ARACHIDONIC ACID TURNOVER IN THE GUINEA PIG ENDOMETRIUM

by

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Signed :

Contents

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ABSTRACT OF THESIS (Regulation 7.9)

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Title of Thesis TURNOVER OF ARACHIDONIC ACID IN THE GUINEA PIG ENDOMETRIUM

Prostaglandin (PG) $F_{2\alpha}$ output from the guinea pig uterus increases after Day 11 of the oestrous cycle and oestradiol (whose output from the ovary increases after Day 10) acting on a progesterone-primed uterus is probably the physiological stimulus for this increase in uterine $PGF_{2\alpha}$ release. The availability of free arachidonic acid in the endometrium is the rate-limiting step in $PGF_{2\alpha}$ synthesis. The aim of the present studies was to determine the source of arachidonic acid for endometrial $PGF_{2\alpha}$ synthesis. Initially, the endometrium was maintained in tissue culture in the presence of tritiated arachidonic acid (^{3}H-AA) and the radioactive contents of the various lipids was subsequently measured. The uptake of ^{3}H-AA into phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was significantly higher on Day 15 (day of high $PGF_{2\alpha}$ synthesis) than on Day 7 (day of low $PGF_{2\alpha}$ synthesis). Arachidonic acid could be incorporated into these phospholipids by an increase in the mass amounts of phospholipids. However results indicated that there was no significant difference in the amount of phospholipids present on Day 7 and Day 15. Arachidonic acid could be incorporated into the phospholipids either by an increase in de novo synthesis or by the addition to lysophospholipid. The rates of PC synthesis and phosphatidylinositol synthesis did not differ on Days 7 and 15, indicating that the measured uptake of arachidonic acid into phospholipid is not due to increased de novo synthesis but rather to increased addition to lysophospholipid. Oestradiol may be the stimulus for this latter process.

A procedure for determining the major pool (s) of release of arachidonic acid in the guinea pig endometrium was carried out, utilising the same tissue culture technique. The release of radioactivity from endometrial tissue lipids prelabelled with ^{3}H-AA for 24hr, was measured over a 24hr time period. There was no significant release of ^{3}H-AA from any endometrial lipid class on Day 15; but on Day 7 there was a significant release of ^{3}H-AA from diglyceride and monoglyceride. In order to stimulate a greater release of arachidonic acid (which is more easily monitored) calcium ionophore A23187 was used since A23187 stimulates $PGF_{2\alpha}$ release from the guinea pig uterus. This stimulus however did not cause any significant decrease in ^{3}H-AA content of any lipid class. In the tissue culture system used, oestradiol stimulates and progesterone inhibits $PGF_{2\alpha}$ output from the guinea pig endometrium. Therefore, the effects of these steroids on arachidonic acid release from the various lipids have been measured also. After labelling the lipids with ^{3}H-AA, Day 7 and Day 15 endometrial tissue was incubated for a further 48hr in fresh medium containing no steroids, 10ng/ml oestradiol or 1000ng/ml progesterone. The radioactive content of the various lipids were measured. There was no significant changes in the release of ^{3}H-AA from any lipid class (in the absence of steroids) on Day 7 or Day 15 except that on Day 15 a significant decrease in ^{3}H-AA content of PC and PE was observed after 48hr. The presence of steroids had no further significant effect.

The overall data for the uptake and release studies suggest that PE and PC may be the source of arachidonic acid for increased synthesis of $PGF_{2\alpha}$ in the guinea pig endometrium after Day 10 of the cycle.

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GENERAL INTRODUCTION

History

An ubiquitous family of compounds known under the general term of prostanoids has been identified over the last 50 years. Its scientific origins can be traced back to 1930 when New York gynaecologist Raphael Kurzrok and pharmacologist Charles Lieb reported that an unknown substance in human semen caused uterine contractions in some women and relaxation in others. Experiments *in vitro* with semen added to strips of human uterus resulted in similar observations and also revealed the interesting observation that a single tissue sample would contract or relax when treated with different semen samples.

