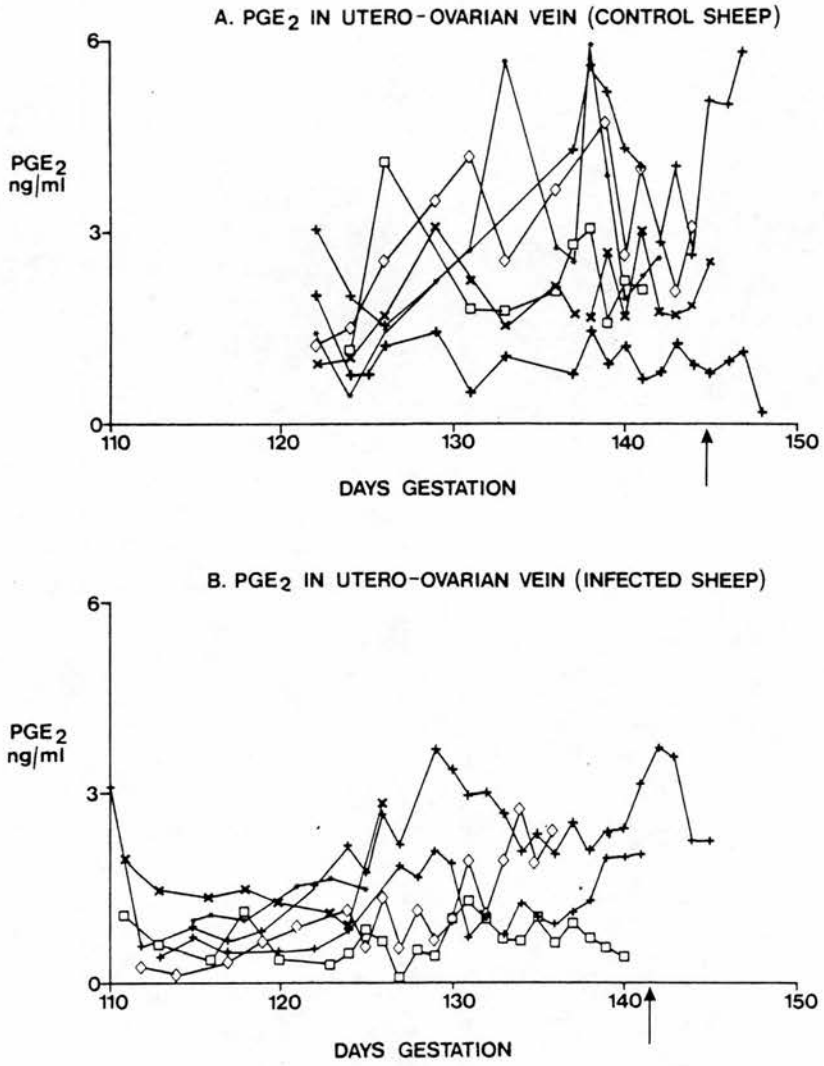


Fig. 6.3
Prosataglandin E_2 in allantoic fluid of control and Chlamydia-infected sheep. Allantoic fluid was withdrawn by catheter from A. control sheep or B. sheep infected with an ovine abortion strain of C. psittaci (4.5×10^5 ELD_{50}) on day 115 of parturition. The PGE_2 concentrations are the mean of triplicate determinations, carried out on allantoic fluid of individual sheep. Serial results from individual sheep are joined. The mean day of parturition is indicated by an arrow.

Gestation (days)	111–121	122–126	127–135	136–parturition
Control	–	2.56±0.46(7)	2.68±2.52(7)	8.13±2.15(17)
Infected	2.08±0.22(21)	3.00±0.38(11)	6.04±1.11(30)	9.75±1.61(23)

Table 6.3

Mean PGE_2 concentration in allantoic fluid of six Chlamydia-infected and six control sheep during late gestation. The mean PGE_2 concentration in sheep whose individual PGE_2 profiles are shown in figure 6.3, in ng ml^{-1} of utero-ovarian venous plasma ± SEM for n samples of utero-ovarian venous blood, withdrawn from indwelling catheters in control sheep or in sheep infected with 4.5×10^5 ELD_{50} of an ovine abortion strain of C. psittaci during the stated period of gestation.

controls. The concentration of PGE_2 in the allantoic fluid of infected sheep between days 111 to 126 of gestation was 2.33 ± 0.196 ng ml^{-1}(n=32), increasing to 6.04 ± 1.12 ng ml^{-1}(n=30) in the same sheep between days 127 to 135 of gestation. This increase was significant, P<0.01 (n=62), using the unpaired t-test. The concentration of PGE_2 in the allantoic fluid of the same sheep, taken between days 136 of gestation and the day of parturition, was 9.75 ± 1.61 ng ml^{-1}(n=23). This was not significantly higher than the PGE_2 concentration between days 127 to 135 of gestation in the same sheep (see table 6.3). The concentration of PGE_2 in the allantoic fluid of the infected sheep between days 127 to 135 of gestation was 6.04 ± 1.11 ng ml^{-1}(n=30), and in the control sheep, 2.68 ± 2.52 ng ml^{-1}(n=7). The difference between these groups was not significant, due to the increase in PGE_2 concentrations, during this period. However, when the PGE_2 concentrations in the allantoic fluid of individual infected and control sheep, taken on the same day of gestation, between days 127 to 135 were paired and analysed using the paired t-test, the PGE_2 concentrations in infected sheep were significantly higher than the PGE_2 concentrations in the allantoic fluid of control sheep taken on the same day of gestation (P<0.02).

6.3.4 Progesterone in Peripheral Plasma during Chlamydial Infection

The mean peripheral plasma progesterone concentrations of six control sheep and of six sheep experimentally infected with *C. psittaci* on day 90 of gestation are shown in figure 6.4. Parturition was significantly earlier (P<0.05) in the infected group. The profiles of progesterone release in the two populations showed the following differences. There was a greater variation in

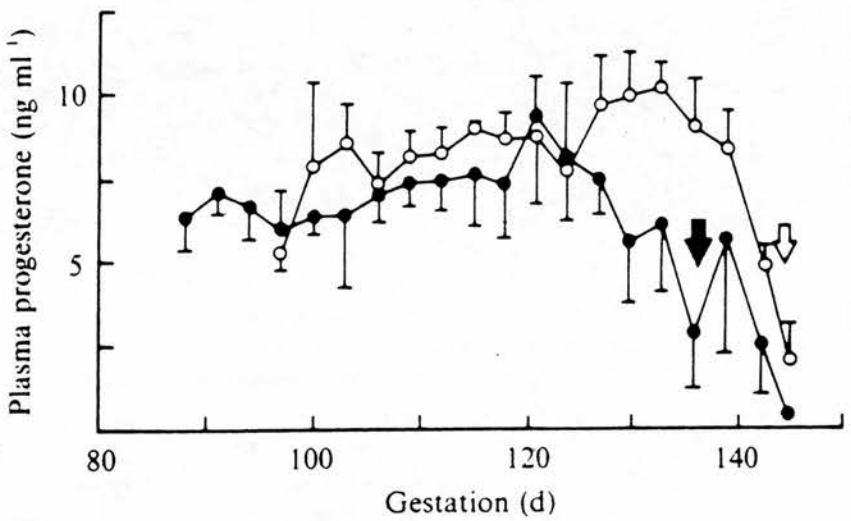


Figure 6.4
Progesterone in peripheral plasma of control and C. psittaci-infected sheep. Ewes were infected by subcutaneous injection on day 90 of gestation with 5×10^5 ELD_{50} of C. psittaci strain S26/3. The filled arrow indicates the mean day of parturition in infected, and the open arrow, in control ewes (the mean duration of gestation in control sheep was 144.7 ± 1.08, n = 6, and in C. psittaci-infected sheep, 137 ± 1.6, n = 6). All animals had a single lamb. Progesterone concentrations are the means of three to six determinations of plasma progesterone from individual control (○) or infected (●) sheep on separate days of gestation.

the concentration of plasma progesterone in infected animals. The mean plasma progesterone concentration in infected animals, compared on 8 days between days 100 to 130 of gestation, was 80 $\pm$ 5.3% of the mean plasma progesterone concentration in control sheep. The concentration of progesterone in the plasma of control sheep dropped sharply during the eight days before parturition, while that of infected sheep showed a more gradual decline, commencing 13 days before parturition, on day 125 of gestation. The difference between the mean progesterone concentrations, paired between day 100 and day 144 of gestation and analysed using the paired Student's t-test, was highly significant ($P<0.01$, no. of paired concentrations = 22). The difference between the curves was significant well before delivery; when plasma progesterone levels from individual infected sheep were paired randomly with progesterone concentrations in controls on the same day of gestation, and were compared between day 125 to 135 of gestation, using the paired t-test, a significant difference was observed ($P<0.05$, no. of paired concentrations = 9). The progesterone concentrations in infected and control sheep were also analysed in relation to the day of parturition, using the paired t-test for samples collected between 0 and 8 days before parturition, and between 9 and 20 days before parturition. There was a significant ($P<0.05$) difference in plasma progesterone concentrations between 16 paired samples from infected and control sheep in the earlier (day -9 to -20) period. However, there was no significant difference in the plasma progesterone of 16 paired samples from infected and control sheep during the period of progesterone decline immediately before parturition (day -8 to 0).

6.3.5 Oestradiol 17β in Peripheral Plasma, Utero-Ovarian Vein Plasma and Amniotic Fluid during Chamydial Infection

In the first group of ewes assayed for oestradiol 17β, the duration of gestation in the infected group was 137 ± 1.6 (n=6), range 132-142 days and in the control group the mean gestation period was 144.7 ± 1.08 days (n=6), range 144-149 days. Parturition was significantly earlier ($P<0.05$) in the infected group.

In the C. psittaci-infected group, a pre-partum increase in plasma oestradiol 17β was detected in the one sheep sampled at 24 h before parturition (24 pg ml^{-1}, compared with 9.2pg ml^{-1} and 12.9pg ml^{-1} in two control sheep). The five Chlamydia-infected sheep sampled on the day of parturition had a mean plasma oestradiol concentration of 7.2 ± 4.8 pg ml^{-1}, which was significantly ($P<0.05$) lower than that of control sheep (45 ± 7.3 pg ml^{-1},n=4).

Peripheral plasma oestradiol 17β was analysed daily in a second group of ewes, consisting of four infected and five control sheep during the two days prior to parturition (table 6.4). The duration of gestation in the infected group (139.8 ± 1.1 d, n=4) was not significantly different from that of the control group (143.8 ± 1.1 d, n=5). Abnormalities in the timing of oestradiol 17β release were observed in the sheep infected with C. psittaci compared with controls. The characteristic rise in plasma oestradiol 17β, which was observed on the day of parturition in control animals, was detected 24 h earlier in Chlamydia-infected sheep. In contrast with the control group, the mean peripheral plasma oestradiol concentration in the Chlamydia-infected group 24 h before parturition was higher than the mean concentration of oestradiol 17β

Sample	Sheep		Oestradiol 17β concentrations (mean ± SE pg ml^{-1}) on indicated days before parturition#		
			-2	-1	0
Peripheral plasma	Control	(n = 5)	9.15±2.16	21.7 ±5.7*	63.1 ±10.3
	Infected	(n = 4)	16.6 ±0.80	56.3 ±7.05*	26.4 ±15.9
Utero-ovarian venous plasma	Control	(n = 6)	10.1 ±0.58	18.3 ±1.82	29.5 ± 7.36
	Infected	(n = 6)	6.37±0.17	12.4 ±4.90	32.0 ± 5.31
Amniotic fluid	Control	(n = 6)	5.86±0.86*	5.67±0.87*	9.31± 0.96
	Infected	(n = 6)	17.6 ±5.28*	12.9 ±3.07*	24.3 ±17.3

Table 6.4
Oestradiol 17β in peripheral plasma, utero-ovarian venous plasma and amniotic fluid of C. psittaci-infected and control sheep. * Indicates significant differences between control and infected groups ($P<0.02$), using the Student's unpaired t-test.

#The concentrations of oestradiol 17β in sheep infected subcutaneously with C. psittaci (strain S26/3,5 x 10^5 ELD_{50}), and of control ewes injected with sterile saline.

on the day of parturition.

As uterine prostaglandin release is controlled by oestradiol 17ß (Liggins et al., 1972; Olson et al., 1984), the effect of chlamydial infection on the concentration of oestradiol 17ß within the uterus of these sheep was analysed by cannulation of the utero-ovarian vein and amniotic sacs. The oestradiol 17ß concentrations in the utero-ovarian plasma and amniotic fluid were determined (table 6.4). The sheep used for these oestradiol-17ß measurements were the same as those used for the PGE_2 determinations. The oestradiol 17ß concentrations in utero-ovarian venous plasma of control sheep were similar to previously reported values (Bedford et al., 1972). There was no significant difference in the oestradiol 17ß concentrations released into the utero-ovarian vein by infected, compared with control uteri. However, significantly higher local intrauterine concentrations of oestradiol 17ß were observed in the amniotic fluid of Chlamydia-infected animals compared to controls (table 6.4), indicating that chlamydial infection may have compromised local intrauterine diffusion barriers.

6.4 Discussion

A characteristic of infection with both C. psittaci and C. trachomatis during pregnancy is a period of latency, followed by the onset of placental infection of the foetal membranes and placenta during the last quarter of gestation (Stamp et al., 1950; Studdert, 1968; Novilla & Jensen, 1970; Helm et al., 1987; Aitken, 1986; Sweet et al., 1987). We have found premature increases in PGE_2 concentrations in two intrauterine compartments monitored during late gestation in sheep infected with an ovine abortion strain of

C. psittaci. Increases in amniotic fluid PGE_2 associated with C. psittaci infection were observed from day 122 of gestation, and this was thirteen days before any changes in PGE_2 in amniotic fluid of control sheep (figure 6.1). In the allantoic fluid, the concentration of PGE_2 increased from day 127 of gestation, nine days before the increase observed in controls (figure 6.3). It should be noted that the chlamydial infection established in our catheterised sheep was relatively mild. The mean period of gestation of the infected group (141.3 $\pm$ 1.8 d) was not significantly different from the control group (144.5 $\pm$ 1.1 d) in the animals used for PGE_2 and utero-ovarian vein and amniotic fluid oestradiol-17β measurements, and 80% of the lambs from infected sheep survived.

The difference between PGE_2 profiles in infected and control groups was greatest in the amniotic fluid. This was the fluid space in closest contact with the focus of C. psittaci infection (Mellor, 1980) which spreads from the chorionic membrane to caruncular tissue (Studdert, 1968). The amniochorion is also the primary site of infection of both C. psittaci and C. trachomatis in humans (Johnson et al., 1985; Alger et al., 1988). Our studies indicated that the localised infection of C. psittaci was associated with the release of PGE_2 from the amniochorion. Phospholipase A_2 with specificity for arachidonic acid in the sn-2 position of phospholipids has been observed (Okazaki et al., 1978) in foetal membranes, as well as arachidonic acid specific diacylglycerol and monoacylglycerol lipase activity (Okazaki et al., 1981). Foetal membranes also contain phosphatidylinositol - specific phospholipase C activity (Di Renzo et al., 1981) and foetal membrane lysosomes in humans have also been

found to contain phospholipase A2 activity (Schwarz et al., 1976). It is possible that the tissue necrosis that occurs during chlamydial infection may release or activate these enzymes, resulting in increased PGE_2 production which would be evident in the amniotic fluid.

A comparison of the PGE_2 profiles in the utero-ovarian venous plasma in infected and control sheep suggested that C. psittaci infection disrupted the local caruncular exchange of PGE_2. The concentration of PGE_2 detected in the utero-ovarian vein of infected sheep was 69% that of control animals. It is possible that the impaired exchange of metabolites at this site, which is responsible for approximately 83% of uterine blood flow (Makowski et al., 1968), may be a cause of the low birth weight associated with chlamydial infection (Sweet et al., 1987; Studdert, 1968). It is also possible that impaired vascular function masked the increase in PGE_2 release in infected sheep indicated by the amniotic and allantoic fluid PGE_2 profiles. The increase in utero-ovarian venous PGE_2 concentrations detected in infected sheep occurred more gradually, over a longer time span than the increase in control sheep. Evidence of increased prostaglandin release into the peripheral circulation was recently reported by Fredriksson et al., (1988), who detected a premature increase in peripheral PGF metabolite concentrations in sheep infected with C. psittaci.

In the amniotic and allantoic fluid, the concentrations of PGE_2 detected during the period of premature PGE_2 release in sheep infected with C. psittaci, reached concentrations which were not significantly different from the concentrations of PGE_2 released by

the uteri of control sheep prior to parturition. This indicated that the PGE_2 released during chlamydial infection may be active in initiating events associated with premature labour. It is possible that the inflammatory leucocytes infiltrating the uterus at the sites of C. psittaci infection (Stamp et al., 1950; Studdert, 1968; Johnson et al., 1985; Aitken, 1986; Leaver et al., 1988) may contribute to the PGE_2 pool in intrauterine fluids of infected sheep. However, when the magnitude of the premature increases in PGE_2 which we detected in intrauterine fluids of infected sheep (between three and four ng ml^{-1} of amniotic or allantoic fluid) were compared with the concentrations of PGE_2 synthesised by inflammatory leucocytes (Kurland & Brockman, 1978; Lewis, 1983), the premature release of PGE_2 in amniotic and allantoic fluids was found to be an order of magnitude greater than the amount of PGE_2 sythesised by leucocytes. This suggested that most of the PGE_2 detected in the fluids of infected sheep was of uterine, rather than of leucocyte, origin.

The uterine synthesis of prostaglandins is stimulated by oestrogens (Thorburn & Challis, 1979), and there have been two reports of abnormal release of oestrogens during C. psittaci infection, showing impaired oestrogen production (Martel et al., 1983; Fredriksson et al., 1988). The premature release of oestradiol 17β we observed in peripheral plasma and the increased levels in the amniotic fluid of infected sheep may partly induce the increased levels of PGE_2 observed in amniotic and allantoic fluids in infected sheep.

In addition to controlling the release of PGE_2, there is evidence that oestradiol 17β may facilitate the infectivity of C. trachomatis

in vivo and in vitro (Rank et al., 1982; Bose & Goswanmi, 1986; Sugarman & Agbor, 1986). The premature release of oestradiol 17β from the uterus, and the elevated intrauterine concentrations associated with C. psittaci infection, may induce metabolic and vascular changes enhancing chlamydial growth during late gestation, and it is likely that these effects are at least partly mediated by PGE_2. The decline in peripheral plasma progesterone concentrations started around day 122 of gestation, this coincided with the increases in PGE_2 we observed in the amniotic and allantoic fluids of infected sheep. Since a decline in progesterone levels is associated with an increase in prostaglandin levels (Taylor et al., 1982), the decrease in progesterone observed during chlamydial infection may be a further cause of increased prostaglandin levels in infected sheep.

The pathogenesis which we describe may be relevant to other mammalian species, because the placenta and foetal membranes are major sources of steroid hormones and prostaglandins in most species, including humans, during late pregnancy. There are considerable inter - species differences in the pattern of hormone secretion, and in the relative importance of the stimuli which initiate labour (Allen, 1975). However, in all mammalian species, pregnancy is dependent on maintained progesterone secretion, and oestradiol and prostaglandins stimulate uterine responses at the time of parturition (Thorburn & Challis, 1979; Bedford et al., 1972). Premature progesterone withdrawal can initiate labour and increase oestradiol synthesis in the sheep (Mitchell et al., 1983). The abortifacient agent Actinobacillus seminis has been reported to cause a decline in plasma progesterone in pregnant ewes

(Smith & Hughes, 1974), although this occurred earlier in pregnancy than the inhibition which we observed in Chlamydia-infected sheep (figure 6.4). Premature increases in oestradiol 17β and prostaglandin $F_{2\alpha}$ synthesis have been observed during infectious abortion due to intrauterine surgery (Bedford et al., 1972; Silver et al., 1986) or endotoxin infusion (Roberts et al., 1975) respectively. There has been a recent report of decreased plasma progesterone and oestradiol 17β concentrations and elevated peripheral $PGF_{2\alpha}$ metabolite concentrations in four sheep infected with C. psittaci (Fredriksson et al., 1988). There has only been one report on PGE_2, which is thought to play a role in the initiation of labour (Bleasdale & Johnston, 1982), in infectious abortion (Romero et al., 1988). Our report is the first description of the effect of C. psittaci infection on the intrauterine distribution of oestradiol 17β and prostaglandin E_2 during pregnancy.

In conclusion, mild infection with C. psittaci was associated with abnormalities in the intrauterine concentrations of PGE_2 in amniotic fluid, utero-ovarian vein, and allantoic fluid. In the two intrauterine sacs, we detected the premature secretion of PGE_2, which reached the concentrations of PGE_2 which were found just prior to parturition in the control sheep. In the utero-ovarian vein, there was evidence of impaired secretion of PGE_2. These results suggest that chlamydial infection causes the release of PGE_2 from intrauterine tissues. This release may have been partly facillitated by premature increases in oestradiol 17β and decreases in progesterone that were also associated with infection by C.

psittaci. It is likely that the changes in placental steroid and prostaglandin synthesis observed here, contribute to the premature labour associated with C. psittaci infection in sheep.