A short time later Goldblatt (1935) in England and Von Euler (1935,1937) at the Karolinska Institute in Sweden extended this work. They discovered that the unidentified substance(s) in human seminal fluid not only stimulated the contraction of a variety of smooth muscles but also affected the blood pressure of animals when injected intravenously. In his paper published in 1935, Von Euler suggested the name prostaglandin for the unknown factor because he had found trace amounts of it in prostate gland tissue. Four years later he concluded that prostaglandin was a lipid soluble fatty acid probably containing a double bond and a hydroxyl group (Von Euler, 1939)

Inadequate analytical techniques and the intervention of World War II slowed prostaglandin research for nearly 15 years. However with the encouragement of Von Euler, Bergström

resumed efforts at the Karolinska Institute after the war in order to purify the unknown factors from seminal fluid, using techniques such as paper chromatography, gas-liquid chromatography and countercurrent distribution. Bergström and Sjöval (1957) were then able to isolate one prostaglandin 'factor' (PGF) in crystalline form. By 1960 they were able to demonstrate the structures of prostaglandins F and E using techniques such as X-ray crystallography (Bergström and Sjöval, 1960 a, b). This firmly established that prostaglandin was not a single compound but a group of chemically related compounds known under the general term of prostanoids. The use of mass spectrometry (Bergström, Ryhage, Samuelsson and Sjöval, 1963) confirmed the identities of both prostaglandins $F_{1\alpha}$ and E_1.

Other groups of the prostanoid family include those of the A, B, C and D series. A mixture of prostaglandins A and B (PGA and PGB) and their 19-hydroxylated derivatives were initially measured in human seminal fluid (Bygdeman, Svanborg and Samuelsson, 1969). Since these earlier studies were performed on semen stored under haphazard conditions, controversy developed as to whether PGA and its isomer, PGB,were formed naturally by the seminal vesicles or by dehydration of PGE during the extraction techniques prior to analysis.

Later studies were performed on freshly collected semen or on semen samples immediately frozen after collection at -10°C. Also, prior to extraction, the unstable β-ketol systems in the PGE compounds were protected by oximation thus ensuring minimal degradation to the A or B series. Data from such

studies indicated that the PGA and PGB and their 19-hydroxylated derivatives were not detectable indicating that the PGA and PGB compounds monitored in the earlier studies had been formed by the decomposition of the PGE and 19-hydroxylated PGE compounds in semen (Taylor and Kelly, 1974)

There is no evidence as yet that PGA can be synthesised in the body from PGE. However PGA can be converted enzymatically to PGC. PGC is degraded to PGB by isomerisation in the body (Jones, Cammock and Horton 1972). Prostaglandins of the D series are isomers of the PGE compounds (Granstrom, Lands, and Samuelsson, 1968). Formerly PGD was regarded as an inactive byproduct of prostaglandin biosynthesis. This view has changed after the identification of several biological effects, including its ability to inhibit platelet aggregation (Smith, Silver, Ingerman and Kocsis, 1974).

Intermediates in PGE, PGF and PGD biosynthesis, namely PGG_2 and PGH_2 were isolated by Nugteren and Hazelhof (1973) and by Hamberg and Samuelsson (1973). Both groups used crude enzyme preparations from sheep vesicular glands in their biosyntheses. These intermediates have since been shown to be precursors to two other groups of compounds - the thromboxanes (TXs) (Hamberg, Svensson and Samuelsson, 1973) and prostacyclin (PGI) (Gryglewski, Bunting, Moncada, Flower and Vane, 1976).

Chemistry

The prostaglandins (PGs) are unsaturated hydroxy acids with a

five membered ring in a twenty carbon atom skeleton. Their structure is based on a monocarboxylic acid given the general name prostanoic acid, which is not known to be a naturally occurring substance. A large number of variants of the original substance have now been identified and they may be classified into nine types (PGA to PGI inclusive). They differ only in the position of double bonds and/or varying functional groups occurring usually within the cyclopentane ring, (see Fig.1.). The number of double bonds in the side chains are designated by a subscript numeral e.g. PGE_2 which has two double bonds in its side chains. PGs of the "1" series invariably have only one double bond in the trans Δ^{13-14} position. Members of the "2" series have another double bond in the cis Δ^{5-6} position whilst members of the "3" series have a further double bond in the trans Δ^{17-18} position. In the F series of PGs, an additional subscript refers to whether the C-9 hydroxyl group is above (α) or below (β) the plane of the cyclopentane ring i.e. denotes its isomeric form.

The degree of unsaturation of PGs is determined by the fatty acid precursor. For the "1" series this is cis-8,11,14-eicosatrienoic acid (dihomo-γ-linoleic acid, DHLA); for the "2" series this is arachidonic acid(cis-5,8,11,14-eicosatetraenoic acid, AA) and for the "3" series this is cis-5,8,11,14,17-eicosapentaenoic acid.

Since the "2" series are the major PGs found in most biological systems (the major exception being primate seminal fluid where PGs of the "1" and "2" series are found in equal quantities, see Kelly, 1978) it will be necessary to concentrate the discussion on the "2" series.

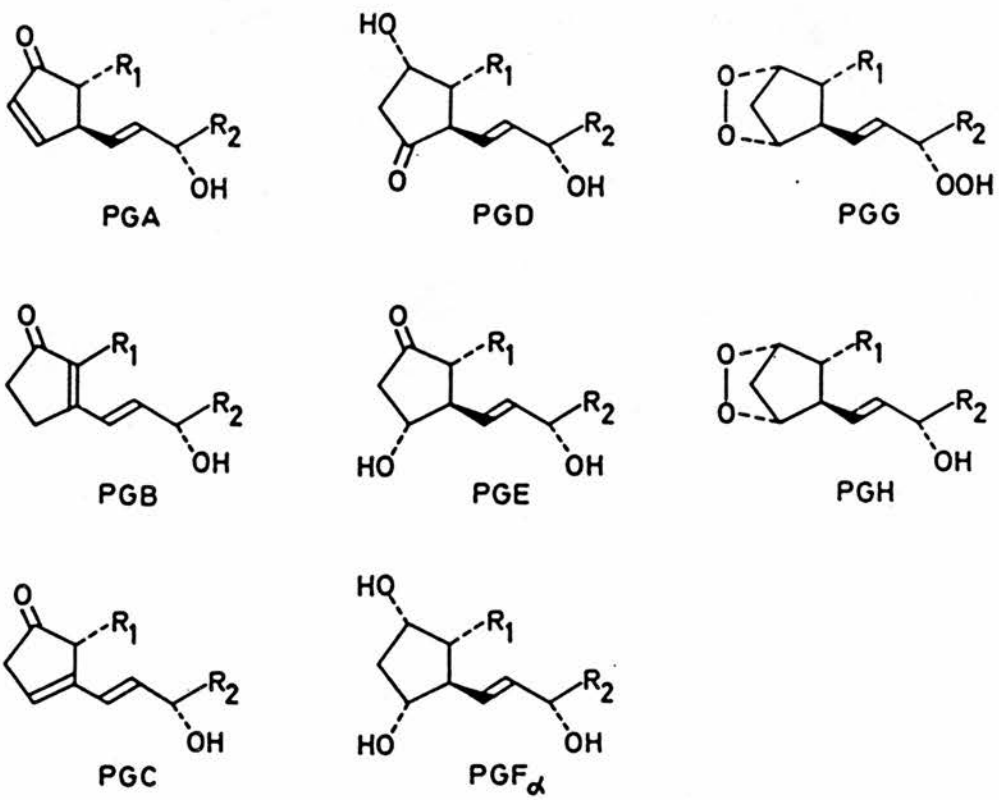


Figure 1. Nomenclature of prostaglandins A to H inclusive. Prostacyclin (PGI_2) is depicted in Fig. 2.

$R_1 = C_7 H_{11} O_2$ and $R_2 = C_5 H_{10}$

Biosynthesis

PGs are not stored within the cell so biosynthesis of them must precede their release. Biosynthesis of the "2" series PGs is dependent upon the availability of the precursor, namely arachidonic acid. Once released the arachidonic acid is converted to prostaglandins and thromboxanes by prostaglandin synthetase, a term commonly used to describe a microsomal complex of enzymes. The first step is catalysed by cyclooxygenase which converts arachidonic acid to the cyclic endoperoxide, 15-hydroperoxide compound, namely PGG_2 (by the dioxygenase component of the enzyme). The cyclic endoperoxide is rapidly converted to its 15-hydroxy analogue, PGH_2 (by the peroxidase component of the enzyme). PGH_2 is the key intermediate in the biosynthesis of the prostaglandins and thromboxanes (Fig. 2). The precise nature of the products formed depends upon the tissue under study. For instance in platelets thromboxane A_2 (TXA_2) is the major product (Hamberg et al., 1975) whereas in aortic tissue prostacyclin (PGI_2) is formed primarily (Gryglewski et al., 1976).