Chapter 7

General Discussion

Since Quackenbush first demonstrated in 1942 that normal parturition will not occur in rats fed α-linolenic acid as the main dietary source of EFA, it has been known that n3 fatty acids will inhibit parturition. Due to the importance of prostaglandins E_2 and $F_{2\alpha}$ in the initiation and maintenance of labour it has been suggested that the n3 effect on parturition may be acting through disruption of prostaglandin production. A fish oil diet high in n3 fatty acids has been shown to decrease PGE_2 production by intrauterine tissues (Leaver et al, 1986). Prior to the studies described in this thesis, however, a number of questions regarding the incorporation and metabolism of the n3 and n6 fatty acids in the uterus remained unanswered. It was not known to what extent the n3 fatty acids would be incorporated into the uterus or whether the n6 fatty acids would be specifically retained in response to a diet containing predominantly n3 fatty acids. Inhibition of elongation and desaturation of n6 fatty acids by n3 fatty acids could result in low conversion of 18C n6 precursors to the 20C n6 prostaglandin precursors and incorporation of 20:5n3 could result in competition with arachidonic acid for cyclooxygenase enzymes, so inhibiting formation of PGE_2 and $PGF_{2\alpha}$. Prior to the current investigation no measurement of 3-series prostaglandin production in the uterus had been made.

Infection by the intracellular parasite Chlamydia psittaci instead of impairing parturition, may result in premature labour or abortion. As with the n3 fatty acids, this effect could be mediated

through abnormal prostaglandin production, we therefore measured PGE_2 production in pregnant sheep infected with C. psittaci and compared this to levels in control uninfected sheep. In this final chapter the overall conclusions of findings presented in this thesis shall be discussed and summarised and some ideas for future investigations that have arisen as a result of the present study shall be suggested.

7.1 Effects of Dietary n3 and n6 EFA on Uterine Fatty Acid Composition

After weaning only three weeks of feeding a diet high in n3 or n6 EFA was necessary to induce significant changes in the fatty acid composition of the rat uterus. The same dietary induced changes were seen after feeding for up to 500 days. Uteri of rats fed a FO diet with a high n3 fatty acid content contained higher proportions of n3 fatty acids than rats fed a control pelleted diet or an EPO diet, and the opposite was true for n6 fatty acids. The proportion of arachidonic acid was significantly higher ($p < 0.05$) in the EPO group than in the FO group of adult rats (mean age 231 days) and in both EPO ($p < 0.05$) and control ($p < 0.05$) groups of phospholipids in young rats (mean age 44 days). 20:5n3 proportions, however, were significantly higher in FO groups in both total lipid in adult rats and phospholipid in young rats than in EPO and control groups ($P < 0.001$ for control adult rats, others $P < 0.05$).

The control diet contained higher proportions of n3 fatty acids than the FO diets, however, uteri from rats on FO diets contained higher proportions of n3 fatty acids than those from control rats. This may have been due to the control diet having a far lower n3/n6 ratio

than the FO diets (0.183 compared to 2.899 in adult rats and 3.522 in young rats respectively) and suggests that the uterus will only incorporate n3 fatty acids in relatively high proportions when sufficient levels of n6 fatty acids are not available. Huang et al. (1987) reported similar results in rat plasma and liver.

D6 desaturase activity in the rat uterus appeared to be low, illustrated by a comparison of proportions of 20:3n6 and arachidonic acid in the total lipid of adult rats fed control and EPO diets. The uteri of control rats contained significantly higher ($p < 0.05$) proportions of linoleic acid than uteri of rats on the EPO diet (11.12% compared to 3.82% respectively). However, the uteri of EPO fed rats contained higher proportions of arachidonic acid (9.92% compared to 7.72% respectively). The diets and uteri of control and EPO rats contained similar proportions of 18:3n6 and 20:3n6 so the difference was unlikely to have arisen from conversion of these fatty acids to arachidonic acid. However, n3 fatty acids present in the control diet and tissue may have inhibited desaturation of n6 fatty acids. Accumulation of 18:3n6 or 20:3n6 was not observed in rats on any of the diets in any of the lipid fractions. This suggests active D5 desaturase actvity in the uterus.

In rats of all diet groups a general trend of high 22C to 20C n3 fatty acids was observed, whereas the opposite was true for the n6 fatty acids. This indicates that either the D4 desaturase was more active on n3 fatty acids than n6 fatty acids or that preferential incorporation of the 22C n3 above the 20C n3 fatty acids was taking place. Comparisons of total proportions of arachidonic acid, 20:5n3 and 22:6n3 in the EPO, FO and control diets suggest that 20:5n3 and

22:6n3 compensate for low arachidonic acid levels in the uterus. However, this compensation is purely structural and not functional, at least concerning the process of parturition.

Individual lipid fractions within the uterus were found to respond differently to changes in n3 and n6 dietary fatty acid content, with the neutral lipid fractions, with the exception of the free fatty acids, having higher n3/n6 ratios than the phospholipids, in response to a diet containing α-linolenic acid as the major EFA source. Free fatty acid, PC and PI lipid fractions were affected least by high n3 fatty acid diet content, maintaining low n3/n6 ratios of 0.26 ± 0.14, 0.3 ± 0.12 and 0.23 ± 0.05 respectively. PE was more susceptible to the high n3 dietary content than the other phospholipids, giving an n3/n6 ratio of 0.47 ± 0.12.

Very low incorporation of 20C and 22C EFA into triglyceride and cholesterol esters was observed in rats on all of the diets, suggesting that these lipids do not form quantitatively major pools of eicosanoid precursors. Relatively high proportions of the 18C EFA were, however, found in the triglyceride-cholesterol ester lipid fractions. This lipid fraction was found to be very susceptible to changes in dietary fatty acid content, with a high uterine n3/n6 fatty acid ratio of 1.39 resulting after three weeks of feeding rats a diet containing 97.6% n3 EFA (in the form of α-linolenic acid ethyl ester).

The monoglyceride, diglyceride and free fatty acid components of the neutral lipid fraction contained higher proportions of the 20C EFA than the triglyceride-cholesterol ester fraction. The activity of

inositol-specific phospholipase C, diacylglycerol lipase and monoacylglycerol lipase specific for monoacylglycerol with arachidonic acid in the sn-2 position have been identified in human uterine decidua and foetal membranes (Di Renzo et al., 1981; Okazaki et al., 1981), so these neutral lipid fractions are possible sources of arachidonic acid for prostaglandin synthesis.

In addition to a dietary induced change in uterine fatty acid composition, an age related change in fatty acid composition of newly weaned rats compared to adult rats was also observed. Newly weaned rats were found to contain a significantly higher ($P < 0.01$) proportion of fatty acids with less than 18 carbon atoms. The high proportion of short chain fatty acids in the newly weaned rats may have resulted from high levels of these fatty acids in the mothers milk (Ross et al., 1985). However, the adult rats on the semi-synthetic diets consumed large quantities of short chain fatty acids compared to those on the control diet with little effect on the proportions of short chain fatty acids in the uterus. This suggests that dietary levels of short chain fatty acids have little influence on the incorporation of these fatty acids in the adult rat.

7.2 Uterine Arachidonic Acid Conservation

Arachidonic acid was the fatty acid most conserved in all of the dietary groups in phospholipid of young rats and total lipid of adult rats, including the rats on the high n3 fatty acid diets. Tissue/diet arachidonic acid ratios in EPO, FO and control diet fed rats were higher than those for any other fatty acids tested in total uterine lipid of adult rats and phospholipid of young rats. There are three possible explanations for the mechanism behind the

high tissue arachidonic acid concentrations; (1) Arachidonic acid was specifically incorporated into the tissue, (2) Arachidonic acid levels were increased by production from precursors, and (3) Arachidonic acid was specifically retained despite low dietary levels.

The studies have shown little evidence for specific incorporation of arachidonic acid directly from the diets, with dietary levels of arachidonic acid having little direct bearing on tissue arachidonic acid proportions, however, the arachidonic acid content of all the diets was very low. There was, however, some evidence for specific incorporation of n6 fatty acids in preference to n3 fatty acids, illustrated by the fact that the control diet contained higher proportions of both 18:3n3 and 20:5n3 than the FO diet, however, the uteri from the FO fed rats contained higher proportions of these fatty acids than the control diet rats, indicating that when n6 fatty acids were available, less n3 fatty acids were incorporated. Evidence for the production of arachidonic acid from precursors came from the fact that, despite the EPO diet containing a lower proportion of arachidonic acid than either the FO or control diets, the tissue levels of arachidonic acid were highest in the EPO group. This may have been due to there being no n3 fatty acids present in the EPO diet to inhibit conversion of n6 precursors to arachidonic acid. Arachidonic acid was present in the lipid of rats fed the FO and α-linolenic acid diets that contained very low levels of n6 fatty acids, indicating that the arachidonic acid was selectively retained in these rats. There was also evidence for conservation of n3 fatty acids in the uterus as the rats on the EPO and linoleic

acid diets maintained n3/n6 tissue ratios far higher than those in their corresponding diets.

Arachidonic acid was found to be most conserved in the phospholipid fractions of phosphatidylcholine and phosphatidylinositol, with phosphatidylethanolamine being more susceptible to dietary change. PI, PC and PE have all been suggested to be possible sources of free arachidonic acid in uterine tissues involved in prostaglandin synthesis at parturition (Leaver et al., 1981; Ning et al., 1983; Okita et al., 1982). In the free fatty acid fraction the proportion of arachidonic acid and eicosapentaenoic acid in relation to the other EFA was higher than in the other lipid groups, suggesting specific release of the eicosanoid precursors. Enzymes specific for arachidonic acid release from PE and monoglyceride have been identified in uterine decidua and foetal membranes (Okazaki et al., 1978; Okazaki et al., 1981b). Evidence for selective release of arachidonic acid during labour has also been found (Ogburn et al., 1980; Okita et al., 1982).

7.3 The Effect of Dietary Fatty Acids on Uterine Prostaglandin Production

Inhibition of 2-series prostaglandin production has been shown to occur in uterine tissues in response to high dietary n3 fatty acid intake (Leaver et al., 1986). However, it was not known if uterine cyclooxygenase enzymes would metabolise n3 fatty acids to 3-series prostaglandins. In this study evidence was presented for the presence of 3-series prostaglandins in uteri of rats fed diets containing different levels of n3 and n6 fatty acids. Using a very sensitive method of mass spectrometry, synthesis of PGE_3 and $PGF_{3\alpha}$

was detected in the calcium ionophore, A23187, stimulated uteri of rats fed a control pelleted diet (82.6% n6 EFA), a linoleic acid diet (99.9% n6 EFA) or an α-linolenic acid diet (97.6% n3 EFA). In unstimulated uteri PGE_3 and $PGF_{3\alpha}$ were produced only in the α-linolenic acid fed group. A23187 induced increased production of total prostaglandin as well as causing an increase in the proportion of 3-series to 2-series prostaglandins. This suggested that the phospholipases activated by A23187 were less specific for arachidonic acid than those in the unstimulated uterus. The phospholipases activated by A23187 may act on a different fatty acid pool or may be present in a different part of the uterus from phospholipases active in the unstimulated uterus. Leaver et al. (1981) reported different levels of prostaglandin synthesis in the endometrium and myometrium of guinea pig uterus, and in the rabbit kidney the existence of two fatty acid pools for prostaglandin synthesis has been suggested (Schwartzman and Raz, 1979; Schwartzman and Raz, 1981).

In rats maintained on diets containing high concentrations of n3 fatty acids growth and gestation will occur normally (Leat and Northrop, 1981) it is only parturition that is inhibited. If there is a similar increase in the proportion of 3-series to 2-series prostaglandins produced at parturition as was observed in A23187 stimulated uteri compared to unstimulated uteri, this could explain why only parturition is affected by the n3 diet. The increase in 3-series prostaglandin production observed on stimulation with A23187 suggests that only a limited degree of substrate specificity in prostaglandin synthesis is at the level of the cyclooxygenase.

Rats fed the α-linolenic acid diet synthesised lower total levels of prostaglandins than rats fed the linoleic acid diet. This, combined with the increase in proportions of 3-series to 2-series prostaglandins in the linolenic compared to the linoleic acid fed rats may cause the inhibition of parturition associated with high dietary n3 fatty acid intake.

7.4 The Effect of Infection by Chlamydia Psittaci on Uterine Prostaglandin E_2 and Steroid Hormone Production

Premature labour and abortion in sheep often results from infection by Chlamydia psittaci (Studdert and McKercher, 1968; Novilla and Jensen, 1970). Ovine abortion strains of C. psittaci may also cause abortion in women (Roberts et al., 1967; Beer et al., 1982; Johnson et al., 1985). Prostaglandins and steroid hormones act closely together to control the timing, initiation and maintenance of parturition. It is therefore possible that chlamydial infection may cause abortion through effects on production of the steroid hormones and prostaglandins. In this thesis, changes were reported in patterns of production of steroid hormones and PGE_2 in sheep experimentally infected with an ovine abortion strain of C. psittaci compared to uninfected sheep. In response to a relatively mild infection, PGE_2 concentrations were found to significantly increase thirteen days early in the amniotic fluid and nine days early in the allantoic fluid, compared to uninfected sheep ($P < 0.001$ and $P < 0.01$ respectively). The increased concentrations of PGE_2 seen in the infected sheep were similar to those in uninfected sheep at parturition. In contrast to the premature increases in PGE_2 concentrations observed in the amniotic and allantoic fluids, in the

utero-ovarian vein, PGE_2 concentrations in infected sheep were only 69% of levels in control sheep. This suggested that infection by C. psittaci disrupted the transfer of PGE_2 into the utero-ovarian vein.

In sheep, increased oestradiol 17β is associated with increased prostaglandin synthesis (Thorburn and Challis, 1979), whereas high progesterone concentrations are associated with low prostaglandin concentrations (Taylor et al., 1982). A decrease in progesterone concentrations on day 122 of gestation in infected sheep was observed, which coincided with the increase in PGE_2 in the amniotic fluid. Premature increases in oestradiol 17β were observed in peripheral plasma and amniotic fluid of infected sheep. The changes in the steroid hormone concentrations may be partly responsible for the elevated levels of PGE_2 seen in infected sheep, however, tissue necrosis caused by C. psittaci infection may also result in increased PGE_2 concentrations. The results suggest that premature labour resulting from infection by C. psittaci may be initiated by alterations in PGE_2 and placental steroid release.

7.5 Future Studies

This study has demonstrated how the fatty acid composition and prostaglandin production of the uterus may be altered by dietary fatty acid composition and microbial infection by C. psittaci. In the course of the present investigation a number of questions have arisen that will require to be addressed in the future. These questions will now be discussed.

Certain lipid pools were found to be more susceptible to changes in dietary fatty acids than others. Differences in incorporation and

release of fatty acids from individual phospholipid species has been observed in a number of cells and tissues and more interest is now being centred on differences between diacyl and other phospholipid species. These were not analysed in the present study but would be worthwhile analysing in a future study. As well as analysing different lipid classes, the uterus could be divided into separate tissues and fatty acid content, fatty acid release and prostaglandin synthesis measured in the individual tissues. At parturition various tissues are involved to different degrees in the synthesis of prostaglandins so analysis of the uterus as a whole does not necessarily give the most accurate information on the source of fatty acids for prostaglandin synthesis.

Levels of fatty acids in serum, amniotic fluid and uterine and foetal tissues involved in prostaglandin synthesis have been shown to vary with gestational age, and before, during and after labour (Ogburn et al., 1980; Olund and Lunell, 1980; Das et al., 1975; Schwarz et al., 1975; Schwartz et al., 1977), and it has been suggested that arachidonic acid is specifically incorporated into phospholipids late in pregnancy. The effect that the n3 fatty acids have on this specific incorporation has not yet been analysed and could provide further information on the mechanism of the impairment of parturition caused by the n3 fatty acids.

From the information obtained on the fatty acid contents of the different lipid fractions, possible sources of fatty acids for prostaglandin synthesis were suggested, however, further studies on the fatty acid composition of the different lipid groups before and after tissue stimulation is necessary for a more positive

identification of the lipid groups involved. The results in chapter 5 suggested a different source of fatty acids may be used for prostaglandin synthesis in the unstimulated uterus compared to the uterus stimulated with the calcium ionophore A23187. It has been suggested that different agonists may induce release of fatty acids from different sources (Hsueh et al., 1981; Schwartzman and Raz, 1981; Hong and Deykin, 1979) and that the proportion of the fatty acids released may also vary with the type of stimulus. We observed increased release of 3-series prostaglandins in response to A23187. However, A23187 may cause a less specific release of fatty acids than other stimuli (Hong and Deykin, 1979) such as progesterone, oestrogen and oxytocin, that are involved in the control of prostaglandin synthesis during pregnancy and parturition. It would therefore be a worthwhile study to investigate the fatty acids released and prostaglandins synthesised in response to these hormones in animals maintained on diets with varying n3 and n6 EFA content. If 3-series prostaglandins are produced in similar quantities at parturition as they are when the uterus is stimulated with A23187 then they may be partly responsible for the n3 fatty acid induced inhibition of parturition, possibly by antagonising the effects of the 2-series prostaglandins. The effects of the 3-series prostaglandins on the uterus are not known, however, in view of the current findings this clearly requires investigation.

Injection of arachidonic acid into the amniotic fluid of pregnant women has been shown to cause abortion (MacDonald et al., 1974). It is possible that eicosapentaenoic acid administered in a similar way could delay labour. If this was the case then eicosapentaenoic acid

could be used in the management of preterm labour. It is also possible that similar treatment could delay or prevent the premature delivery and abortion caused by infection with C. psittaci and an interesting experiment would be to observe the effects of a diet high in n3 fatty acids on pregnant sheep infected with C. psittaci.

The effect of dietary n3 and n6 fatty acids on uterine fatty acid content and the effect of these fatty acids and of infection by the intracellular parasite, Chlamydia psittaci, on uterine prostaglandin production have been investigated in this thesis. Further work is required to investigate the incorporation of n3 and n6 EFA into the diacyl and plasmalogen phospholipid species as well as incorporation into specific uterine tissues. The sources of the prostaglandin precursors released in response to different agonists also requires investigation.

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Publications

The following publications are a result of work presented in this thesis. The publishers and co-authors permission has been obtained for the inclusion of photocopied publications.

Leaver H.A., Howie A., Appleyard W., Aitken I.D., & Hay L.A. (1987). Altered steroid hormone and prostaglandin metabolism during chlamydial infection in sheep. Biochem. Soc. Trans. 15, 479.

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Altered steroid hormone and prostaglandin metabolism during chlamydial infection in sheep

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Chlamydial infection is associated with premature labour in sheep, and causes necrosis of placental tissue. The effect of *Chlamydia* infection on the synthesis of progesterone was studied, by measuring serum progesterone in seven infected and seven control animals. Progesterone inhibits the release of prostaglandin (PG) E_2 and $PGF_{2\alpha}$ from the pregnant uterus (Taylor *et al.*, 1978). Local inflammation at the site of infection may also stimulate intrauterine PGE_2 synthesis (Thorburn & Challis, 1979).

The amniotic and allantoic sacs and the utero-ovarian vein were cannulated in seven *Chlamydia*-infected sheep and seven uninfected controls. Infection was by subcutaneous inoculation of $10^{4.5}$ infectious particles of an ovine abortion strain of *Chlamydia psittaci*. PGE_2 was detected by radioimmunoassay of uterine fluids (Leaver & Seawright, 1982) without prior extraction, as parallel binding curves were obtained in the presence and absence of fluid. However, PGE_2 in utero-ovarian plasma was immediately extracted, as an interfering factor, and PGE_2 degradation, were detected in plasma. Anti-PGE_2 antiserum was purchased from the Institut Pasteur, Paris, and anti-rabbit IgG was donated by the Scottish Antibody Production Unit.

The plasma progesterone of *Chlamydia*-infected sheep was not significantly different from control values until day 120 of gestation (see Fig. 1). The concentration of progesterone decreased significantly between day 119 and day 135 of gestation in *Chlamydia*-infected animals, but not in control, uninfected sheep. The decline in plasma progesterone in control sheep occurred between day 139 and 145 of pregnancy. Therefore, the decline in circulating progesterone was observed 20 days earlier in *Chlamydia*-infected sheep, and 16 days before delivery.

The concentration of PGE_2 in amniotic and allantoic fluids in control animals was low (2–4 ng/ml), between day 15 and day 128 of gestation. In contrast, an elevated concentration of PGE_2, and pulsatile release of this prostaglandin, was observed in *Chlamydia*-infected sheep from day 119, which gradually increased until delivery. The release of PGE_2 into the utero-ovarian vein just before parturition, observed in control animals, was impaired or inhibited in *Chlamydia*-infected sheep. The magnitude of the increase in PGE_2 observed in *Chlamydia*-infected amniotic fluid (over 2 ng/ml), suggested that the PGE_2 was of uterine, rather than leucocyte, origin. The relation of the timing of PGE_2 release, to the decline in plasma progesterone, also suggested an endocrine control of this PGE_2 release.

Abbreviation used: PG, prostaglandin.

In summary, changes in circulating steroid hormones, and intrauterine PGE_2, were detected in *Chlamydia*-infected sheep. These changes may precipitate the premature labour associated with *Chlamydia* infection.

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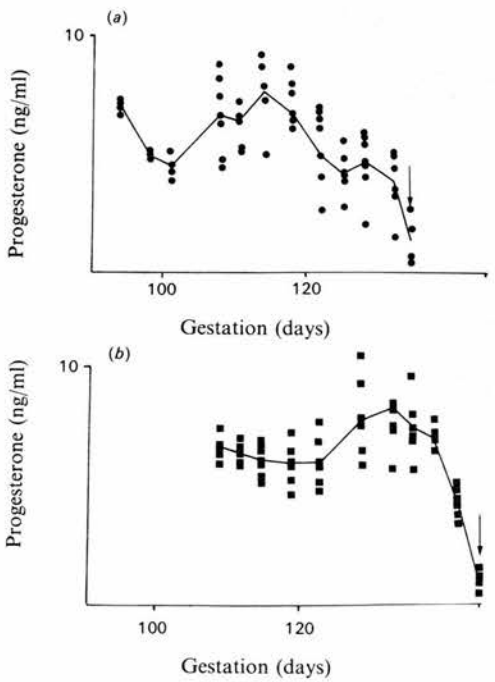

Fig. 1. *Plasma progesterone in Chlamydia-infected (a) and control (b) sheep during late pregnancy*

The mean date of lambing is indicated with an arrow. Progesterone was extracted from plasma using diethylether, and results were corrected for efficiency of solvent extraction (70 ± 6%), and detected using the antiserum of Scaramuzzi *et al.* (1974).