Prostaglandin cyclooxygenase is common to all tissues, so all tissues in the body have the capacity for synthesising PGs. Studies on the oxygenation process of this enzyme demonstrated an unusual kinetic pattern (Smith and Lands, 1972). Utilisation of arachidonic acid accelerated until a maximum velocity was reached and then it dropped to zero velocity gradually. Addition of fresh enzyme to the reaction mixture caused further biosynthesis of PGs. The deceleration and cessation of biosynthesis indicated an inactivation of

the enzyme i.e. the enzyme was "self-limiting" or "suicidal" in behaviour. Further investigations revealed that the prosthetic group, haem, contained within the enzymes was destroyed at a much slower rate than the enzyme (Hemler and Lands, 1980). Therefore the inactivation of the enzyme was due to some direct action on the enzyme rather than destruction of haem per se. In addition glutathione peroxidase was shown to inhibit cyclooxygenase activity (Smith and Lands, 1972). This finding, together with the observation that the induction period required for the oxygenation of arachidonic acid was reduced by the incorporation of hydroperoxy acids in the medium, implied that PGG_2 (a hydroxyperoxy acid) acted as a cofactor in its own biosynthesis (see Lands, 1981).

The major class of inhibitors of cyclooxygenase i.e. the non-steroidal, anti-inflammatory drugs such as aspirin and indomethacin, cause reversible inhibition of the cyclooxygenase enzyme by competing with the substrate (arachidonic acid) for the catalytic site (Vane, 1971). Aspirin also inhibits the cyclooxygenase by transferring an acetyl group to a serine residue at the active site; the action was shown to be irreversible (Roth and Majerus, 1975). Indomethacin also has a time dependent irreversible action, which is dependent upon the presence of a free carboxyl moiety and of a halogen atom in the indomethacin molecule (Rome and Lands, 1975).

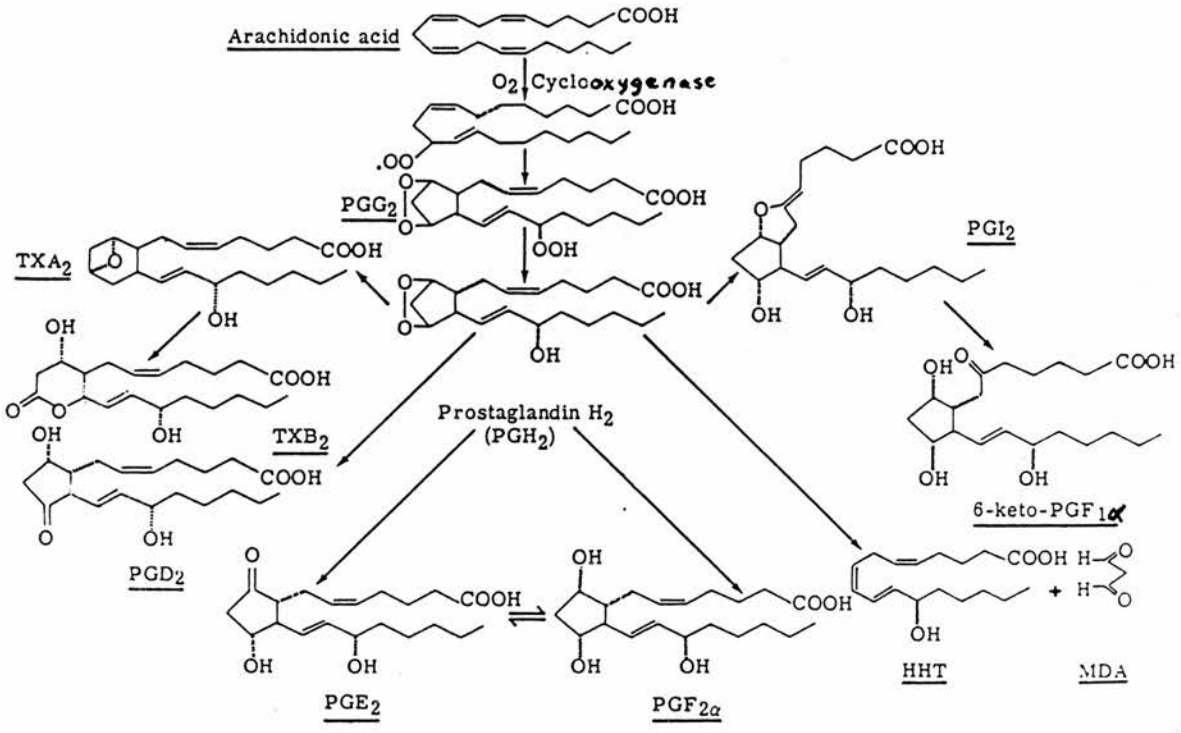


Figure 2 Major routes of biosynthesis of prostaglandins (PGs) and thromboxanes (TX) of the 2-series. 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA) may also be formed from arachidonic acid.

Both cyclic endoperoxide intermediates resulting from cyclooxygenase activity are stable for several months in dry acetone at -20°C but they decompose rapidly upon addition of hydroxylic solvents. The half life of PGH_2 and PGG_2 in aqueous medium at 37°C is about 5 min (Hamberg, Svensson, Wakabayashi and Samuelsson, 1974).

Action of an isomerase enzyme on PGH_2 results in PGE_2 while a reduction step, possibly non enzymatic, on PGH_2 yields $PGF_{2\alpha}$. The enzyme thromboxane A_2 synthase converts PGH_2 to TXA_2, malondialdehyde (MDA) and 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (Hammarstrom and Falardeau, 1977). In the presence of water TXA_2 is rapidly converted to the relatively inactive but stable thromboxane B_2 (TXB_2) (Hamberg et al., 1975). Another enzyme, prostaglandin I_2 synthase, converts PGH_2 to the labile but biologically active PGI_2. This prostanoid is unstable in aqueous solution and rapidly yields the biologically inactive but stable 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$F_{1\alpha}$). PGD_2, an isomer of PGE_2 (Granstrom et al., 1968) is also formed enzymatically from PGH_2 (Fig. 2)

The synthesis and release of prostaglandins from many intact tissues has been examined and one organ which has received much attention is the uterus. The main reason for this is that in many non-primate mammalian species, the uterus controls the life-span of the corpus luteum.

Uterine Luteolytic Hormone (luteolysin)

In 1923, Loeb carried out investigations into the importance of the uterus in controlling the length of the oestrous cycle. He demonstrated that hysterectomy in the guinea pig prevented regression of the corpus luteum resulting in a prolonged oestrous cycle of 60 to 120 days. Loeb suggested that "it is possible that the uterus, in particular its mucosa, produced an internal secretion which exerted a specific, abbreviating effect on the life of the corpus luteum " (Loeb, 1927). It was hypothesised therefore that, in the guinea pig, there existed a uterine luteolytic hormone with the ability to cause the corpus luteum to regress.

The total removal of the uterus with subsequent prolonged luteal function has also been demonstrated in the cow (Wiltbank and Casida, 1956 ; Anderson, Neal and Melampy, 1962), the mare (Hughes, Stabenfeldt and Evans, 1977), the sheep (Wiltbank and Casida, 1956), the pig (Spies, Zimmerman, Self and Casida, 1958), the pseudopregnant rat (Bradbury,1937), the rabbit (Asdell and Hammond, 1933) and the hamster (Caldwell, Mazer and Wright, 1967).

Treatment resulting in the destruction of the endometrium in the pig often caused luteal maintenance (Anderson, Butcher and Melampy, 1961) suggesting that the luteolytic hormone was probably secreted by the endometrial cells of the uterus.

In certain species such as the Macacus rhesus monkey (Burford and Diddle, 1936) and most notably the human (Jones and Telinde, 1961; Beavis, Brown and Smith, 1969) the removal of

the uterus had no effect on cyclic ovarian function. It is probable that in these primate species the uterus exerts no control over the life span of the corpus luteum.

Between 1929 and 1969 attempts to isolate the luteolytic principle from the uterus of those species which apparently produced it were unsuccessful. However several important characteristics of the uterine - luteal relationship were established. One of the most striking features was that in many species the uterine horn exerted an influence over its adjacent ovary only. Thus in the hemi-hysterectomised animal, the corpora lutea were maintained in the ovary adjacent to the hemi-hysterectomy and normal luteal regression occurred in the ovary ipsilateral to the retained horn. The local effect of the luteolysin was demonstrated in the ewe (Inskeep and Butcher, 1966), guinea pig (Bland and Donovan, 1966), pseudopregnant rat (Barley, Butcher and Inskeep, 1966) and hamster (Orsini, 1968). An exception was found in the cycling pig where normal bilateral regression continued to occur after hemi-hysterectomy. However, when only one quarter of one horn was left intact corpora lutea on the other side did not now regress (Anderson et al., 1961). The local nature of the uterine luteolytic hormone seemed to indicate that the pituitary was not involved in the uterine - luteal relationship (Bland and Donovan, 1966).