Evidence that sequential solubilization of the bile canalicular membrane occurs during the onset of bile acid-induced cholestasis

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Under normal physiological conditions bile phospholipid is almost entirely composed of palmitoyl-linoleoyl or palmitoyl-oleoyl phosphatidylcholine. It is generally believed that this phospholipid is solubilized from biliary tract membranes by bile acids yet, surprisingly, these membranes contain very little of this type of phospholipid as their structure comprises mainly of stearoyl-arachidonoyl-phosphatidylcholine, sphingomyelin and phosphatidylethanolamine (together with large amounts of cholesterol relative to other hepatic membranes). To explain this phenomena Barnwell *et al.* (1984) proposed that a microtubule-mediated bile acid-dependent process carries biliary phospholipid vesicles from a storage site in the endoplasmic reticulum to the bile canalicular membrane (BCM) and, furthermore, as suggested by Lowe *et al.* (1984), these vesicles fuse with the BCM forming fluid microdomains of biliary phospholipid preferentially solubilized by bile acids.

Since inhibition of the transport of biliary phospholipids to the BCM with microtubule- disrupting agents causes cholestasis (Barnwell *et al.*, 1984), it is likely that this process forms a protective and/or membrane repair function. In the present study the possibility that the breakdown of this process leads to the onset of bile acid-induced cholestasis was investigated.

The experimental procedure involved the intravenous infusion of various bile acids (cholic acid, chenodeoxycholic acid, deoxycholic acid or their glycine and taurine conjugates) into anaesthetized male Sprague–Dawley rats (200–250g) fitted with a bile fistula. Administering bile acids in stepwise increasing doses first of all increased bile flow (up to maximum of 200% of control values) and then decreased bile flow after maximum bile acid secretion rate had been achieved. When bile flow had declined to 50% of that before bile acid infusion, livers were removed and pure BCM and microsomal membranes isolated. Bile phospholipids, from 10 min samples collected throughout the experiments, were separated by t.l.c., as were hepatic membrane phospholipids. Quantification of individual phospholipid species was followed by a detailed analysis of their fatty acid composition by capillary column g.l.c.

The results of these analyses indicated that the quantitative changes in biliary and hepatic membrane phospholipid during the onset of cholestasis were essentially very similar. In the early stages of bile acid infusion, when bile flow was increasing, bile phospholipids were comprised almost entirely of phosphatidylcholine and contained significant amounts of palmitic, linoleic and oleic acids only. Bile samples collected when bile flow and bile acid secretion were maximal still contained only phosphatidylcholine; however the fatty acid profile of this phospholipid now included increasing amounts of stearic and arachidonic acids. Subsequent bile samples collected as bile flow declined contained sphingomyelin (up to 20% of the total phospholipid) while those collected when bile flow was at its minimum also contained phosphatidylethanolamine. Both sphingomyelin and phosphatidylethanolamine were found to have long-chain fatty acids, typical of those found in BCM phospholipids, in their composition. Interestingly, a comparison of the total amounts of each lipid subclass secreted during the bile acid infusion, showed little variation between individual animals or with the individual bile acid infused.

Microsomal and BCMs were found to be considerably depleted in total phospholipids (up to 50%) but not cholesterol when compared with controls. Unlike the BCM, in which no overall change in the proportion of each phospholipid component had taken place, the microsomal membrane contained significantly less phosphatidylcholine-derived palmitic, linoleic and oleic acids (i.e. biliary type phosphatidycholine).

Taken together these observations are suggested to indicate that a series of common events take place during the onset of the cholestasis induced by a variety of different bile acids. Initially it is envisaged that the transport of biliary type phosphatidylcholine increases with bile acid until the rate of new synthesis can no longer keep pace with demand and/or the preformed pool of this material is completely depleted. With continued bile acid transport into bile without biliary phospholipid it is likely that solubilization of the BCM structure occurs. Samples of bile collected during the onset of cholestasis first of all contained phosphatidylcholine followed by sphingomyelin and then phosphatidylethanolamine, thereby suggesting a sequential solubilization of these phospholipids from the BCM. Nevertheless, the final composition of the BCM during cholestasis was changed only in its total phospholipid and not individual phospholipid content. It is suggested that the consequence of the breakdown of BCM repair is that BCM phospholipids are solubilized by bile acids resulting in a greatly increased cholesterol/phospholipid ratio (0.35 : 1.2) and a subsequent failure in BCM secretory function.

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Abbreviation used: BCM, bile canalicular membrane.

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Pulmonary surfactant biosynthesis: studies *in vivo* and *in vitro*

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Pulmonary surfactant phospholipids are responsible for reducing surface tension at the alveolar surface and are essential to normal lung function. The phospholipids of surfactant are synthesized in the endoplasmic reticulum of the type II cells of the lung and stored in lamellar inclusion bodies before release on to the alveolar surface (Chevalier & Collet, 1972; Van Golde, 1976).

The successful isolation and maintenance of alveolar type II cells in culture (cf. Longmore & Brown, 1984) has provided a readily available homogenous cell population in which to study surfactant biosynthesis. However, studies by Mason & Dobbs, (1980) have indicated that isolated cell preparations may gradually loss their ability to synthesize

Changes in Progesterone, Oestradiol 17β, and Intrauterine Prostaglandin E_2 during Late Gestation in Sheep Experimentally Infected with an Ovine Abortion Strain of *Chlamydia psittaci*

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The placenta is the primary site of infection of *Chlamydia psittaci* and is also intimately involved in the control of parturition. Changes in the pattern of placental hormone secretion were investigated in ewes infected with *C. psittaci* and in saline-injected controls. The concentration of progesterone in peripheral plasma of infected sheep was significantly lower than in control sheep ($P<0{\cdot}01$). A gradual decline in plasma progesterone occurred in *Chlamydia*-infected sheep, beginning on day 125 of gestation, in comparison with the sharper decline commencing on day 139 of gestation in the control population. The release of oestradiol 17β, which was greatest on the day of parturition in control sheep, was significantly ($P<0{\cdot}02$) increased on the day before parturition in *Chlamydia*-infected sheep. The concentrations of prostaglandin E_2 in amniotic and allantoic fluids were low during late pregnancy in 12 control sheep, but were significantly raised ($P<0{\cdot}05$) in four out of 12 samples obtained from *Chlamydia*-infected sheep over the same period. The changes in progesterone and prostaglandin E_2 were temporally related to the morphological and histochemical changes characteristic of trophoblast infection. These findings suggest that *C. psittaci* infection may precipitate premature labour by altering placental steroid and prostaglandin release.

INTRODUCTION

Premature labour and abortion are major complications associated with *Chlamydia psittaci* infection of sheep (Studdert & McKercher, 1968; Novilla & Jensen, 1970). Ovine abortion strains of *C. psittaci* have been demonstrated to infect women and are associated with abortion in these subjects (Roberts *et al.*, 1967; Beer *et al.*, 1982; Johnson *et al.*, 1985). In both sheep and humans, the perinatal complications caused by *C. psittaci* appear to be more severe than those associated with *Chlamydia trachomatis*, but the pathophysiology of both infections is similar with respect to the site and timing of infectivity and the effect of initiating premature labour (Sweet *et al.*, 1987; Studdert, 1968). The widespread distribution of chlamydial infection in sheep has been known for many years (McEwen *et al.*, 1951; Studdert & McKercher, 1968). However, recent applications of monoclonal antibodies and gene probe techniques have indicated a much wider distribution of chlamydial infection than had been previously suspected in the human population (Sweet *et al.*, 1987).

The endocrinology of infectious abortion has been the subject of intermittent attention (Smith & Hughes, 1974; Roberts *et al.*, 1975; Minkoff, 1983; Lamont *et al.*, 1985; Helm *et al.*, 1987).

Abbreviations: ELD_{50}, egg LD_{50}; PGE_2, prostaglandin E_2.

However, there has been little investigation of the effects of chlamydiae on the factors which play a role in controlling parturition (Martel *et al.*, 1983; Rank *et al.*, 1982; Fredriksson *et al.*, 1988). This is surprising, since pathological studies indicate that the infectivity of chlamydiae is closely related to the stage of pregnancy, and disorders in the initiation of parturition result from this infection.

The primary focus of chlamydial infection during pregnancy is the placenta. Three placental products are important in the control of parturition, viz. progesterone, oestradiol 17β and prostaglandin E_2 (PGE_2). Progesterone is important in the maintenance of pregnancy (Bedford *et al.*, 1972; Taylor *et al.*, 1982), and progesterone synthesis during late pregnancy in both sheep and women occurs in the placenta (Linzell & Heap, 1968). The placenta is also a major source of oestrogens in these species. The secretion and distribution of oestradiol 17β during gestation, and its effect on prostaglandin synthesis, have been characterized (Allen, 1975; Liggins *et al.*, 1972). Plasma progesterone and oestradiol 17β concentrations are indicators of the placental production and secretion of these steroids. The concentrations of oestradiol 17β in the amniotic fluid and the utero-ovarian vein reflect the intrauterine production and diffusion of oestradiol 17β.

Changes in the concentrations of oestradiol 17β and progesterone influence the release of PGE_2 at parturition (Thorburn & Challis, 1979; Taylor *et al.*, 1982; Olson *et al.*, 1984). The primary site of placental infection of *C. psittaci* is the chorionic membrane, which is also a major site of placental PGE_2 synthesis. PGE_2 is also released in significant quantities by leucocytes, particularly by inflammatory macrophages (Lewis, 1983). The prostaglandins exert a range of effects within the uterus at parturition. Prostaglandins act on the myometrium, where they stimulate and co-ordinate myometrial contraction (Wickland *et al.*, 1984). In the cervix, prostaglandins induce ultrastructural changes resulting in an increase in cervical patency (Keirse *et al.*, 1983). Stimulation of the chorionic membrane causes release of arachidonic acid and prostaglandin E_2 from the foetal membranes (Grieves & Liggins, 1976). A role for prostaglandin E_2 in initiating labour has been proposed (Bleasdale & Johnston, 1984). The secretion of prostaglandin E_2 and its metabolites increases during late pregnancy, and prostaglandin E_2 is synthesized in greater quantities than prostaglandin $F_{2\alpha}$ during early labour (Dray & Frydman, 1976). The intrauterine concentrations of PGE_2 indicate the locally active concentrations of this metabolically labile compound close to its site of action.

In this study, the relationship between the timing of changes in placental hormone metabolism and morphological and histochemical changes in the placenta during chlamydial infection was analysed in order to establish the sequence of pathophysiological events associated with premature labour.

METHODS

Experimental infection of sheep. Twenty-two Scottish Blackface ewes were infected by subcutaneous inoculation with between $0{\cdot}45 \times 10^6$ and $1{\cdot}6 \times 10^6$ egg LD_{50} (ELD_{50}) of ovine abortion strain S26/3 of *C. psittaci* between days 90 and 115 of gestation. Twenty-three pregnant ewes were used as controls. The number of foetuses was determined in all animals between days 60 and 80 of gestation using ultrasonography, and at birth. These experiments were part of a study of the pathogenesis of *C. psittaci* infection during pregnancy, as this is a serious cause of mortality in lambs and morbidity (infectious abortion) in pregnant sheep. In addition, *Chlamydia* infection has been demonstrated to be a cause of abortion in pregnant women in contact with sheep. The study was reviewed by the Ethics Committee of the Institution where the experiments were conducted before commencement.

Peripheral plasma samples for progesterone assays. Six ewes were infected on day 90 of gestation by subcutaneous injection with 5×10^5 ELD_{50} of *C. psittaci* strain S26/3, and six control ewes were injected with sterile saline. The mean duration of gestation $\pm$ SE in control sheep was $144{\cdot}7 \pm 1{\cdot}08$ d ($n = 6$), range 144–149 d, and in *C. psittaci*-infected sheep, $137 \pm 1{\cdot}6$ d ($n = 6$), range 132–142 d. Only sheep with single lambs were used, as twin pregnancies are associated with higher progesterone levels, which are not directly proportional to the number of lambs (Bedford *et al.*, 1972; H. A. Leaver, unpublished observations). Blood was withdrawn at 3 d intervals from the jugular vein of infected and control sheep up to day 110 of gestation and at 2 d intervals after day 110, and placed into tubes containing 2 IU preservative-free heparin ml^{-1} (Evans Medical). Plasma was prepared by centrifugation of the heparinized sample at 2000 *g* for 10 min. All plasma samples were stored at -40 °C.

Progesterone concentrations were expressed as the means ± SE of results from samples of plasma taken from three to six individual control or infected sheep on the same day of gestation.

Progesterone radio-immunoassay. Peripheral plasma progesterone was extracted using ethyl acetate (efficiency 71 ± 3%). Progesterone was determined by a radio-immunoassay using the antiserum and technique of Scaramuzzi *et al.* (1974), and [1,2,6,7,16,17-^{3}H]progesterone radiotracer (Amersham, batch no. 10/H/4723). Antibody-bound progesterone was precipitated using dextran charcoal. The precision of progesterone determination was 10·2% for within-assay duplication (inter-assay coefficient of variation), and 9·6% for between-assay replication (intra-assay coefficient of variation), for two plasma samples analysed six times within the same assay (n = 6), and two plasma samples analysed in six different assays (n = 6), respectively (Hunter, 1978).

Peripheral plasma samples for oestradiol 17β assays. Oestradiol 17β was analysed in peripheral plasma samples collected for progesterone analysis (see above), and in peripheral plasma samples from a second group of animals, consisting of four ewes infected on day 113–115 of gestation by subcutaneous injection with 0·5 × 10^6 ELD_{50} of *C. psittaci* strain S26/3, and five control ewes injected with sterile saline. This second group were catheterized 2 d after injection, on day 115-117 of gestation, for intrauterine sampling (see below). The mean durations of gestation ± SE in this second group were 143·8 ± 1·1 d (n = 5), range 141–147 d, in control sheep and 139·8 ± 1·1 d (n = 4), range 137–141 d, in *C. psittaci*-infected sheep. Two of the control ewes had twins, and three had single lambs. All seven lambs survived. Two of the infected ewes bore twins, and two bore single lambs. Two out of six lambs of the *C. psittaci*-infected sheep were born dead: one of twin lambs was dead on delivery, and another, single lamb, died of asphyxia during delivery. In group two, blood was withdrawn from the jugular vein at 24 h intervals during the last 3 d of gestation, and placed into tubes containing 2 IU preservative-free heparin ml^{-1}. Plasma was prepared by centrifugation (see above) and stored at −40 °C. Oestradiol 17β concentrations were expressed as pg ml^{-1} ± SE in the plasma of control or infected sheep, sampled on separate days before parturition.

Utero-ovarian venous plasma, amniotic fluid and allantoic fluid samples from catheterized animals for oestradiol 17β assays. The intrauterine distribution of oestradiol 17β during chlamydial infection was analysed in the six control sheep injected with saline, and in the six sheep infected with 5 × 10^5 ELD_{50} of *C. psittaci* strain S26/3 on day 113–115 of gestation, whose peripheral plasma oestradiol 17β was determined. This third group consisted of the sheep used for peripheral plasma oestradiol (see above), plus two additional infected sheep and one additional control sheep. The mean durations of gestation were 144·5 ± 1·1 d (n = 6), range 141–148 d, in the control group, and 141·3 ± 1·8 d (n = 6), range 137–141 d, in the infected group. In the control group, three of the ewes had twins and three had single lambs, and in the infected group, four of the ewes bore twins, and two bore single lambs. In the *C. psittaci*-infected group, two out of ten lambs were born dead: one of twin lambs was dead on delivery, and another, single lamb, died of asphyxia during delivery. In the control group, all nine lambs survived.

Ewes were implanted with indwelling catheters into the amniotic and allantoic cavities and into the utero-ovarian vein on day 115–117 of gestation (Mellor, 1980). Amniotic and allantoic sacs of each foetus were catheterized using Foley two-way balloon catheters (size 12 Ch, with 30–40 ml balloon; Eschmann, Sussex, UK). Small samples (0·5–2 ml) of amniotic and allantoic fluids were withdrawn using minimal suction at 24 h intervals from day 137 of gestation in control sheep and from day 133 of gestation in infected sheep. Sterility within each two-way tap was maintained by twice-daily flushing with thiomersal solution, consisting of thiomersal (BDH; 0·1% in ethanol)/acetone/ethanol (1:500:500, by vol.). Amniotic and allantoic fluids were placed immediately into 10 ml of 'analytical-reagent'-grade methanol, and stored at −40 °C.

A utero-ovarian vein was also catheterized using 1·4 mm external diameter vinyl tubing (Portex Ltd). A two-way luer stopcock was attached to each catheter. Vascular catheters were sampled daily and flushed with a heparin saline solution (80 IU preservative-free heparin ml^{-1}) twice daily. Blood was placed into tubes containing 2 IU preservative-free heparin ml^{-1}; plasma was prepared by centrifugation (see above), and stored at −40 °C.

Oestradiol 17β radio-immunoassay. Oestradiol 17β radio-immunoassay was carried out on samples taken from amniotic fluid, allantoic fluid, the utero-ovarian vein, and peripheral plasma, using a kit (Steranti Research; batch no. S703). The addition of up to 50 μl of fluids did not significantly change the binding curve of the anti-oestradiol serum, unless methanol was present. The aqueous-methanol amniotic and allantoic fluid samples were taken to dryness, and resuspended in 50 μl standard human serum containing <0·01 pg oestradiol ml^{-1}. The anti-oestradiol serum was raised in rabbits, and the secondary precipitating goat anti-rabbit IgG was coupled to a solid phase. The accuracy of the assays was controlled by using human serum with three known oestradiol concentrations, viz. 27·7 ± 3·68, 60·2 ± 6·60 and 176 ± 13·1 pg ml^{-1}. The sensitivity of the assay was 3·1 pg oestradiol ml^{-1} at 2·5 standard deviations from the mean, and the inter-assay and intra-assay coefficients of variation were 11·4% and 6·83%, respectively (n = 6).

Amniotic and allantoic fluid samples for PGE_2 assays. A fourth group of animals was used in these experiments. Twelve ewes were infected on day 90 of gestation by subcutaneous injection with 0·45 × 10^6 ELD_{50} of *C. psittaci* strain S26/3; 12 control ewes were injected with saline. Four ewes (two infected animals and two controls) were killed on each of the following days of gestation: 97, 103, 109, 115, 120 and 125. Amniotic fluid, allantoic fluid, and placental tissues were removed at necropsy. Amniotic and allantoic fluids were withdrawn, using a syringe and

gauge 16 needle, during aseptic delivery of foetuses 5 min (range 3–8 min) after killing of the ewes. Care was taken during sampling to withdraw fluid as far as possible from the site of the puncture, as PGE_2 may be released during rupture of foetal membranes, or from blood or endothelial cells (Leaver *et al.*, 1988). Blood-stained or meconium-stained samples were discarded (Leaver *et al.*, 1988). Three allantoic and two amniotic samples from the control group, and one allantoic and two amniotic samples from the infected group, were rejected on this basis. All samples (3–10 ml) were placed immediately in methanol (10 ml), and stored at −40 °C prior to PGE_2 radioimmunoassay. In both control and *C. psittaci*-infected ewes, five animals were carrying single lambs, and seven had twins. The local PGE_2 concentrations in amniotic and allantoic fluids of individual foetuses were analysed. The PGE_2 concentrations of individual foetal sacs were expressed as ng per ml of amniotic fluid or allantoic fluid on day 97–125 of gestation.

Prostaglandin E2 radio-immunoassay. Prostaglandin E_2 radio-immunoassay was carried out on samples of amniotic and allantoic fluids, either taken to dryness and resuspended in buffer, or extracted using ethyl acetate. There was no significant difference in the PGE_2 concentration in ethyl acetate-extracted and in methanol-treated samples, after correcting for the efficiency of extraction. Addition of amniotic or allantoic fluid to PGE_2 standards did not influence the binding curve of the anti-PGE_2 antiserum. Radio-immunoassay of PGE_2 was carried out using anti-PGE_2 antiserum (Institut Pasteur, Paris; batch no. D7), and [5,6,8,11,12,14,15(n)-^{3}H] PGE_2 radiotracer (Amersham; batch no. 60) under previously described conditions (Leaver *et al.*, 1987). Antiserum was precipitated using donkey anti-rabbit IgG donated by the Scottish Antibody Production Unit, Carluke, Lanarkshire, UK. The precision of PGE_2 determination was 12·6% for within-assay duplication, and 9·6% for between-assay replication ($n = 6$).

Pathology of placentae. Placentae were removed from 12 infected and 12 control ewes (fourth group of animals used for PGE_2 assays). Two infected and two control sheep were killed on each of the following days of gestation: 97, 103, 109, 115, 120, 125. Six cotyledons from each uterine horn bearing a foetus were examined in paraffin section, stained by haematoxylin and eosin. *C. psittaci* inclusions were identified using an immunoperoxidase method (Finlayson *et al.*, 1985).

Statistics. Results were expressed as mean ± standard error (SE) of the mean for n determinations. The normality of distribution of each group being tested was analysed using the standard score (z test), and the statistical significance of differences between paired and unpaired data was analysed using the paired and unpaired Student's t-tests, respectively (Moroney, 1951). The unpaired t-test incorporated the Behrens Fisher statistic and, therefore, did not assume that the variances of the population groups being compared were the same.

RESULTS

Plasma progesterone in sheep infected with C. psittaci

The mean peripheral plasma progesterone concentrations of six control sheep and of six sheep experimentally infected with *C. psittaci* on day 90 of gestation are shown in Fig. 1. Parturition

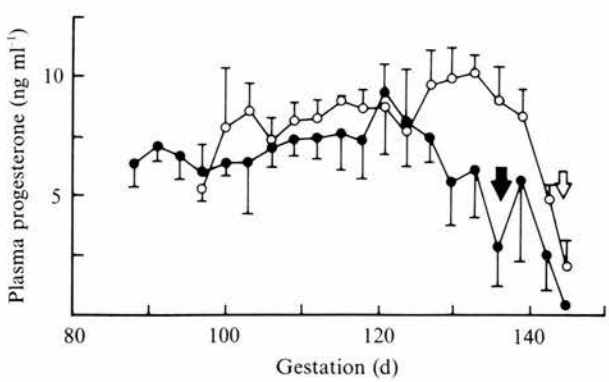

Fig. 1. Progesterone in peripheral plasma of control and *C. psittaci*-infected sheep. Ewes were infected by subcutaneous injection on day 90 of gestation with 5×10^5 ELD_{50} of *C. psittaci* strain S26/3. The filled arrow indicates the mean day of parturition in infected, and the open arrow, in control ewes (the mean duration of gestation in control sheep was 144·7 ± 1·08, $n = 6$, and in *C. psittaci*-infected sheep, 137 ± 1·6, $n = 6$). All animals had a single lamb. Progesterone concentrations are the means of three to six determinations of plasma progesterone from individual control (○) or infected (●) sheep on separate days of gestation.

Table 1. *Oestradiol 17β in peripheral plasma, utero-ovarian venous plasma and amniotic fluid of C. psittaci-infected and control sheep*

Sample	Sheep	Oestradiol 17β concentrations (mean ± SE pg ml^{-1}) on indicated days before parturition†		
		−2	−1	0
Peripheral plasma	Control (n = 5)	9·15 ± 2·60	21·7 ± 5·70*	63·1 ± 10·3
	Infected (n = 4)	16·6 ± 0·80	56·3 ± 7·05*	26·4 ± 15·9
Utero-ovarian venous plasma	Control (n = 6)	10·1 ± 0·58	18·3 ± 1·82	29·5 ± 7·36
	Infected (n = 6)	6·37 ± 0·17	12·4 ± 4·90	32·0 ± 5·31
Amniotic fluid	Control (n = 6)	5·86 ± 0·86*	5·67 ± 0·87*	9·31 ± 0·96
	Infected (n = 6)	17·6 ± 5·28*	12·9 ± 3·07*	24·3 ± 17·3

* Indicates significant differences between control and infected groups ($P < 0·02$), using the Student's unpaired t-test.

† The concentrations of oestradiol 17β in sheep infected subcutaneously with *C. psittaci* (strain S26/3, 5 × 10^5 ELD_{50}), and of control ewes injected with sterile saline.

was significantly ($P < 0·05$) earlier in the infected group. The profiles of progesterone release in the two populations showed the following differences. There was a greater variation in the concentration of plasma progesterone in infected animals. The mean plasma progesterone concentration in infected animals, compared on eight days between days 100 and 130 of gestation, was 80 ± 5·3% of the mean plasma progesterone concentration in control animals. The concentration of progesterone in the plasma of control sheep dropped sharply during the 8 d before parturition, while that of infected sheep showed a more gradual decline, commencing 13 d before parturition, on day 125 of gestation. The difference between the mean progesterone concentrations, paired between day 100 and day 144 of gestation and analysed using the paired Student's t-test, was highly significant ($P < 0·01$, no. of paired concentrations = 22). The difference between the curves was significant well before delivery: when plasma progesterone levels from individual infected sheep were paired randomly with progesterone concentrations in controls on the same day of gestation, and were compared between days 125 to 135 of gestation, using the paired t-test, a significant difference was observed ($P < 0·05$, no. of paired concentrations = 9). The progesterone concentrations in infected and control sheep were also analysed in relation to the day of parturition, using the paired t-test for samples collected between 0 and 8 d before parturition, and between 9 and 20 d before parturition. There was a significant ($P < 0·05$) difference in plasma progesterone concentrations between 16 paired samples from infected and control sheep in the earlier (day −9 to −20) period. However, there was no significant difference in the plasma progesterone concentration of 16 paired samples from infected and control sheep during the period of progesterone decline immediately before parturition (day −8 to 0).

Oestradiol 17β in C. psittaci-infected and control sheep

The oestradiol 17β concentrations in the peripheral plasma of the sheep whose progesterone secretion is shown in Fig. 1 were assayed at 48 h intervals. In the *C. psittaci*-infected group, a pre-partum increase in plasma oestradiol 17β was detected in the one sheep sampled at 24 h before parturition (24 pg ml^{-1}, compared with 9·2 pg ml^{-1} and 12·9 pg ml^{-1} in two control sheep). The five *C. psittaci*-infected sheep sampled on the day of parturition had a mean plasma oestradiol concentration of 7·2 ± 4·8 pg ml^{-1}, which was significantly ($P < 0·05$) lower than that of control sheep (45 ± 7·3 pg ml^{-1}, n = 4).

Peripheral plasma oestradiol 17β was analysed daily in a second group of ewes, consisting of four infected and five control sheep during the 2 d prior to parturition (Table 1). The duration of gestation in the infected group (139·8 ± 1·1 d, n = 4) was not significantly different from that of the control group (143·8 ± 1·1 d, n = 5). Abnormalities in the timing of oestradiol 17β release

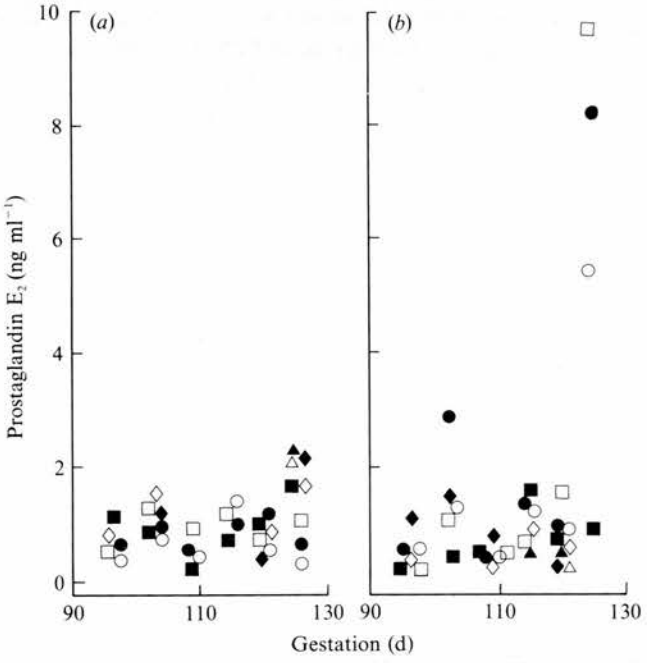

Fig. 2. Prostaglandin E_2 in amniotic and allantoic fluid in (*a*) control and (*b*) *C. psittaci*-infected sheep. Twelve ewes were infected by subcutaneous injection on day 90 of gestation with $4{\cdot}5 \times 10^5$ ELD_{50} of *C. psittaci* strain S26/3, and 12 controls were injected with sterile saline. Four ewes (two infected and two control) were killed on each of the following days of gestation: 97, 103, 109, 115, 120 and 125. Amniotic and allantoic fluids were withdrawn during aseptic delivery of foetuses, 5 min post mortem. In both the control and *C. psittaci*-infected groups of ewes, five animals were carrying single lambs, and seven had twins. Five and three samples, respectively, were rejected from the control and infected groups because they were stained with blood or meconium. The PGE_2 concentrations in amniotic fluid (open symbols) and allantoic fluid (filled symbols) of individual foetuses were analysed. Amniotic fluid and allantoic fluid from the same animal are shown as like pairs of open and filled symbols.

were observed in the sheep infected with *C. psittaci* compared with controls. The characteristic rise in plasma oestradiol 17β, which was observed on the day of parturition in control animals, was detected 24 h earlier in *C. psittaci*-infected sheep. In contrast with the control group, the mean peripheral plasma oestradiol 17β concentration in the *C. psittaci*-infected group 24 h before parturition was higher than the mean concentration of oestradiol 17β on the day of parturition.

As uterine prostaglandin release is controlled by oestradiol 17β (Liggins *et al.*, 1972; Olson *et al.*, 1984), the effect of chlamydial infection on the concentration of oestradiol 17β within the uterus of these sheep was analysed by cannulation of the utero-ovarian vein and amniotic sacs. The oestradiol 17β concentrations in the utero-ovarian plasma and amniotic fluid were determined (Table 1). The oestradiol 17β concentrations in utero-ovarian venous plasma of control sheep were similar to previously reported values (Bedford *et al.*, 1972). There was no significant difference in the oestradiol 17β concentrations released into the utero-ovarian vein by infected, compared with control uteri. However, significantly higher local intrauterine concentrations of oestradiol 17β were observed in the amniotic fluid of *C. psittaci*-infected animals compared to controls (Table 1), indicating that chlamydial infection may have compromised local intrauterine diffusion barriers.

Intrauterine prostaglandin E_2 in C. psittaci-infected and control sheep

The concentrations of prostaglandin E_2 in the amniotic fluid and allantoic fluid of 24 ewes, killed between day 97 and day 125 of gestation, were determined, in order to investigate whether

premature release of prostaglandins occurred during late gestation (see Fig. 2). The concentrations of prostaglandin E_2 in amniotic and allantoic fluids were low (0·89 ± 0·12 ng ml^{-1}, $n = 12$), between days 97 and 125 in the 12 control sheep, but were significantly raised in four out of 12 samples obtained from *C. psittaci*-infected sheep over the same period (6·46 ± 1·49 ng ml^{-1}, $P<0·05$). The PGE_2 concentrations in the amniotic and allantoic fluids of the same animals showed a highly significant correlation ($P<0·01$) in the infected group ($n = 12$), but not in the control sheep ($n = 12$). The distribution of intrauterine PGE_2 concentrations was wider in infected sheep than the distribution of PGE_2 in the corresponding control population (Fig. 2).

Placental pathology of chlamydial infection

The histopathology of placentae during chlamydial infection was investigated, in order to study the histological distribution of *C. psittaci* inclusions, the associated necrosis and the extent of leucocyte infiltration into the placenta of the 24 sheep used for PGE_2 determinations (see above). Placental lesions consistent with those seen in cases of ovine chlamydial abortion (Stamp *et al.*, 1950; Studdert, 1968; Novilla & Jensen, 1970) were observed in the cotyledons of all animals in the infected group killed on days 125 and 120 of gestation and in one of the two infected animals examined on day 115 of gestation. The placental cotyledons of these sheep showed foci of infection, which consisted of *C. psittaci* inclusions in the cytoplasm of trophoblast cells, identified by immunoperoxidase staining, associated with cellular necrosis and phagocyte infiltration. The severity of the lesions increased during the period (days 115–125 of gestation) when infection was detected. No lesions were detected in the placentae from infected ewes killed between days 97 and 110 of gestation or in any of the placentae of control sheep examined on days 97–125 of gestation.

DISCUSSION

A characteristic feature of infection with both *C. psittaci* and *C. trachomatis* during pregnancy is a period of latency, followed by the onset of infection of the foetal membranes and placenta during the last quarter of gestation (Stamp *et al.*, 1950; Studdert, 1968; Novilla & Jensen, 1970; Johnson *et al.*, 1985; Aitken, 1986; Sweet *et al.*, 1987). The severity of infection and the perinatal mortality associated with *C. psittaci* is greater than for *C. trachomatis* in both women and sheep (Sweet *et al.*, 1987; Studdert, 1968). However, both chlamydial species are associated with the initiation of premature labour.

The chorionic membrane of the placenta is the primary focus of intrauterine *C. psittaci* infection, which then spreads to the caruncular regions of the placenta (McEwen *et al.*, 1951; Studdert, 1968). We observed a disruption of placental steroidogenesis, and a stimulation of foetal membrane prostaglandin synthesis during the early stages of chlamydial infiltration into the uterus.

In addition to evidence of changes in steroid hormone and prostaglandin synthesis by *C. psittaci*-infected tissues, we detected changes in the intrauterine distribution of oestradiol 17β and PGE_2. Similar concentrations of oestradiol 17β and PGE_2 were detected in the amniotic and allantoic fluid of infected, but not of control sheep, and this may reflect changes in foetal membrane integrity during chlamydial infection.

The pathogenesis which we describe may be relevant to other mammalian species, because the placenta and foetal membranes are major sources of steroid hormones and prostaglandins in most species, including humans, during late pregnancy. There are considerable inter-species differences in the pattern of hormone secretion, and in the relative importance of the stimuli which initiate labour (Allen, 1975). However, in all mammalian species, pregnancy is dependent on maintained progesterone secretion, and oestradiol and prostaglandins stimulate uterine responses at the time of parturition (Thorburn & Challis, 1979; Bedford *et al.*, 1972). Premature progesterone withdrawal can initiate labour and increase oestradiol synthesis in the sheep (Mitchell *et al.*, 1983). The abortifacient agent *Actinobacillus seminis* has been reported to cause a decline in plasma progesterone in pregnant ewes (Smith & Hughes, 1974), although this occurred earlier in pregnancy than the inhibition which we observed in *Chlamydia*-infected

sheep (Fig. 1). Premature increases in oestradiol 17β and prostaglandin $F_{2\alpha}$ synthesis have been observed during infectious abortion due to intrauterine surgery (Bedford *et al.*, 1972; Silver *et al.*, 1986) or endotoxin infusion (Roberts *et al.*, 1975) respectively. There has been a recent report of decreased plasma progesterone and oestradiol 17β concentrations and elevated peripheral $PGF_{2\alpha}$ metabolite concentrations in four sheep infected with *C. psittaci* (Fredriksson *et al.*, 1988). There has only been one report on PGE_2, which is thought to play a role in the initiation of labour (Bleasdale & Johnston, 1984) in infectious abortion (Romero *et al.*, 1988). Our report is the first description of the effect of *C. psittaci* infection on the intrauterine distribution of oestradiol 17β and prostaglandin E_2 during pregnancy.

The effect of the hormonal environment on chlamydial infectivity during pregnancy has recently been investigated *in vivo* and *in vitro*. It was found that oestradiol 17β enhanced the growth of *C. trachomatis* in a guinea-pig model (Rank *et al.*, 1982), and in cultured human lymphoma cells (Bose & Goswami, 1986; Sugarman & Agbor, 1986). The early increases in plasma oestradiol 17β concentrations, and the high intrauterine concentrations of this steroid which we detected, may induce the metabolic and vascular changes which enhance chlamydial growth at a specific stage of late gestation.

In conclusion, experimental infection of ewes with *C. psittaci* was associated with changes in placental steroid and prostaglandin synthesis. It is likely that the premature decline in progesterone, and the premature rise in oestradiol 17β and prostaglandin E_2 concentrations which we report, contribute to the initiation of premature labour in *C. psittaci*-infected sheep.

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PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS

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The Effect of Chlamydial Infection on the Initiation of Premature Labour: Serial Measurements of Intrauterine Prostaglandin E_2 in Amniotic Fluid, Allantoic Fluid and Utero-Ovarian Vein, Using Catheterised Sheep Experimentally Infected with an Ovine Abortion Strain of *Chlamydia Psittaci*

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Summary — The initiation of premature labour by an ovine abortion strain of *Chlamydia psittaci* was studied in relation to Prostaglandin E_2 (PGE_2), which plays a major role in parturition. The local intrauterine concentration of PGE_2 was monitored after experimental infection with *C. psittaci*, during late gestation, using a catheterised sheep model. Indwelling catheters were implanted into the amniotic and allantoic cavities of six control sheep, and into six sheep infected with an ovine abortion strain of *C. psittaci*. The release of PGE_2 into the utero-ovarian vein of these sheep was also monitored. Infection with *C. psittaci* was associated with a premature rise in PGE_2 in the amniotic fluid between days 122 and 135 of gestation ($P<0.01$). A premature increase in PGE_2 was defected between days 127 and 136 of gestation ($P<0.05$) in the allantoic fluid of sheep infected with *C. psittaci*, but not in the control uninfected sheep. Chlamydial infection significantly decreased the secretion of PGE_2 into the utero-ovarian vein: The mean concentration of PGE_2 in the utero-ovarian vein of infected sheep was 68.8 ± 5.2% of the PGE_2 concentration of control sheep between days 122 to 141 of gestation ($P<0.01$). The release of PGE_2 into the utero-ovarian vein increased between days 126 to 136 of gestation in infected and control sheep ($P<0.01$). The results from the catheterised sheep model indicate that *C. psittaci* infection is associated with a local intrauterine release of PGE_2. The magnitude of this release was similar to the PGE_2 release in control sheep prior to parturition. The PGE_2 released during chlamydial infection may be active in initiating premature labour.

Abbreviations: ELD_{50}, egg LD_{50}; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

Introduction

There is increasing evidence of an involvement of the *Chlamydiae* in premature labour. In humans, placental *C. trachomatis* infection is associated with prematurity and low birthweight (1), while *C. psittaci* has been reported to cause infectious abortion and premature labour in women and sheep (2, 3, 4). The severity of infection and the perinatal mortality associated with the *C. psittaci* species is greater in both women and sheep (1, 5, 6). However, both chlamydial species are associated with the initiation of premature labour.

The primary focus of *C. psittaci* infection of the placenta, the chorionic foetal membrane, is also a major site of prostaglandin synthesis at parturition, and stimulation of the fetal membranes releases prostaglandins and initiates labour (7, 8, 9, 10). The prostaglandins E_2 and $F_{2\alpha}$ which are released within the uterus play a major role in mammalian parturition (11, 12), stimulating uterine contraction, controlling the tone and responsiveness of uterine musculature (13), and increasing the compliance of the uterine cervix before labour (14).

The synthesis of PGE_2 increases during late gestation in both sheep and women (7, 15, 16). The inhibition of prostaglandin synthesis delays premature delivery in sheep (8), and infusion of PGE_2 and $PGF_{2\alpha}$ into the aorta of pregnant sheep stimulates uterine contractions similar to those detected at term (12). An increase in uterine PGE_2 concentration in infectious abortion associated with bacterial endotoxin has been reported in women (17, 18), and there has been a recent report of elevated $PGF_{2\alpha}$ metabolite concentrations in the plasma of four sheep infected with *C. psittaci* (19).

The predominant prostaglandin produced by sheep and human chorion is PGE_2 (7, 20, 15, 21). In contrast to $PGF_{2\alpha}$, the concentration of PGE_2 increases during late gestation and early labour, and there is evidence that PGE_2 has a role in the initiation of labour, while $PGF_{2\alpha}$ is involved in the progression and the coordination of labour after it has been initiated (9).

The timing of *C. psittaci* infection has led to investigation of endocrine factors which might play a role during this period of gestation. The infection of the placenta by the Chlamydiae does not begin until a relatively advanced stage of gestation. In the case of *C. psittaci* infection of sheep, placental infiltration is detected between days 100 to 120 of gestation, commencing in the chorioallantoic membrane, and proceeding to infiltrate the caruncular region of the placenta (6, 22). It has been reported that Oestradiol 17B acts as a "growth factor" for the *Chlamydiae* in several species (23, 24, 25), and we recently demonstrated that *C. psittaci* infection of sheep was associated with a premature decline in circulating Progesterone and premature release of Oestradiol 17B (26). We also detected abnormally high PGE_2 concentrations in the amniotic fluid of some chlamydia infected sheep.

The PGE_2 synthesised by the fetal membranes is converted to $PGF_{2\alpha}$, and both PGE_2 and $PGF_{2\alpha}$ are metabolised, at a variable rate, to a range of inactive oxygenated metabolites. The variability of the activities of the enzymes of PG metabolism, together with the contribution of blood and vascular cells to the circulating PG pool, combine to make the measurement of peripheral PG an indirect indicator of uterine PG synthesis (27). In contrast, the intrauterine concentrations of PGE_2 indicate the local concentrations of biologically active PGE_2. Therefore, we used a catheterised sheep model to investigate the pattern of PGE_2 release within the uterus during *C. psittaci* infection.

Methods

Twelve Scottish Blackface ewes were implanted with indwelling catheters into the amniotic and allantoic cavities and into the utero-ovarian vein on day 113 of gestation (28, 29). Amniotic and allantoic sacs of each foetus were catheterised using Folex two-way balloon catheters (size 12 Ch, with 30–40 ml balloon; Eschmann, Sussex, England). A utero-ovarian vein was also catheterised using 1.4 mm external diameter vinyl tubing (Portex Ltd, Kent, England). A two-way luer stopcock was attached to each catheter. Vascular catheters were sampled daily and flushed with a heparin saline solution (80 I.U. ml^{-1} preservative-free heparin, Evans Medical, Dunstable, Beds) twice daily. Blood was placed into tubes containing 2 I.U. ml^{-1} preservative-free heparin, and plasma was prepared by centrifugation and stored at −40°C. Small samples (0.5–2 ml) of amniotic and allantoic fluids were withdrawn using minimal suction. Sterility within each 2-way tap was maintained by twice daily flushing with thiomersal solution, consisting of thiomersal 0.1% alcohol (British Drug Houses, Poole, Dorset):

acetone:ethanol = 1:500:500, v/v/v. On day 115 of gestation, six ewes were infected by subcutaneous injection of 4.5×10^5 ELD_{50} of an ovine abortion strain of *C. psittaci*, and six controls injected with sterile saline. Single samples of amniotic fluid, allantoic fluid or utero-ovarian vein were taken from each sheep on alternate days up to day 130 of gestation, and daily from day 130 until parturition. Occasionally, it was not possible to withdraw samples on the required day, particularly from the allantoic cavity, due to catheter blockage. Amniotic fluid and allantoic fluid was placed immediately into 10 ml of "analytical reagent" grade methanol, and stored at −40°C. Samples which were stained with blood or meconium (30) were discarded. Utero ovarian plasma (0.2 ml) was immediately extracted using 2 ml of redistilled diethylether, vortex mixed, then immersed in acetone/dry ice until the aqueous layer was frozen. The organic layer was decanted, and the extraction was repeated three times. The extraction of PGE_2 was 94–99% using this technique. Samples were stored at −40°C before radioimmunoassay. The mean duration of gestation was 144.5 ± 1.1 days in the control group, and 141.3 ± 1.8 days in the infected group, and the difference between these gestation periods was not significant. In the control group, three of the ewes had twins and three, single lambs, and in the infected group, four of the ewes bore twins, and two, single lambs. In the chlamydia infected group, two out of ten lambs were born dead: one of twin lambs was dead on delivery, and another, single lamb, died of asphyxia during delivery. In the control group, all nine lambs survived.

Prostaglandin E_2 radioimmunoassay

Radioimmunoassay of PGE_2 was carried out using antiserum from the Institut Pasteur, Paris (Batch nos D2, D7, D12 and D13), standard PGE_2 (Upjohn, Crawley, Sussex), and ^3H PGE_2 radiotracer [5,6,8,11,12,14,15 (n) − ^3H], batch no. NET 428 (Amersham, Buckinghamshire), under previously described conditions (10). Samples were taken to dryness, and resuspended in 20 mM HEPES buffer pH 7.4, containing 0.01% azide, and 1% gelatine. The donkey anti-rabbit immunoglobulin serum used to precipitate the hapten- IgG immune complex, and the normal rabbit serum used in the assay were donated by the Scottish Antibody Production Unit, Carluke, Lanarkshire. The sensitivity of the assay was 0.28 pg ml^{-1} of plasma, and 0.401 pg ml^{-1} of amniotic or allantoic fluid. The precision profile of the assay was monitored as described by Hunter (31), using the mean coefficient of variation of two amniotic fluid samples, five utero-ovarian venous plasma samples, and seven allantoic fluid samples, analysed from triplicate determinations within the eight radioimmunoassays carried out (intra-assay coefficient of variation); and seven allantoic fluid samples, analysed five times within two different assays (inter-assay coefficient of variation). Also, two standard PGE_2 samples (Upjohn, 10–40 pg per tube) were included at the beginning and end of each assay in addition to the standard curve. The inter-and intra-assay coefficients of variation of the three types of sample controls were not significantly different. The mean inter-assay coefficient of variation of sample controls was 13.9% ± 1.98 (n=7), and the mean intra-assay coefficient of variation was 10.8% ± 1.04 (n=15).

Statistical analysis of data

Results described in the text are expressed as the mean PGE_2 concentration ± standard error of the mean for n samples of amniotic fluid, allantoic fluid, or utero-ovarian venous plasma. Results described in the Figures illustrate the mean PGE_2 concentration in fluid/plasma of each individual sheep, sampled on a specific day of gestation, and sequential results from individual sheep are joined. The difference between the means was tested using parametric statistics after analysing the normality of distribution of the data, using the standard score (z score) of each population tested (32). Of the 38 population pairs analysed, only one population (utero-ovarian venous plasma PGE_2 in infected and control sheep between day 136 of gestation and labour) showed a distribution which was significantly different from the normal distribution ($Z=1.862$, $P<0.05$), and, therefore, parametric statistics were not used to analyse this sample group. In the remaining normally distributed data, the variances of data groups being compared were also analysed, using Snedecors' Variance Ratio test. In 31 of these 38 data groups, the variances of the populations being compared were not significantly ($P<0.05$) different from each other, and the *t* test was used to test the Null Hypothesis for two sets of populations with normal distributions. Seven of the 38 data groups analysed showed significantly different variances, and the unpaired

Students' t test for two sets of normal populations with different variances, incorporating the Behrens-Fischer statistic, was used to analyse this data. The sample groups which showed significantly different variances are identified in the results section. Paired data was analysed using the paired Students' t test, after testing the data for normality of distribution. When more than one sample was available on the same day of gestation (in allantoic fluid samples, comparing infected and control sheep, see results), data was paired randomly, i.e. samples were taken for statistical analysis in the order in which the sampling from individual sheep was originally carried out.

Results

Prostaglandin E_2 in amniotic fluid during chlamydia infection

The concentration of PGE_2 in the amniotic fluid of six catheterised control sheep is shown in Figure 1A. Amniotic fluid PGE_2 increased during late gestation in the control group. The concentration of PGE_2 between 122 to 135 days of gestation in control animals (2.72 ± 0.27 ng/ml, n=29 samples) increased significantly from day 136 of gestation to the day of parturition (7.23 ± 0.72 ng/ml, n=41 samples), in samples taken from the same animals, compared using the unpaired Students' test for sample groups with different variances ($P<0.01$, n=67).

In the six sheep infected with *C. psittaci*, the concentration of PGE_2 in the amniotic fluid increased earlier in gestation (Fig. 1B). A significant increase in PGE_2 concentration was observed after day 122 of gestation in the chlamydia-infected group, when samples from the same sheep were compared using the unpaired

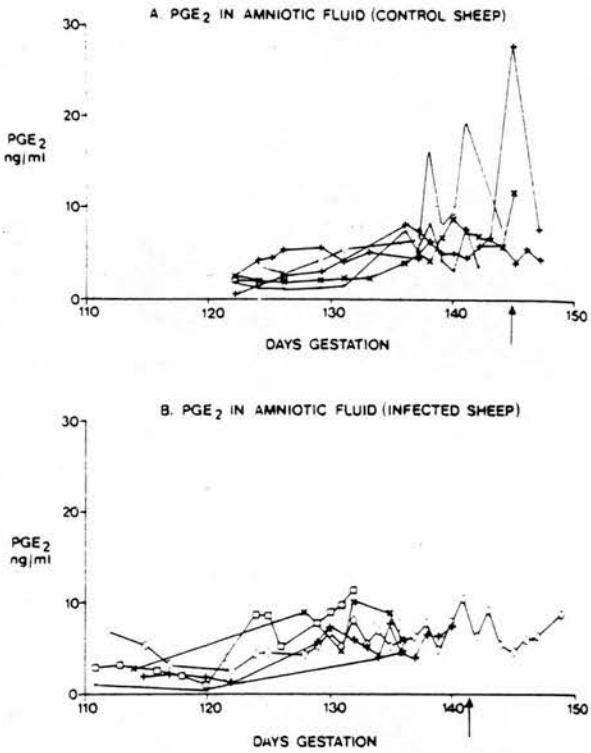

Fig 1 Prostaglandin E_2 in Amniotic Fluid of Control and Chlamydia Infected Sheep. Amniotic fluid was withdrawn by catheter from A. control or B. Sheep infected with an ovine abortion strain of *C. psittaci* (4.5×10^5 ELD_{50}) on day 115 of parturition. The arrow indicates the mean date of parturition. The PGE_2 concentrations are the mean of triplicate determinations carried out on amniotic fluid of each individual sheep. Serial results from each individual sheep are joined.

Students' t test ($P<0.001$, n=43). The mean concentration of PGE_2 was 2.55 ± 0.45 ng ml^{-1} (n=14) on days 111 to 121 of gestation, compared with 6.42 ± 0.44 ng ml^{-1} (n=21) in

Table 1 Mean PGE_2 concentration in amniotic fluid of six control and six chlamydia infected sheep during late gestation. The mean PGE_2 concentrations in sheep whose individual PGE_2 profiles were shown in Figure 1, in ng/ml of amniotic fluid ± SEM for n samples of amniotic fluid, withdrawn from indwelling catheters in control sheep or sheep infected with 4.5×10^5 ELD_{50} and ovine abortion *C. psittaci* strain on the stated days of gestation.

Gestation (days)	*111–121*	*122–135*	*136-parturition*
Control	—	2.72 ± 0.27(29)*	7.23 ± 0.72(41)
Infected	2.55 ± 0.45(14)	6.42 ± 0.44(21)*	6.48 ± 0.42(21)

*The concentration of PGE_2 in the amniotic fluid of chlamydia infected sheep, between days 122 to 135 of gestation, was significantly greater than that of control sheep, using the unpaired Students' t test for sample groups with different variances ($P<0.01$, n=58).

the same sheep on days 122 to 135 of gestation. There was no significant increase in the concentration of PGE_2 in the amniotic fluid between days 122 to 135 of gestation, compared with day 136 of gestation onwards, in the chlamydia infected group.

Prostaglandin E_2 in utero-ovarian vein during chlamydia infection

The concentration of PGE_2 in the utero-ovarian vein of six control sheep showed a pulsatile release of PGE_2, increasing in amplitude from day 126 of gestation (Fig. 2A). The mean concentration of PGE_2 in utero-ovarian plasma of control sheep between days 122 to 125 of gestation was 1.34 ± 0.205 ng ml^{-1} (n=12), and this increased to a concentration of 2.38 ± 0.241 ng ml^{-1} (n=28) in the same sheep between days 126 to 136 of gestation. This increase in PGE_2 concentrations was significant using the unpaired t test ($P<0.01$, n=36). The PGE_2 concentrations in the utero-ovarian venous plasma showed a similar pattern of release in chlamydia infected sheep (Fig. 2B). The greatest increase in PGE_2 was observed after day 122 of gestation. The concentration of PGE_2 in utero-ovarian plasma of infected sheep was 0.922 ± .123 ng ml^{-1} (n=28) between days 110 to 121 of gestation, increasing to 1.52 ± 0.153 ng ml^{-1} (n=53) in the same ewes between days 122 to 135 of gestation. This increase in PGE_2 was significant using the unpaired *t* test for sample groups with different variances ($P<0.01$, n=81). The increase in PGE_2 concentrations in the utero-ovarian vein of infected sheep (from 1.08 ± 0.149 ng ml^{-1} on days 122 to 125, n=14; to 1.55 ± 0.140 ng ml^{-1} on days 126 to 136, n=43), was not significant. There was a further increase in PGE_2 concentrations in utero-ovarian vein of chlamydia infected sheep late in gestation. The concentration of PGE_2 from day 137 of gestation

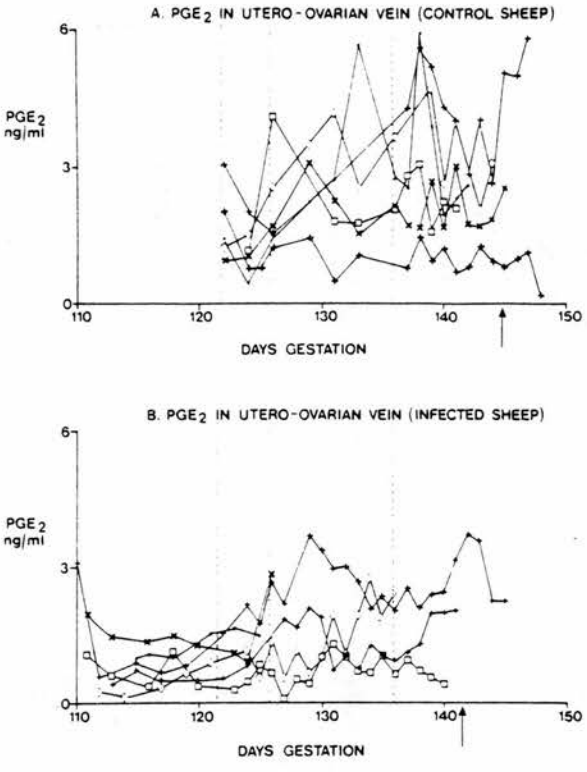

Fig 2 Prostaglandin E_2 in Utero-Ovarian Vein of Control and Chlamydia Infected Sheep. Utero-ovarian plasma was withdrawn by catheter from A. Control Sheep or B. Sheep infected with an ovine abortion strain of *C. psittaci* (4.5×10^5 ELD_{50}) on day 115 of parturition. The arrow indicates the mean date of parturition. The PGE_2 concentrations are the mean of triplicate determinations, carried out on utero-ovarian plasma of six individual sheep. Serial results from individual sheep are joined.

in the chlamydia-infected sheep increased to 1.96 ± 0.228 ng ml^{-1} (n=31) of utero-ovarian venous plasma. This was significantly greater than the PGE_2 concentration detected in the same ewes between days 122 to 125 of gestation, using the

Table 2 Mean PGE_2 concentration in utero-ovarian venous plasma of six chlamydia infected and six control sheep during late gestation. The mean PGE_2 concentration in sheep whose individual PGE_2 profiles were shown in Figure 2, in ng ml^{-1} of utero-ovarian venous plasma ± SEM for n samples of utero-ovarian venous blood, withdrawn from indwelling catheters in control sheep or sheep infected with 4.5×10^5 ELD_{50} of an ovine abortion strain of *C. psittaci* on the stated period of gestation.

Gestation (days)	*111–121*	*122–125*	*126–136*	*137- parturition*
Control	-	1.34 ± 0.205(12)	2.38 ± 0.241(28)	2.51 ± 0.19(53)
Infected	0.922 ± 0.123(28)	1.08 ± 0.149(14)	1.55 ± 0.140(43)	1.96 ± 0.23(31)

unpaired t test for sample groups with different variances ($P<0.01$, $n=32$).

The increase in utero-ovarian venous PGE_2 concentrations in infected sheep occurred more gradually, over a longer time span, compared with the increase in utero-ovarian venous plasma (see Table 2). In the utero-ovarian vein, the concentration of PGE_2 increased sharply after day 126 of gestation. The mean PGE_2 concentration in the utero-ovarian venous plasma of control sheep increased by 78%, if samples collected in the period between days 126 to 136 of gestation were compared with samples collected between days 122 to 125. In contrast, the increase in mean PGE_2 over the same period in the infected group (44%) was proportionately lower than the increase in the control group.

The mean concentrations of PGE_2 in the utero-ovarian vein of infected sheep were compared with the mean PGE_2 concentrations detected in control sheep on the same day of gestation throughout the period when infected and control sampling overlapped (between days 122 to 141 of gestation). Over this period, the mean PGE_2 concentration in the utero-ovarian vein of infected sheep was 68.8 ± 5.2% the mean PGE_2 concentration of control sheep (range 52.6% to 96.8% for 12 data pairs). The mean PGE_2 concentrations in the utero-ovarian venous plasma of infected sheep were significantly lower than the mean PGE_2 concentrations detected in control sheep, using the paired t test for samples with different variances ($P<0.01$, $n=12$). This suggested that the transfer of PGE_2 into the utero-ovarian vein was compromised by chlamydial infection.

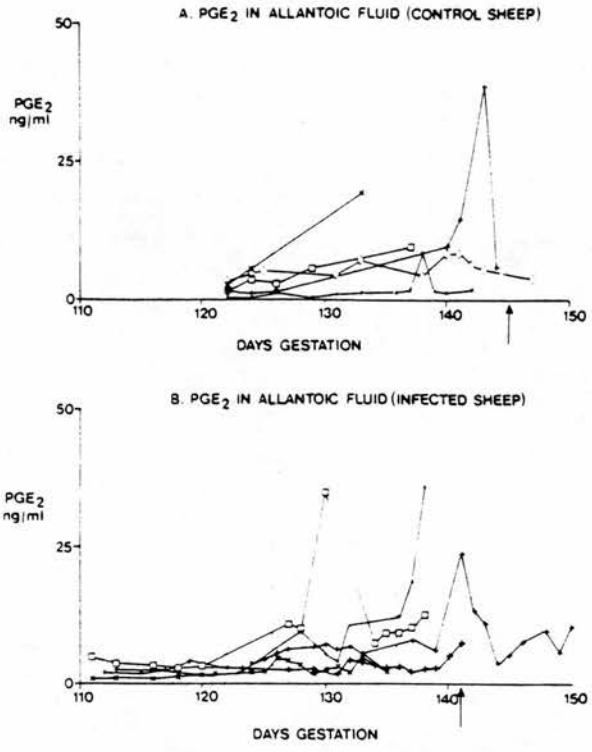

Fig 3 Prostaglandin E_2 in Allantoic Fluid of Control and Chlamydia Infected Sheep. Allantoic fluid was withdrawn by catheter from A. Control Sheep or B. Sheep infected with an ovine abortion strain of *C. psittaci* (4.5×10^5 ELD_{50}) on day 115 of parturition. The PGE_2 concentrations are the mean of triplicate determinations, carried out on allantoic fluid of individual sheep. Serial results from individual sheep are joined. The mean day of parturition is indicated by an arrow.

Prostaglandin E_2 in allantoic fluid during chlamydial infection

The sampling of allantoic fluid was infrequent after day 130 of gestation, due to catheter blockage caused by particulate matter, and to the increase in allantoic fluid viscosity which occurs during this period. This problem was most acute during the five days before parturition, when the allantoic fluid was decreasing in volume (29). The concentration of PGE_2 in the

Table 3 Mean PGE_2 concentration in allantoic fluid of six chlamydia-infected and six control sheep during late gestation. The mean PGE_2 concentration in sheep whose individual PGE_2 profiles were shown in Figure 3, in ng ml^{-1} of utero-ovarian venous plasma ± SEM for n samples of utero-ovarian venous blood, withdrawn from indwelling catheters in control sheep or in sheep infected with 4.5×10^5 ELD_{50} of an ovine abortion strain of *C. psittaci* during the stated period of gestation.

Gestation (days)	*111–121*	*122–126*	*127–135*	*136-parturition*
Control	—	2.56 ± 0.46(7)	2.68 ± 2.52(7)	8.13 ± 2.15(17)
Infected	2.08 ± 0.22(21)	3.00 ± 0.38(11)	6.04 ± 1.11(30)	9.75 ± 1.61(23)

allantoic fluid of the six control sheep increased during late gestation (Fig. 3A). The concentration of PGE_2 in allantoic fluid between days 122 to 135 of gestation was 3.66 ± 0.922 ng ml^{-1} (n=21), increasing to 8.13 ± 2.15 ng ml^{-1} (n=17) in the same sheep between day 136 and the day of parturition. This increase was significant, when analysed using the unpaired *t* test ($P<0.05$, n=38). The concentration of PGE_2 in the allantoic fluid of six sheep infected with *C. psittaci* is shown in Figure 3B. The increase in allantoic fluid PGE_2 occurred earlier in infected sheep than in controls. The concentration of PGE_2 in the allantoic fluid of infected sheep between days 111 to 126 of gestation was 2.33 ± 0.196 ng ml^{-1} (n=32), increasing to 6.04 ± 1.12 ng ml^{-1} (n=30) in the same sheep between days 127 to 135 of gestation. This increase was significant, $P<0.01$ (n=62), using the unpaired *t* test. The concentration of PGE_2 in the allantoic fluid of the same sheep, taken between days 136 of gestation and the day of parturition, was 9.75 ± 1.61 ng ml^{-1} (n=23). This was not significantly higher than the PGE_2 concentration between days 127 to 135 of gestation in the same sheep. The concentration of PGE_2 in the allantoic fluid of the infected sheep between days 127 to 135 of gestation was 6.04 ± 1.11 ng ml^{-1} (n=30), and in the control sheep, 2.68 ± 2.52 ng ml^{-1} (n=7). The difference between these groups was not significant, due to the increase in PGE_2 concentrations, during this period. However, when the PGE_2 concentrations in the allantoic fluid of individual infected and control sheep, taken on the same day of gestation, between days 127 to 135 were paired and analysed using the paired *t* test, the PGE_2 concentrations in infected sheep were significantly higher than the PGE_2 concentrations in the allantoic fluid of control sheep taken on the same day of gestation ($P<0.02$).

Discussion

In this paper we report premature increases in PGE_2 concentrations in two intrauterine compartments monitored during late gestation in sheep infected with an ovine abortion strain of *C. psittaci*. Increases in amniotic fluid PGE_2 associated with *C. psittaci* infection, were observed from day 122 of gestation, and this was thirteen days before any changes in PGE_2 in amniotic fluid of control sheep (Fig. 1). In the allantoic fluid, the concentration of PGE_2 increased from day 127 of gestation, nine days before the increase observed in controls (Fig. 3). It should be noted that the chlamydial infection established in our catheterised sheep was relatively mild. The mean period of gestation of the infected group (141.3 ± 1.8 d) was not significantly different from the control group (144.5 ± 1.1 d), and 80% of the lambs from infected sheep survived.

The difference between PGE_2 profiles in infected and control groups was greatest in the amniotic fluid. This was the fluid space in closest contact with the focus of *C. psittaci* infection (29) which spreads from the chorionic membrane to caruncular tissue (6). The amniochorion is also the primary site of infection of both *C. psittaci* and *C. trachomatis* in human (2, 5). Our studies indicated that the localised infection of *C. psittaci* was associated with the release of PGE_2 from the amniochorion.

A comparison of the PGE_2 profiles in the utero-ovarian venous plasma in infected and control sheep suggested that *C. psittaci* infection disrupted the local caruncular exchange of PGE_2. The concentration of PGE_2 detected in the utero-ovarian vein of infected sheep was 69% that of control animals. It is possible that the impaired exchange of metabolites at this site, which is responsible for approximately 83% of uterine blood flow (33), may be a cause of the low birth weight associated with chlamydial infection (1, 6). It is also possible that impaired vascular function masked the increase in PGE_2 release in infected sheep indicated by the amniotic and allantoic fluid PGE_2 profiles. The increase in utero-ovarian venous PGE_2 concentrations detected in infected sheep occurred more gradually, over a longer time span than the increase in utero-ovarian venous plasma. Evidence of increased prostaglandin release into the peripheral circulation was recently reported by Fredriksson *et al.*, (1988), who detected a premature increase in peripheral PGF metabolite concentrations in sheep infected with *C. psittaci*.

In the amniotic and allantoic fluid, the concentrations of PGE_2 detected during the period of premature PGE_2 release in sheep infected with *C. psittaci*, reached concentrations which were not significantly different from the concentrations of PGE_2 released by the uteri of control sheep prior to parturition. This indicated that the PGE_2 released during chlamydial infection may be active in initating events associated with premature labour. It is possible that the inflam-

matory leucocytes infiltrating the uterus at the sites of *C. psittaci* infection (34, 6, 2, 22, 26) may contribute to the PGE_2 pool in intrauterine fluids of infected sheep. However, when the magnitude of the premature increases in PGE_2 which we detected in intrauterine fluids of infected sheep (between three and four ng ml^{-1} of amniotic or allantoic fluid) were compared with the concentrations of PGE_2 synthesed by inflammatory leucocytes (35, 36), the premature release of PGE_2 in amniotic and allantoic fluids was found to be an order of magnitude greater than the amount of PGE_2 sythesised by leucocytes. This suggested that most of the PGE_2 detected in the fluids of infected sheep was of uterine, rather than of leucocyte, origin.

The uterine synthesis of prostaglandins is stimulated by oestrogens (12), and there have been three reports of abnormal release of estrogens during *C. psittaci* infection, showing impaired oestrogen production (37, 19) and premature oestradiol release (26) respectively. In addition to controlling the release of PGE_2, there is evidence that oestradiol 17β may facilitate the infectivity of C. trachomatis *in vivo* and *in vitro* (23, 24, 25). The premature release of oestradiol 17β from the uterus, and the elevated intrauterine concentrations associated with *C. psittaci* infection (26), may induce metabolic and vascular changes enhancing chlamydial growth during late gestation, and it is likely that these effects are at least partly mediated by PGE_2.

A characteristic of infection with both *C. psittaci* and *C. trachomatis* during pregnancy is a period of latency, followed by the onset of placental infection of the fetal membranes and placenta during the last quarter of gestation (34, 6, 4, 3, 22, 1). In the sheep model of *C. psittaci* infection which we used, we detected histochemical evidence of *C. psittaci* infiltration between days 110 and 120 of gestation (22, 26). Therefore, the histochemical evidence of *C. psittaci* infection either slightly preceded, or occurred simultaneously with, the evidence of increased PGE_2 release which we detected from day 122 of gestation. This indicated that the intrauterine release of PGE_2 was sensitive to early fetal membrane infection with *C. psittaci*.

In conclusion, mild infection with *C. psittaci* was associated with abnormalities in the intrauterine concentrations of PGE_2 in amniotic fluid, utero-ovarian vein, and allantoic fluid. In the two intrauterine sacs, we detected the premature secretion of PGE_2, which reached the concentrations of PGE_2 which were found just prior to parturition in the control sheep. In the utero-ovarian vein, there was evidence of impaired secretion of PGE_2. These results suggest that chlamydial infection causes the release of PGE_2 from intrauterine tissues. The resulting localised increase in PGE_2 may stimulate the induction of premature labour in chlamydia-infected sheep.

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15

Effects of n-3 Fatty Acids and Fish Oil on Fatty Acid Turnover in the Uterus in Relation to the Signal for Parturition

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INTRODUCTION

Premature labour is a major cause of perinatal mortality. Little is known about the events which initiate and control parturition, although in most species a balance of estrogen and progesterone plays a role in the control of prostaglandin production by the uterus. Large amounts of arachidonic acid and prostaglandins are released during parturition (MacDonald et al, 1974; Ogburn et al, 1980; Olund & Lunel 1979; Leaver et al, 1987), and the importance of prostaglandin biosynthesis at parturition is demonstrated by the effect of prostaglandin infusions and prostaglandin synthetase inhi-

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bitors. In almost every species, inhibitors of prostaglandin synthesis block parturition, and infusions of prostaglandin $F_{2\alpha}$ or E_2 initiate it (Aitken, 1972; Thorburn & Challis, 1979; Lewis & Schulman, 1983).

In this paper, we will discuss the effect of the n3 fatty acids and fish oil on prostaglandin and arachidonic acid metabolism in the uterus. In addition, other possible roles of the essential fatty acids on parturition will be considered, and we will indicate how this information throws light on the signal which triggers parturition. Health implications will also be discussed. The paper will be divided into the following sections:

i) Physiological effects of the n3 fatty acids on parturition.
ii) Metabolism of the n3 and n6 fatty acids in the uterus.
iii) Uterine prostaglandins and the role of the n3 and n6 fatty acids.
iv) Other roles of n3 and n6 fatty acids, and the signal to parturition.
v) Health implications of an n3 fatty acid or a fish oil diet during pregnancy.

The effects of the n3 and n6 fatty acids will be discussed in relation to the triggering of parturition, and we will emphasise recent investigations of the effects of the fish oils. In each section, we will present original, unpublished data from our laboratory.

The crucial role of the n6 essential fatty acids in parturition was established by Quackenbrush et al (1942). In these and subsequent studies the predominant effect of the n3 fatty acids was an inhibition of the initiation of labour (Table 1). Quackenbrush et al found that rats fed a diet depleted of essential fatty acids and supplemented with the n3 C18 fatty acid, linolenic acid, showed only slight abnormalities in weight, implantation and gestation. However, the n3 linolenic acid fed rats had major defects in initiating and sustaining labour. The uterus was thin, flaccid and lacking tone and vaginal bleeding was observed. As a result of the protracted labour, fetal mortality was high, but if Caesarian Section was carried out early in labour, live animals were delivered (Leat

Table 1
Effects of n3 Fatty Acids at Parturition

Species	n3 Source	Primary Effect	Reference
Rats	Linolenic Acid	Parturition	Quackenbrush (1942)
Rats	Ara & C18	Gestation	Waltman (1978)
Rats	Linolenic Acid	Parturition	Leat (1981)
Rats	Columbinic Acid	Parturition	Houtsmuller (1981)
Rats	Fish Oil	Parturition	Leaver (1986)
Women	Fish Oil	Gestation	Olsen (1987)

and Northrop, 1980). In contrast, rats fed the essential fatty acid deficient diet supplemented with the n6 C18 fatty acid linoleic acid, experienced normal labour and delivered healthy offspring. The relationship between the C18 and C20 n6 fatty acids linoleic acid and arachidonic acid was recognised by Quackenbrush et al, who showed that both n6 fatty acids supported normal parturition.

In 1979, Leat and Northrop confirmed the inhibitory effect of linolenic acid feeding on parturition, and they suggested that the n3 linolenic acid in the diet reduced, by competetive inhibition, the conversion of n6 linoleic acid to arachidonic acid. They suggested that, under these conditions, there was impaired production of the prostaglandins. An inhibition of parturition was also observed when the linolenic acid isomer, columbinic acid (c18; 3 n6 9,13 trans) was fed to rats (Houtsmuller, 1981), who postulated that an inhibition of prostaglandin synthesis caused impaired labour in the columbinic acid fed rats.

An inhibition of parturition was also observed when rats were fed purified essential fatty acids and a normal pelleted diet which was not depleted of essential fatty acid (Waltman et al, 1979). Essential fatty acid fed rats showed a remarkable prolongation of gestation (of between 5 and 13 days). This study differed from previous reports (Quackenbrush et al, 1942; Leat & Northrop, 1979; Houtsmuller et al, 1979) in fail-

ing to show any specificity for the n3 fatty acids. A further difference lay in the administered dose of essential fatty acids used in this study, which was very low (5-10 mg/kg). This amount would supply less than 0.2% of the energy intake of the pregnant rat, which represents only approximately 20% of the essential fatty acids which these rats would consume in their pelleted diet (see below).

We analysed the fatty acid composition of a commercial pelleted diet (CRM Diet, BSS Ness, Edinburgh) using the techniques described in Table 2, and we found that the essential fatty acid content of the commercial pelleted diet consisted of approximately 0.8% n6 fatty acids and 0.01% n3 fatty acids as a proportion of the total calorific value of the diet.

Table 2
Fatty Acid Composition of Dietary Fats administered to Rats on a Semisynthetic Diet.

Fatty Acid	HCO	FO[1]	FO[2]	EPO
12:0	55.0	—	—	—
14:0	18.9	14.51	12.06	0.985
14:1	0.17	0.52	1.61	0.635
16:0	7.94	15.39	18.9	8.247
16.1	0.322	10.37	10.4	<0.01
18:0	7.0	3.20	3:24	2.33
18:1	2.4	10.37	14.2	7.90
18:2n6	0.76	0.924	2.40	68.00
18:3n3	0.034	0.447	0.729	<0.01
18:3n6	<0.01	<0.01	0.377	8.756
18:4n3	<0.01	1.355	<0.01	<0.01
20:0	0.016	1.07	<0.01	<0.01
20:1	0.058	0.918	1.71	<0.01
20:4n6	0.058	0.266	0.506	<0.01
20:5n3	<0.01	21.45	15.62	<0.01
22:1	<0.01	<0.01	<0.01	0.319
22:5n3	<0.01	2.06	1.54	<0.01
22:6n3	<0.01	4.13	6.51	<0.01

Hydrogenated Coconut Oil: HCO (Pilsbury's, Birmingham, U.K.), Fish Oil: FO[1] (Hi-EPA, Efamol Ltd., Guildford, U.K.), FO[2] (Seven Seas Health Care, Hull, U.K.), and Evening Primrose Oil: EPO (Efamol Ltd., Guildford, U.K.) were subject to alkaline hydrolysis and methylation (Leaver & Poyser, 1981). Gas chromatography was carried out using a Carbowax bonded capillary column DB wax (J & W Scientific, Rancho Cordova, California, USA) at 180-230°C (4°C/min) and a Pye 204 gas chromatograph with flame ionisation detector, using heptadecanoic acid as internal standard

The Effect of Fish Oil on Parturition

In 1985, we reported that fish oil, given as the predominant dietary essential fatty acid source (>99.7% of dietary essential fatty acid), inhibited the initiation and the progression of labour in rats (Leaver *et al*, 1986). In these experiments and in the experiments reported in this paper we fed a semisynthetic diet, low in essential fatty acids, with daily oral supplementation with predominantly n3 fatty acids (fish oil) or predominantly n6 fatty acids (evening primrose oil). The diet consisted of fat 16.5 en%, of which 13.5 en% was saturated fat (hydrogenated coconut oil) and 3.0 en% was essential fatty acid supplement (fish oil or evening primrose oil). The fatty acid composition of the dietary fat given to these rats is shown in Table 2. The n3/n6 ratio of essential fatty acids in the fish oil diet, the evening primrose oil diet and the pelleted diet is shown in Table 6. The essential fatty acid content of the diet corresponded to the minimum recommended essential fatty acid intake for man, although less than half of this dose will cure signs of essential fatty acid deficiency in the rat and other animals (Holman, 1968). The other components of the diet were protein (fat free Casein, BDH, Poole, UK) 15.7 en%, carbohydrate (D-glucose) 68.7% and non-digestible fibre (cellulose 11.02 g/kcal, kaolin 5.5g/kcal). The diet was supplemented with DL methionine (2.6g/kcal), Vitamin premix (2.76 g/kcal) and mineral premix (12.9 g/kcal) all from Special Diet Services, Cambridge UK. In order to avoid oxidation, essential fatty acid supplements were stored under nitrogen and administered orally at a dose of 1.5 ml/kg rat body weight/d. In each of our diet experiments, two groups of rats received semisynthetic diet supplemented with either fish oil (predominantly n3 fatty acid) or evening primrose oil (predominantly n6 fatty acid) and these were compared with a control group receiving a normal pelleted diet.

The consumption of semisynthetic diet, monitored on alternate days, was not significantly different in fish oil and evening primrose oil fed rats. The growth of rats fed the essential fatty acid supplemented diet was not significantly different from the growth of rats fed the control diet in preweaning and post-weaning groups (Fig. 1). On certain days

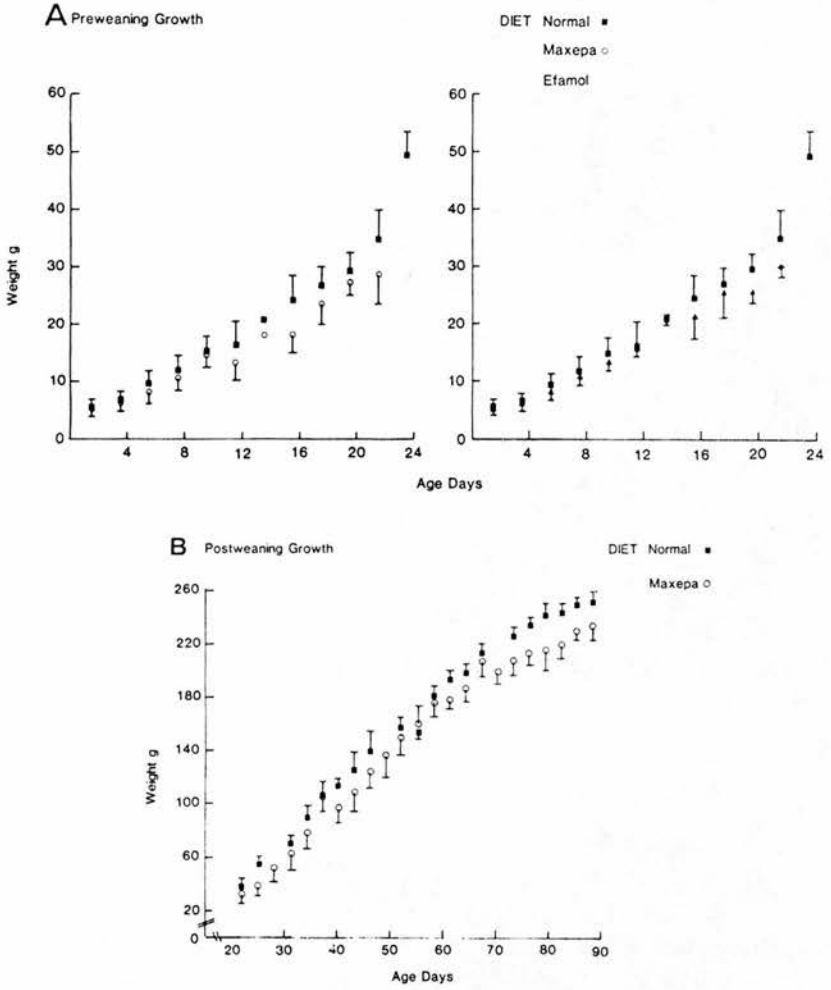


FIG. 1. Growth of Rats fed a Semisynthetic Diet Supplemented with Fish Oil or Evening Primrose Oil or a Normal Pelleted Diet (CRM, BSS Ness Edinburgh, U.K.) A. Preweaning growth B. Postweaning growth.

of pregnancy, the fish oil-fed rats, but not the evening primrose oil-fed group, were significantly lighter than the rats fed a control diet (Leaver et al, 1986). There was, however, little evidence that the fish oil diet affected fetal development during pregnancy. The effect of the fish oil diet on fertilisation and implantation was investigated (Table 4). The number of successful matings was analysed by caging females in estrous (determined by vaginal cytology for at least 16 days before mating, Leaver and Poyser, 1981) with males of the same diet group, and of proven fertility, overnight. Mating was judged to have occurred if sperm was detected in the vagina. The pregnancy rate in the fish oil group was not significantly different from that of rats fed the control pelleted diet. The number of pups per litter in the fish oil-fed rats was not significantly different from the control diet rats (Table 3). The

Table 3
Effect of a Fish Oil Diet on Parturition in Rats

Diet	Days Gestation	Litter Size	Survival	No.
Control	22.6 ± 0.02	9.3 ± 0.38	6.3 ± 0.99	15
EPO	22.8 ± 0.01	9.2 ± 0.42	6.6 ± 0.70	5
FO	23.2* ± 0.05	8.3 ± 0.51	1.4* ± 0.66	20

Rats were fed from weaning for over 60 days on either fish oil (FO) supplemented or evening primrose oil (EPO) supplemented semi-synthetic diet, or on a control pelleted diet (CRM diet, BSS Ness, Edinburgh, UK). *Indicates value significantly different from control ($p < 0.05$ using the unpaired Student' t test). Survival indicates the number of pups which reached weaning age (22d). (From Leaver et al, 1986).

intrauterine development of pups during gestation was normal as judged by birth weight and by survival of pups delivered by Caesarian Section after sacrificing the mother (Table 4).

In contrast with the limited effects of a fish oil diet on gestation, a major effect on the initiation and progression of labour was observed. Labour was delayed in the fish oil fed rats, which showed significantly longer gestation than

Table 4
Fertilisation, Implantation and Placentation of Rats fed Fish Oil (FO), Evening Primrose Oil (EPO) or Control Diet

	Pregnancy Rate %	Birth Weight (g)	Survival
Control	73.1	6.12 ± 0.175 (91)	8.3 ± 0.80 (6)
FO	70.6 (34)	5.98 ± 0.192 (60)	8.1 ± 0.62 (5)
EPO	—	5.82 ± 0.168 (38)	—

Results indicate the mean percentage of successful matings, birth weight of pups or 21 day survival per litter after Caesarean section ± SEM. The figures in parenthesis indicate the number of pregnancies, pups weighed, and litters monitored respectively.

the other dietary groups (Table 3). The duration of labour was prolonged and vaginal bleeding was observed in rats fed fish oil. Perinatal mortality was high in the fish oil group, compared with the group of rats fed the same semisynthetic diet supplemented with evening primrose oil, or with the group fed the control diet. Approximately 83% of pups died within 5 days of parturition, and 5% mothers died in labour during 60 deliveries of fish oil fed rats. In our initial report we attributed the perinatal mortality to the trauma suffered during prolonged parturition. We have investigated whether impaired lactation contributed to the pup mortality in the immediate postnatal period. The postnatal survival rate of rats fed fish oil, compared with a control pelleted diet, is shown in Table 5. Maternal nursing behaviour was not significantly impaired in the fish oil fed rats and strong evidence of lactation (milk lines in pups, milk solids in pup stomachs) was observed in 20—30% of the dead pups. It is also found that the suckling activity of the weaker pups was reduced. Therefore, although partial inhibition of lactation may have contributed to pup mortality, there was no evidence that it was a major factor. The survival rate of the fish oil pups which survived over 5 days was not significantly different from the control group. The effects of this fish oil diet on parturition bear a strong resemblance to the effect of C18 n3 linolenic

Table 5
Postnatal Survival Rate of Litters: Rats Fed Fish Oil or Control Diet

	0 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Fish Oil	8.90±.32	5.37±.71	3.0 ±.72	2.42±.74	2.0 ±.75	1.84±.73
Control	9.42±.47	8.75±.39	7.92±.56	7.41±.69	7.41±.69	7.25±.72

Mean litter size of rats fed for 60 days from weaning on the semi-synthetic diet supplemented with fish oil HIEPA (FO_1, Table 2), or with a normal pelleted diet. There were 19 litters in the fish oil group and 12 litters in the control group.

acid reported by Quackenbrush et al (1942) and Leat & Northrop (1979) and indicate that an n3 fatty acid effect may be acting in a fish oil diet which contains predominantly, but not exclusively, n3 fatty acids (n3 fatty acid: 78-86% of total polyunsaturated fatty acid).

The prostaglandin E_2 content in the intrauterine tissues of rats fed a fish oil diet for 60 days was determined using radioimmunoassay and anti-PGE_2 antibody obtained from the Institut Pasteur, Paris. The prostaglandin E_2 concentration in fetal membranes, obtained during parturition from 6 rats was 2.8 ± 0.7 ng/g in fish oil fed rats and 19.1 ± 4.0 ng/g in rats fed a control diet. In uterine wall (myometrium with attached decidua), 0.8 ± 0.16 ng/g of PGE_2 was detected in fish oil fed rats and 7.5 ± 0.4 ng/g in controls. The prostaglandin E_2 in the fetal membrane and uterine wall of fish oil fed rats was significantly ($p < 0.05$, n=6) lower than that of evening primrose oil fed or control diet fed rats.

We have also investigated the production of the 3 series prostaglandins by the uterus of rats fed an n3 fatty acid diet using mass spectrometry. The methyl-TMS mass spectrum of prostaglandin $F_{3\alpha}$ was characterised by several high molecular weight fragments bearing the n3 double bond which were two mass units smaller than the corresponding fragments of $PGF_{2\alpha}$ (Fig. 5). Our analysis of prostaglandin F_α released by uterine tissue incubates (Purified by silicic

acid chromatography and thin layer chromatography) obtained from rats fed the semisynthetic diet supplemented with 3 en% linolenic acid showed evidence of production of $PGF_{3\alpha}$ (fragmentation of PGF MeTMS at 377, 402, 477, 492 and 582 Daltons). The predominant fragments detected in the mass spectrum of PGF MeTMS in n6 (linoleic acid) fed rats and in $PGF_{2\alpha}$ standard had a mass of 379, 404, 479, 494, 569, 584 Daltons. Further analysis of the 2 and 3 series prostaglandins in rats fed fish oil and linolenic acid are in progress.

n-3 and n-6 Fatty Acids in Uterine Lipids after Fish Oil Feeding

It was likely that the uterine fatty acid composition of the rats fed a fish oil diet influenced uterine activity observed during labour, although few reports describing the effect of dietary fatty acids on uterine lipid composition have been published. We therefore analysed the effect of the fish oil diet on uterine lipid, in order to determine the effect of a fish oil diet on uterine n3 and n6 fatty acid. The incorporation of n3 and n6 fatty acids into the major uterine lipids was analysed. The uterine lipid fatty acid composition of rats fed the fish oil diet, the evening primrose oil diet and the control, pelleted diet was determined, after alkaline hydrolysis and methylation of uterine lipid, by gas chromatography. The proportion of n3 and n6 essential fatty acid in the total uterine lipid of the three dietary groups is shown in Table 6. The n3/n6 es-

Table 6
N3/N6 Fatty Acid Ratio in Uterine Lipids of Rats

Diet Group	Diet Fatty Acid	Tissue Fatty Acid
Control	0.21	0.202 ± 0.273 (n = 16)
EPO	0.004	0.067 ± 0.088 (n = 6)
FO	6.28	1.01 ± 1.12 (n = 24) *

The n3/n6 fatty acid ratio (percent of n3 fatty acid/ percent of n6 fatty acid) was determined in the diet and in the uterine tissue of rats fed the semisynthetic diet supplemented with fish oil (FO) FO_1 HIEPA, see Table 2; or evening primrose oil (EPO), or a controll pelleted diet (control) for 60d. The fish oil had shown abnormal parturition. *Indicates an n3/n6 ratio significantly different ($p. < 0.001$) from control, using the unpaired Students' t test.

sential fatty acid ratio of the uterine lipid of rats on the fish oil diet was significantly higher than the n3/n6 ratio in control and evening primrose oil groups. The uterine lipid of the evening primrose oil group had a higher n6 content and consequently, a lower n3/n6 ratio. The n3/n6 ratio of the uterine lipid of control rats (0.202) was very similar to that of their diet (0.210). However, both rats fed a diet with a high n3/n6 ratio (the fish oil group) and rats fed a diet with a low n3/n6 ratio (the evening primrose oil group), had a tissue fatty acid composition significantly different from the dietary n3/n6 fatty acid ratio, even after feeding the EFA supplemented diet for 60 days. Three generations of fish oil feeding (210 days, n=9 rats) did not significantly change the n3/n6 ratio of uterine lipids in the fish oil group from that achieved after 60 days of fish oil feeding (the n3/n6 fatty acid ratio in uterine lipid after three generations of fish oil feeding was 1.05 ± 0.142). There was no evidence of essential fatty acid deficiency in rats fed the essential fatty acid supplemented semisynthetic diet, as detected by C20:3n9 in uterine lipid. The proportion of C20:3n9 was never greater than 0.1% of total uterine fatty acid in any of the diet groups. The 20:3 n9/20:4 n6 ratio in the fish oil and evening primrose oil fed rats was less than 0.2 (a ratio of greater than 0.4 indicates fatty acid deficiency, Holman, 1960).

The effect of dietary fish oil on the n3/n6 fatty acid ratio in the major uterine lipid pools was analysed (Table 7). Significant changes in the uterine lipid pools of fish oil and evening primrose oil fed rats compared with control diet rats, were observed after only 21 days of dietary supplementation. The increase in n3/n6 ratio in the fish oil group was greater in the phospholipid than in the neutral lipid pool.

The distribution of the polyunsaturated fatty acids among the C18, C20 and C22 species in the uterus was investigat-

Table 7
n3/n6 Fatty Acid Ratio in Uterine Lipids of Rats

Diet	Neutral Lipid	Phospholipid
Control	0.022 ± 0.0118 (4)	0.146 ± 0.029 (4)
EPO	0.0098 ± 0.0061 (6)**	0.0337 ± 0.0176 (5)*
FO	0.076 ± 0.068 (6)	1.026 ± 0.218 (6)*

The n3/n6 ratio of rats fed from weaning for 21 days on control pelleted or semisynthetic EFA deficient diet supplements (with evening primrose oil (EPO) or fish oil (FO). * Significantly different from control $p < 0.02$; ** $p < 0.01$. The number in parenthesis indicates the number of fat uteri analysed.

ed, in order to find if the uterus showed the major changes in distribution of the C18 and C22 unsaturated fatty acids, and the preferential incorporation of C22 compared with C20 n3 fatty acid reported in platelets and erythrocytes after fish oil feeding (Cartwright et al, 1985; Simonsen et al, 1987). Within each diet group, the C22 fatty acids had the highest n3/n6 ratio, and the C18 fatty acids, the lowest n3/n6 ratio (Tables 8 and 9). The greatest change in n3/n6 ratio after fish

Table 8
n3/n6 Fatty Acid Ratio in Total Uterine Lipid

	n	C18	C20	C22
Control	16	0.034 ± 0.005	0.084 ± 0.022	4.00 ± 1.62
EPO	5	0.027 ± 0.009	0.040 ± 0.02	0.301 ± 0.096
FO	24	0.088 ± 0.025	0.658 ± 0.106**	22.6 ± 6.7*

The n3/n6 fatty acid ratio in C18, C20 and C22 classes was calculated for n rats fed control pelleted diet, or semisynthetic diet supplemented with fish oil (FO), FO_1, HIEPA; or evening primrose oil (EPO), for 60 days. * denotes an n3/n6 ratio significantly different from Control using Wilcoxon Rank nonparametric statistics, $p < 0.05$; **$p < 0.001$.

Table 9
n3/n6 Fatty Acid Ratio: Uterine Neutral Lipid and Phospholipid

	n	C18		C20		C22	
		NL	PL	NL	PL	NL	PL
Con	4	0.059±.003	0.024±.009	0.10±.03	0.02±.005	<nd	1.1±.13
EPO	5	0.007±.003	0.039±.017	0.17±.10	0.02±.007	<nd	0.18±.11**
FO	6	0.067±.029	0.074±.055	0.78±.18	0.65±.12**	<nd	159±130

The n3/n6 fatty acid ratio in C18, C20 and C22 classes was calculated for n rats fed control pelleted diet, or semisynthetic diet supplemented with fish oil (FO), FO_1, HIEPA; or evening primrose oil (EPO), for 21 days. ** denotes ratios significantly different from the control group using Wilcoxon Rank nonparametric statistics, $p < 0.01$; <nd: C22 fatty acids were below the level of detection (<0.01% of total fatty acid in NL).

oil feeding was observed in the C20 fatty acid pool, and the most significant change for any individual fatty acid was observed in eicosapetaenoic acid, which increased by 1220% in the fish oil group compared with the control group. There was a modest increase in the uterine content of docosahexanoic acid in the fish oil group (165% of control). The administered fish oil consisted of 22% eicosapentaenoic acid and 7% docosahexanoic acid (Table 2). Therefore, there was little evidence of selective incorporation of the C22, compared with the C20 n3 fatty acids into the major lipid pools of the uterus, when a control diet (n3/n6 ratio 0.0125) was compared with a fish oil diet (n3/n6 ratio 5.2). A quantitatively greater change in the n3 content of the C22 pool was observed at very low dietary n3 levels (the evening primrose oil diet; n3/n6 ratio 0.0026), compared with moderate amounts of n3 fatty acid in the control diet (n3/n6 ratio 0.0125). The n3 content of the uterine C22 pool of rats fed the evening primrose oil diet was significantly depleted, compared with the C22 n3 fatty acids in the control diet group. The amount of uterine polyunsaturated fatty acid esterified to C18, C20 and C22 pools showed relatively small changes associated with fish oil feeding. A significant ($p < 0.01$) decrease of 12% in the amount of poly-

unsaturated fatty acid esterified to C18, and a 22% increase in the C22 class was observed in the uterus of the fish oil group, compared with the control group. These experiments indicate that the incorporation of dietary n3 and n6 fatty acids into the major uterine lipids differs from other tissues (see Kinsella in the volume) in showing a relatively high capacity to esterify C20 fatty acid. Also, there was evidence that the uterine C20 polyunsaturated fatty acid pool was conserved under widely different dietary n3/n6 loads.

The changes in total uterine lipid fatty acid, and in neutral lipid and phospholipid fatty acid composition after fish oil feeding showed that the proportion of n6 fatty acids in the major lipid pools was decreased from 86.5% of essential fatty acids in the normal diet, to 52.1% of essential fatty acids after the fish oil diet. Thus, the fish oil diet resulted in a change of 34.4% in the proportion of n6 fatty acid in the essential fatty acid composition of the major uterine lipids. These changes in essential fatty acid composition in total uterine lipid, neutral lipid and phospholipid are quantitatively smaller than the observed changes in the efficacy of parturition in fish oil fed rats (fetal survival rate, see Table 3) which was decreased by 80%, or the uterine content of prostaglandin E_2, which was decreased by over 80%. However, the major uterine lipid pools include a wide range of molecular species, and it is necessary to consider the activity of specific lipids in relation to localisation, metabolic turnover, and their physiological role in parturition.

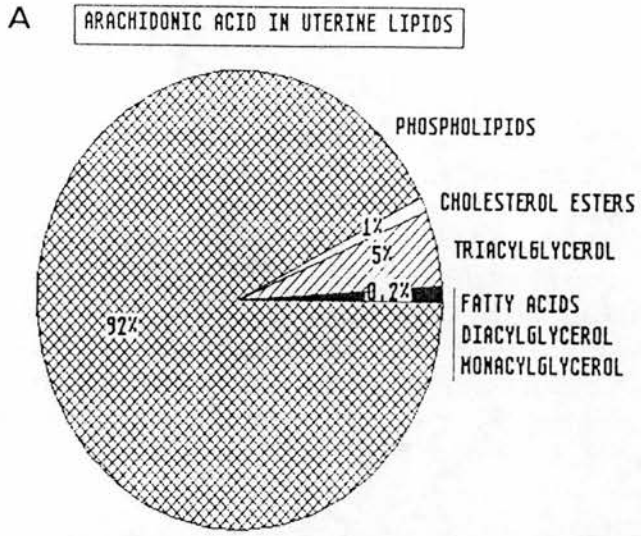
Metabolically Active and Physiologically Significant Lipid Pools in the Uterus

The best characterised role of essential fatty acids at parturition is the release of arachidonic acid and prostaglandins E_2 and $F_{2\alpha}$. The role of prostaglandins will be discussed in the next section. Other possible functions of essential fatty acids in parturition (see Table 11) will also be discussed in relation to the activity of the n3 and n6 fatty acids. In this section, we will consider the release of arachidonic acid from the uterus, and possible sites of n3 fatty acid involvement

in this process.

In considering the physiological role of the uterine lipids, it is important to identify the pools of arachidonate which contribute to, and whose release is controlled by, the signals which regulate uterine function. The physiological signals for parturition are not well defined. Most investigation has centred on hormonal influences, and the steroids and oxytocin have been shown to influence the timing and progress of parturition (Thorburn, 1979, Soloff et al, 1979; Ellwood, 1980; Poyser, 1981). The physiological role of prostaglandins E_2 and $F_{2\alpha}$ in parturition has been as well established in this respect as any other prostaglandin-mediated process, but investigation of the role of other local mediators is only beginning. On the cellular level, current understanding of the triggering events at parturition are poorly defined and, apart from certain relations to hormones and prostaglandins, little is known about the cellular structures and molecular responses which are activated at parturition. The signals which will be considered in relation to uterine lipid pools are outlined in Table 12.

The distribution of arachidonic acid in the lipids of the non-pregnant uterus is shown in Fig. 2. The major pool of



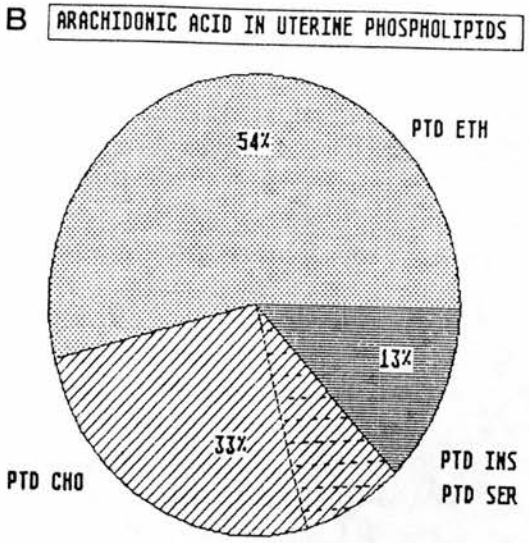


FIG. 2. A. Distribution of Arachidonic Acid in the Lipids of the Non-Pregnant Guinea-Pig Uterus in Animals fed the normal pelleted diet. Lipids were separated by silicic acid chromatography, and the fatty acid composition of each fraction was analysed (Leaver & Poyser, 1981). This distribution did not change significantly during the oestrous cycle. B. Distribution of Arachidonic Acid in the Phospholipids of the Non-pregnant Guinea-Pig uterus. Lipids were separted by thin layer chromatography and analysed by gas chromatography (Leaver & Poyser, 1981). The area of phosphatidylcholine (PtdCho), enclosed by the dotted line indicates the proportion of arachidonate which decreased at estrous. This proportion of arachidonate increased in the phosphatidylinositol (PtdIns), fraction at estrous.

arachidonate lies in the phospholipids, of which phosphatidylethanolamine was the largest component. Further separation of the phosphatidylinositol/phosphatidylserine fraction indicated that over 70% of the arachidonate in this fraction was esterified to phosphatidylinositol. Under the influence of estradiol, the proportion of arachidonic acid esterified to phosphatidylinositol increased, and that in phosphatidylcholine decreased. The incorporation and release of arachidonic acid by these lipid pools was monitored in the nonpregnant uterus and an arachidonate-specific change in the pattern of esterification into the uterine phospholipids, under the influence of estradiol, was observed (Leaver and

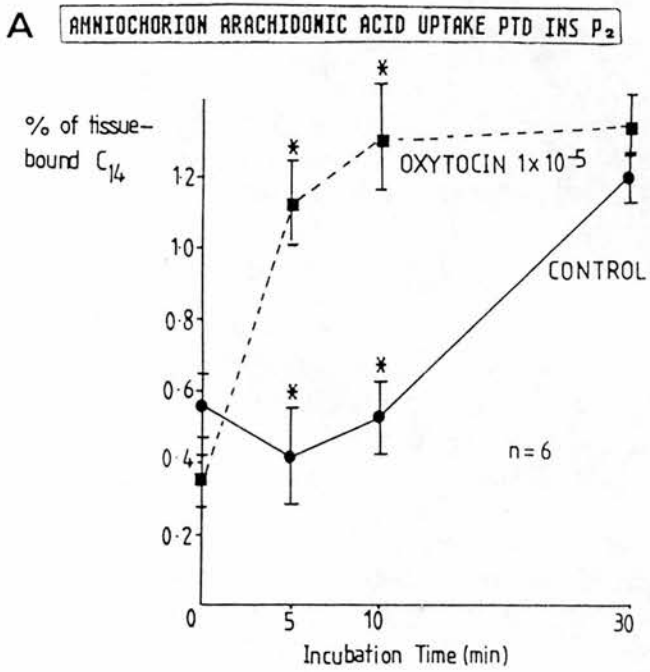
Ning 1981; Ning et al 1983). Both the uptake and the release of arachidonic acid (but not of oleic acid) within uterine phospholipids were increased by estradiol. There was stimulation of endogenous arachidonate incorporation into the phospholipids, particularly PtdIns at estrous (Leaver & Poyser, 1981; Fig. 2B), or under the influence of exogenous estradiol 17b (Grove et al, 1987). The increase in the arachidonic acid content of uterine phospholipids which occurs during late gestation (Schwartz et al, 1975) may also be a result of a similar estrogen stimulated acylation.

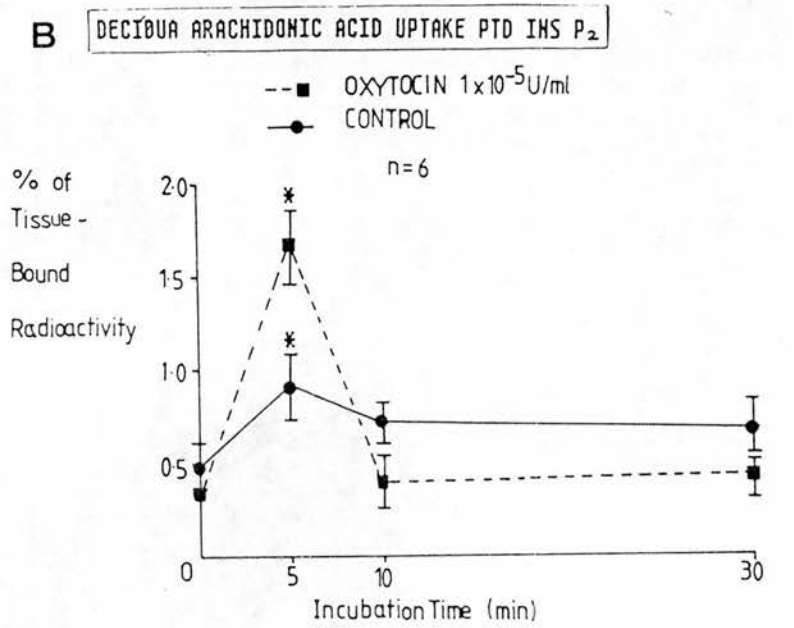
At parturition, the depletion of the arachidonic acid content of the fetal membrane phospholipids with the highest proportion of arachidonate, phosphatidylethanolamine (PtEt) and phosphatidylinositol (PtdIns) suggests that some degree of selectivity for arachidonate is present in the phospholipases acting on the fetal membrane (Okita et at, 1982; Okazaki et al, 1978; Leaver et al, 1983). The pH dependance and the subcellular distribution of the phospholipase activity in human fetal membranes during labour suggest that the lipases involved in early labour are not predominantly lysosomal in origin, as suggested by one of the earliest theories of cell activation at parturition (Gustavii, 1972). The fatty acid composition of diacylglycerol released during parturition resembles that of fetal membrane PtdIns (Okita et al, 1982; Leaver et al, 1983), and the relative decrease in arachidonate in PtdIns and PtdEt indicate that phospholipase A_2 and phospholipase C both release approximately 50% of the fetal membrane bound arachidonate during early labour (Okita et al, 1982; Okazaki *et al* 1981). The activity of these lipases with respect to n3 fatty acids and eicosapentaenoic acid has not been analysed.

The phospholipase A_2 and phospholipase C activity of human fetal membranes is activated by relatively high calcium concentrations (2-4 m mol/l Ca^{++} causes half maximal activation) but decidual phospholipase C is calcium-independent (Okazaki *et al*, 1978; Di Renzo *et al*, 1981). The effect of increasing the intracellular calcium concentration in human and in Guinea Pig endometrium is similar to the

effect of adding arachidonic acid, if prostaglandin $F_{2\alpha}$ synthesis is monitored, (Fig. 6).

A molecule whose role in generating the intracellular calcium signal has been characterised is the polyphosphoinositide, inositol 145 tris phosphate (Ins. 145 P_3; Streb, 1983). The formation of Ins 145 P_3 is catalysed by a receptor-stimulated phospholipase C, acting on phosphatidylinositol bis phosphate (PtdInsP_2; Michell, 1987). We observed rapid turnover of arachidonic acid esterified to PtdIns P_2, and an enhancement of this turnover by oxytocin in fetal membranes and uterine decidua (Fig. 3). We also detected a concurrent





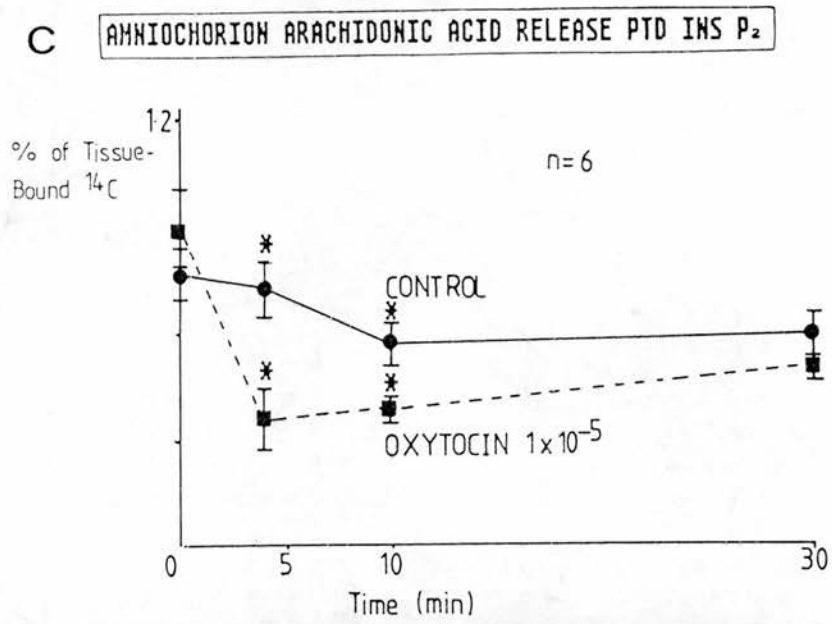


FIG. 3. Uptake and Release of ^{14}C Arachidonic Acid in Human Fetal Membranes and Decidua obtained at Elective Caesarian Section and Incubated with Oxytocin ($1x10^{-5}$u/ml). Tissue was immediately rinsed in ice-cold 0.9% saline, and explants were placed in ^{14}C sodium arachidonate (0.5 μCi/μl) in Tris HCl buffer pH 7.5, 1.8 mM/1 $MgCl_2$, $CaCl_2$ 1.0mM, ATP 7.5 mMol/l at 4°C. "Uptake" incubations were performed in a shaking water bath at 37°C for 0-30 min. "Release" incubations were carried out after 30 min uptake, for 0-30 min. The reaction was terminated with 5% trichloroacetic acid. Tissue $PtdInsP_3$ was extracted using acidified chloroform, and isolated using preparative t.l.c. (Jolles *et al*, 1981). Arachidonic acid incorporated into $PtdInsP_3$ was expressed as a percentage of tissue-bound radioactivity (30-40% of tissue-bound ^{14}C was esterfied).

rapid turnover of inositol and phosphate in a compound co-chromatographing with Ins 145 P_3 (Fig. 4). This release of Ins 145P_3 was calcium independent (release was not stimulated by 5-60 min incubation with the calcium ionophore A23187 5ug/ml and an extracellular calcium ion concentration of 1.8m mol/l). Thus, in human fetal membranes, we iden-

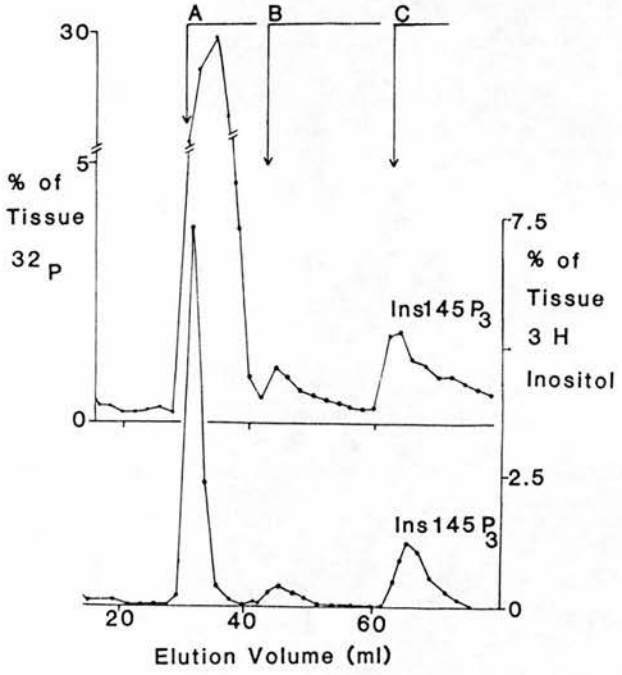


FIG. 4. Ion Exchange Chromatographic separation of phosphoinositides derived from Amniochorion. Amniochorion explants from the same patient at elective

Caesarian section were incubated for 60 min in Li^{++} containing buffer with either 0.25 mCi of ^{32}P phosphate or 2μCi of ^{3}H inositol. Lipids were deacylated, and the water soluble products were separated using a Dowex 1 (formate form) column, and the following elutants: A, 5mM $Na_2B_4O_7$/0.18M ammonium formate; B, 0.1 M formic acid/0.3 M ammonium formate; C, 0.1 M formic acid/0.75 M ammonium formate (Creba *et al*, 1983).

tified the synthesis of a compound known to release calcium within the cell. The arachidonic acid esterified to PtdIns P_2 represented only 1—3.5% of exogenous radiolabelled arachidonic acid added to the fetal membranes, and approximately 0.4% of endogenous total phospholipid-bound arachidonate in the fetal membranes. However, the rapid turnover of arachidonyl PtdIns P_2 indicated that this was a metabolically mobile pool of arachidonate. In the uterus, as in other tissues, direct evidence linking PtdIns P_2-bound arachidonate with prostaglandins in a product-precursor relationship is inconclusive, because the diacylglycerol generated by PtdIns P_2 phosphodiesterase is chemically not clearly different from diacylglycerol resulting from phospholipase C activity on other phosphoinositides. It remains an intruiging possibility that the polyphosphoinositides represent a specifically released pool of arachidonate during the triggering of labour.

There is growing evidence that the acylation and transacylation pathways of synthesis of phosphoinositides and other phospholipids may be important in establishing the fatty acid composition of these lipids (MacDonald *et al*, 1987; Bell, 1986; Bishop *et al*, 1983; Swendsen et al, 1987). The n6/n3 fatty acid selectivity in the pathways of PtdIns synthesis (Chabot et al, 1987; Dudley et al, 1987) may play an important role in modulating the activity of these biologically active lipids (Burn, 1988; Michell, 1988).

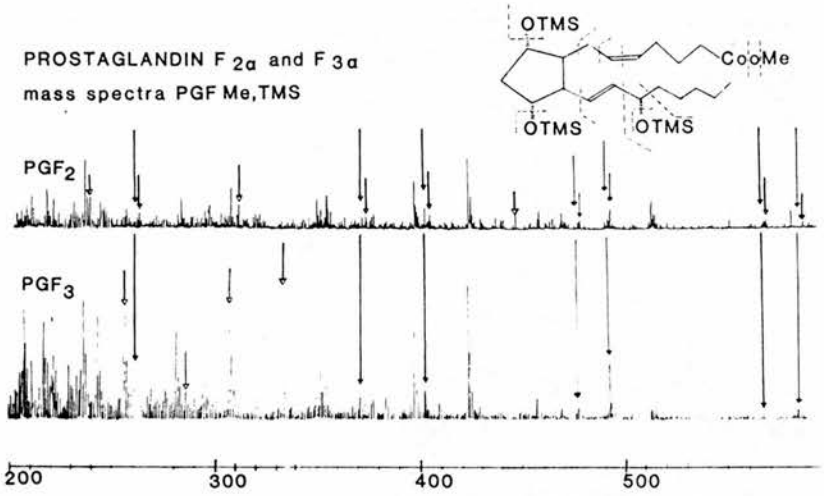


FIG. 5. Fragmentation of Prostaglandin $F_2\alpha$ and Prostaglandin $F_3\alpha$ Me-TMS derivative by Gas Chromatography Mass Spectrometry. PGF released from rat uterine explants in vitro, after 19h incubation in Medium 199 at 37°C, was purified by Sep-Pak (Waters, Millford, Mass, USA) adsorption, and thin layer chromatography, and the Me, TMS ester prepared (Fenwick et al, 1977). The PGF-Me, TMS ester was separated by capillary gas chromatography, using a fused silica DB1 column (J&W Scientific, Rancho Cordova, California, U.S.A.) at 150-270°C (4°C/min), and analysed using a VG Analytical Micromass 7070F mass spectrometer. Standard $PGF_2\alpha$ was obtained from Upjohn (Crawley, Sussex, UK) and $PGF_3\alpha$ from Cayman Chemicals (Ann Arbour, Mich, USA).

A Role for Prostaglandin Synthesis in n3 Fatty Acid Effects at Parturition.

In all of the recent reports on the effects of the n3 fatty acids on parturition, it has been proposed that inhibition of the synthesis, or the actions of the 2 series prostaglandins resulted in impaired parturition. It is surprising that uterine prostaglandin synthesis in the n3 fed subjects has not been closely analysed. The quantitation and the pharmacokinetics of prostaglandin release at parturition also throws light on another aspect of the action of the prostaglandins and n3 fatty acids: The prostaglandins are thought to play a role in other processes in placentation which are relatively unaffected by the dietary n3 fatty acids (see Table 10), including ovulation, implantation, and lactation (Poyser, 1981). Prostaglandin synthesis at parturition differs from other prostaglandin-mediated processes in the quantity of arachidonic acid and prostaglandin is released, which is two to three orders of magnitude greater than the changes reported in ovulation, lactation and implantation (MacDonald *et al*, 1974; Bussman and Deis, 1979; Ogburn *et al*, 1980; Leaver *et al*, 1987; Poyser, 1981). A further difference between the role of prostaglandins at parturition, compared with other aspects of placentation, is the distance between the major sites of synthesis (the fetal membranes and uterine decidua) and target tissues (the myometrium and the uterine cervix; Wickland *et al*, 1984; Ellwood, 1980). Therefore, at parturition prostaglandin E and F appear to act in a more 'hormonal' capacity than in other prostaglandin-mediated processes, diffusing locally into the myometrium and cervix, and coordinating, and possibly inducing, labour by an extracellular, rather than an intracellular, mode of action.

The susceptibility of labour to pharmacological agents which affect prostaglandin concentration, together with the effect of the n3 fatty acids on prostaglandin synthesis, indicate that prostaglandin synthesis is a likely site of action of the n3 fatty acids and of the n3 fatty acids in fish oil at parturition. There is evidence for competetive inhibition of the action of the n6 fatty acids or their metabolites by n3 fatty

Table 10
Prostaglandins in Placentation

Physiological Function	References
Ovulation	Poyser (1981); Thorburn (1979)
Implantation	Poyser (1981)
Parturition Initiation	Aitken (1972); Lewis (1973); MacDonald (1974)
maintenance	Wickland (1984); Leaver (1987)
Lactation	Bussman (1979)
Cervical dilatation	Ellwood (1980)

acids in this process. The proportions of dietary n3 fatty acid in the fish oil diet (78-86%) resembled the extent of inhibition of PGE_2 synthesis concentrations in intrauterine tissue were decreased by over 80% in fish oil fed rats). The 'efficacy' of labour (neonatal survival rate) was also decreased in the fish oil fed group by over 80%. The extent of substrate inhibition of cyclooxygenase by n3 fatty acids, and the extent of production of the 3 series prostaglandins is currently under investigation. In contrast, the n3 substrate in the major uterine lipid pools only increased by 34% in the fish oil fed animals. It is probable that a significant component of the n3 fatty acid pool which competitively inhibits the intrauterine synthesis of PGE_2 arises fromn a quantitatively minor intrauterine lipid pool.

Other Functions of Essential Fatty Acids at Parturition

In addition to the prostaglandins, other activities of the essential fatty acids may play a role in parturition (Table 11).

Table 11
Functions of Essential Fatty Acid in the Uterus

Proposed Molecular Activity	References
Prostaglandins	Thorburn (1979); Leat (1981)
PAF synthesis	Billah (1983); Findlay (1981); Hannahan (1986)
Protein kinase activation	Nishizuka (1988)
Diacylglycerol	Okazaki (1981); Ning (1983); Leaver (1983); McDonald (1987)
Phosphoinositides	DiRenzo (1981); Leaver (1981, 1985)
TNF and IL1	Roberts (1985); Flynn (1985); Romero (1988a,b); Suffys (1987).

In this section, we will consider the effect of the n3 fatty acids on the activity of these metabolites, with reference to arachidonate flux at parturition (Fig. 7), and the triggering of labour (Table 12).

Table 12
Signal to Parturition

Level of Organisation	Molecular Activities	References
Hormonal	Steroids, Oxytocin	Fuchs (1978); Grove (1987)
Mediator	Prostaglandins, PAF, IL1, TNF	Lewis (1973);Leaver (1984, 1987); Billah (1983); Silver (1986)
Intracellular	Lysosomes, Lipase, activation	Gustavii (1972); Okazaki (1978, 1981) Leaver (1986)
	Ca++ mobilisation	Leaver (1982, 1986); Grove (1987)
	Receptor expression	Soloff (1979)
	Enzyme modification	Burn (1988); Nishizuka (1988)
	Membrane modification	Okita (1982), Leaver (1985 and Tables 6-9)

The effect of administered arachidonic acid in inducing parturition, and the release of arachidonate in a hundredfold higher proportion than released eicosanoids (MacDonald, 1974), indicates that arachidonic acid, and possibly other essential fatty acids, may directly stimulate parturition. A cellular target for arachidonic and eicosapentaenoic acid has recently been proposed (Nishizuka, 1987). However, there is currently little evidence that n3 fatty acids act differently to n6 fatty acids in activating protein kinase C.

A better characterised activator of protein kinase C is diacylglycerol. The activity of the diacylglycerol which is mobilised in the plasma membrane in response to certain stimuli has been shown to involve protein kinase C as a responding cellular system, which in turn controls certain contractile proteins and enzyme activities (Preiss *et al*, 1987, Bell *et al*, 1986). A major species of diacylglycerol released at parturition is the 2-arachidonyl diacylglycerol. However, the shorter chain diacylglycerols are more active in stimulating protein kinase C in other tissues. At parturition, there is competition between two other enzymes for diacylglycerol, diacylglycerol kinase and diacylglycerol lipase, both of which show

arachidonate selectivity and are active at parturition (Okazaki at al, 1981; Okita et al, 1982). The effect of the n3 fatty acids on diacylglycerol metabolism and activity is poorly defined.

An increase in the potent lipid mediator, platelet-activating factor (PAF acether) and lyso PAF acether in human amniotic fluid at parturition has been reported (Billah & Johnston, 1983). PAF acether has potent contractile effects on smooth muscle (Findlay et al, 1981), but its' best characterised actions are the activation of platelets and leucocytes. The half-life of PAF acether in the circulation is short, and PAF availability is regulated by a phospholipase A_2 with arachidonate selectivity (Hanahan, 1986). It is possible that the n3 effects on PAF synthesis and on other phospholipase-mediated actions may be exerted indirectly, at the level of phospholipid synthesis.

In the uterus, there is evidence for arachidonate-specific phospholipid synthesis during pregnancy, and stimulation of this activity under the influence of estradiol and oxytocin (MacDonald *et al*, 1974; Leaver and Poyser, 1981; Ning *et al* 1983; Leaver et al, 1984; Fig. 2). The turnover of arachidonic acid in PtdIns P_2 in the fetal membranes and uterine decidua is enhanced by oxytocin (Leaver *et al*, 1984; Fig. 3). Also, PAF stimulates Ins P_3 release in platelets and leucocytes (Shukla et al, 1987). It remains to be established whether the quantity of Ins 145 P_3 (Figs 3 and 4) and PAF generated by the uterus are able to release sufficient calcium to release fatty acids and prostaglandins (Leaver & Seawright, 1982; Fig. 6) at parturition.

The role of two cytokines, Interleukin 1 and Tumor Necrosis Factor (TNF), should also be considered in the context of uterine function at parturition. Microbial infection has recently been identified as a common cause of abortion and premature labour (Silver *et al*, 1986), and bacterial products stimulate the release of intrauterine prostaglandins (Roberts *et al* 1985; Romero *et al*, 1988a) by a process which probably involves these cytokines (Romero *et al*, 1988b). There is evidence that Prostaglandin E_2 acts as a 'second messenger' in

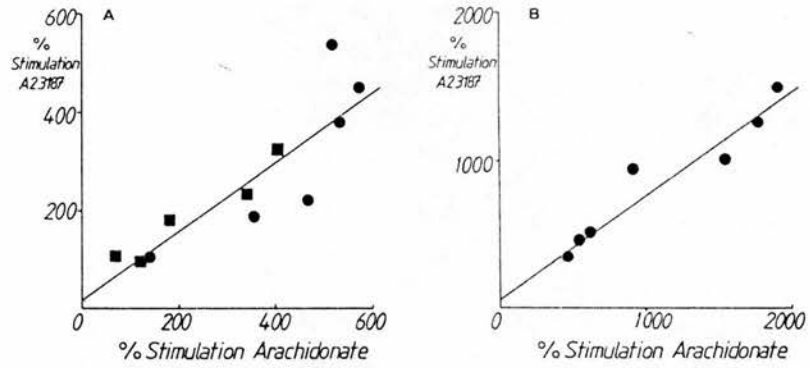


FIG. 6. Effect of Calcium Ionophore A23187 5μg/ml and Arachidonic Acid 100μg/ml on Prostaglandin $F_{2\alpha}$ Synthesis by endometrial explants from A. Guinea Pig and B. Human, incubated in Medium 199 & 10% Foetal Calf Serum at 37°C for 6h. PGF_2 output was expressed as a percentage of $PGF_{2\alpha}$ sythesised in the absence of ionophore or arachidonate.

Interleukin 1 and TNF action (Suffys et al, 1987), and it is interesting in this context, that a fish oil diet inhibits leucocyte eicosanoid release and the severity of inflammation associated with these mediators (Leslie *et al*, 1985). This aspect of TNF and Interleukin 1 action may be important in the treatment of infection during pregnancy with antibiotics, because antibiotics prevent bacterial proliferation, but do not remove, and may actually increase the release of endotoxin from bacterial cell walls (Shenep & Mogan, 1984). There is evidence that the eicosanoids are mediators of endotoxicity

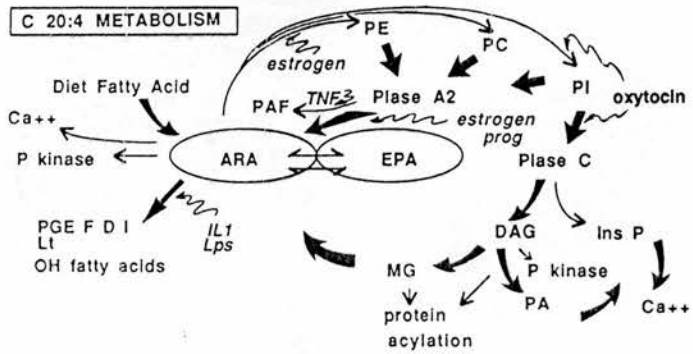


FIG. 7. The metabolism of Arachidonic Acid during early Parturition, indicating quantitatively major pathways and the site of action of extracellular signals.

(Flynn, 1985; Parratt, 1985), and competitive inhibition of their toxic effects (Conte *et al*, 1986) may be an advantage, particularly during pregnancy when susceptibility to endotoxin is increased (Lee et al, 1988).

Health Implications of a Fish Oil Diet During Pregnancy

In the above discussion, we have focussed on the rapidity and extent of the incorporation of the n3 fatty acids in the fish oil diet, and the specificity of the metabolic effects caused by the n3 fatty acids, with particular reference to parturition. These aspects of fish oil ingestion have important implications in the management of pregnancy, because, contrary to the obvious conclusion that fish oil should be avoided during pregnancy, the following findings suggest that the inhibition of parturition is a readily reversible effect: Firstly, a rapid turnover of the uterine lipids which release essential fatty acids at parturition was observed. Secondly, there was rapid incorporation of exogenous essential fatty acids into these uterine lipids at parturition.

High dietary concentrations of fish oil act like other n3 fatty acid preparations which have previously been reported to inhibit parturition. However, there is evidence that this block can be reversed by only two days of n6 fatty acid feeding (Leat & Northrop, 1980). Although the experiments on the fish oil diet and n3 fatty acids which we have described have been performed in rats, there is evidence that a fish oil diet may influence human parturition. Women from the Faroe Islands consuming a diet with a high marine oil content have been reported to have prolonged gestation, and consequently, babies with higher birth weight (Olsen *et al*, 1986). The impairment of parturition which was observed in the Faroe Island women with a high dietary n3 fatty acid intake was less severe than the effect of fish oil feeding with an essential fatty acid depleted diet in our rat experiments, which indicate an extreme state of dietary n3 load. Our experiments indicate that the effects of n3 fatty acids at parturition resemble their effects in platelet aggregation (Sanders *et al* 1982; Sanders and Roshani, 1983) and in inflammation (Leslie *et al*, 1985) in competetively inhibiting the effects of the n6 fatty acids. This suggests that a major factor determining the effect of the n3 fatty acid is the balance of n3/n6 fatty acids in the diet. It is therefore essential to characterise the essential fatty acids in the total diet, in addition to the composition of the 'essential fatty acid supplement'. It is likely that the care with which Quackenbrush *et al* prepared their diets (total diet saponification and lipid extraction, to remove essential fatty acids from the basal diet) allowed the unequivocal demonstration of an n3 fatty acid effect on parturition. In our experiments, the low n6 fatty acid content of coconut oil in the basal diet (Table 2) changed the n3/n6 ratio from 24.4(FO_1) and 7.44(FO_2) in the administered fish oil to 6.28 and 3.68, respectively, in the total diet (Table 6). The lack of detailed n3/n6 fatty acid anaylsis of the diet in the Faroe Island study (Olsen et al, 1986) opens the possibility that dietary components unrelated to the n3 fatty acids may affect the onset of labour (Ackman, 1988). It is therefore important that further controlled epidemiological studies be carried out, in order to establish potential risks of n3 fatty acid overload during the third trimester of pregnancy.

Premature labour often occurs before fetal and uterine maturation, and is associated with an increase in perinatal mortality. A natural product, in the form of fish oil, may be a more acceptable agent for delaying labour that pharmacological intervention, particularly if a beneficial effect of fish oil on fetal development occurs (Clarke *et al*, 1988, and see Fujimoto et al in this volume).

CONCLUSION

In conclusion, we have presented new evidence that dietary n3 fatty acids and fish oil inhibit the initiation of parturition by competing with the n6 fatty acids. It is likely that this inhibition lies at the level of prostaglandin production, although other essential fatty acid containing compounds may also be involved in initiating labour. The incorporation of dietary C20 polyunsaturated fatty acids and the retention

of these fatty acids by the uterus was more pronounced than in other cell types. We observed limited modification of the major lipid pools by the fish oil diet, and the rapidity and specificity of incorporation of exogenous essential fatty acid into the smaller but more rapidly turning over pools of essential fatty acids indicate that the latter pools may be sources of essential fatty acid during early labour. The effect of the n3 fatty acids on parturition indicates that the n6 essential fatty acids play a major role in the initiation of labour in addition to their well characterised role in maintaining labour. It is likely that the n3 fatty acids will play an important role in defining the molecular and cellular events which signal the initiation of parturition (Table 12).

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16

The Effect of Dietary Docosahexaenoate on the Learning Ability of Rats

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INTRODUCTION

Docosahexaenoic acid (DHA or 22:6, n-3) is one of the major n-3 long-chain polyunsaturated fatty acids in fish oils, and its content often reaches more than 35%. It is well known that the tissues of the nervous system involved in sophisticated actions, e.g. the brain,[1] the synaptic junction[2] and the photoreceptors in the retina,[3] are exceptionally rich in DHA. Although the livers of some types of animals show a high content of DHA, the value fluctuates widely among species. In contrast, the DHA content of both the brain and retina is consistently high in all animals. Generally the distribution of polyunsaturated fatty acid types in plant and

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