In 1969, $PGF_{2\alpha}$ was found to mimic <u>in vivo</u> the effect of the luteolytic hormone in the guinea pig (Blatchley and Donovan, 1969), the rat (Pharriss and Wyngarden,1969) and the rabbit (Gutknecht, Cornette and Pharriss, 1969). $PGF_{2\alpha}$ (1mg/kg/day) was infused into the uterine lumen of

pseudopregnant rats for two days (Days 5 and 6) and the progesterone content of the ovaries of these animals was compared to that of animals receiving only saline. It was observed that the progesterone levels were decreased compared to the saline controls (Pharriss and Wyngarden, 1969). Therefore administration of $PGF_{2\alpha}$ at a time when the corpora lutea had become established and functional resulted in morphological regression of the corpus luteum and a decrease in ovarian progesterone levels.

Subsequently, $PGF_{2\alpha}$ was shown to be luteolytic in other non-primate, mammalian species including the sheep (McCracken, Glew and Scaramuzzi, 1970), the cow (Liehr, Marion and Olsen, 1972; Lauderdale, 1972), the pig (Gleeson, 1974) and the mare (Noden, Oxendir and Hafs, 1974)

Evidence That $PGF_{2\alpha}$ Is the Luteolytic Hormone

To determine whether $PGF_{2\alpha}$ was indeed the uterine luteolytic hormone, it was necessary to demonstrate that luteal regression occurredafter $PGF_{2\alpha}$ had been released from the uterus in the normal and experimental situation. The levels of $PGF_{2\alpha}$ in the uterine venous blood were shown to increase towards the end of the oestrous cycle just prior to luteal regression in the sheep (Bland, Horton and Poyser, 1971; McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson, 1972), the guinea pig (Blatchley, Donovan, Horton and Poyser, 1972), the pig (Gleeson and Thorburn, 1973; Gleeson, Thorburn and Cox, 1974) and the cow (Nancarrow, Buckmaster, Chamley, Cox, Cumming, Cummins, Drinan, Findlay, Goding, Restall, Schneider and Thorburn, 1973). The levels

of $PGF_{2\alpha}$ in the uterine venous blood of these four species increased from about Day 11 although this timing was not invariable. Significant increases were usually evident by Day 13 and luteal regression in these species was usually complete by Days 16 to 18. Elevated $PGF_{2\alpha}$ levels were maintained throughout this period.

Bland and Donovan (1966) demonstrated that the insertion of foreign bodies such as glass beads into the uterine lumen of guinea pigs on Days 2 to 4 of the cycle caused luteal regression to occur between Days 9 to 11. In such guinea pigs, an increase in utero-ovarian $PGF_{2\alpha}$ occurred by Day 8 and reached a peak by Day 12 when utero-ovarian plasma progesterone levels were greatly reduced (Blatchley, Donovan and Poyser, 1976). The pattern of $PGF_{2\alpha}$ secretion was similar to and of the same order of magnitude as that found during the normal oestrous cycle of the guinea pig (Earthy, Bishop and Flack, 1975). Consequently, premature regression induced by intra-uterine beads is associated with early release of $PGF_{2\alpha}$ from the uterus. The production of $PGF_{2\alpha}$ by the uterus in response to stimuli such as beads and intra-uterine devices was demonstrated also in the sheep (Spilman and Duby, 1972) rat and hamster (Saksena, Lau and Castracane, 1974).

If $PGF_{2\alpha}$ was indeed the uterine luteolytic hormone, it was necessary to determine whether the inhibition of uterine prostaglandin synthesis prevented normal luteal regression and elongated the oestrous cycle. Inhibition of $PGF_{2\alpha}$ synthesis was brought about by indomethacin, a potent

inhibitor of the cyclooxygenase enzyme (Vane, 1971). A slow-release preparation of indomethacin placed in the uterine lumen increased oestrous cycle length in guinea pigs (Horton and Poyser, 1973; Marley, 1973). Oral or parenteral administration of indomethacin to guinea pigs was not really as effective as local administration of the drug (Marley, 1972; Horton and Poyser, 1973). A subsequent study (Poyser and Horton, 1975) showed that intra-uterine administration of indomethacin maintained high plasma progesterone concentrations for much longer than normal indicating that regression of the corpora lutea had been prevented. Therefore, it was probable that the increase in cycle length induced by indomethacin treatment was due to inhibition of uterine prostaglandin synthesis. Indomethacin treatment was also able to prolong the corpora luteal life span in other species including the rabbit (O'Grady, Caldwell, Auletta and Speroff, 1972) and the rat (Chatterjee, 1973).

Active immunisation against $PGF_{2\alpha}$ resulted in elongation of the oestrous cycle in several species; it is probable that the antibodies effectively neutralised $PGF_{2\alpha}$ when released from the uterus into the vascular system. In the guinea pig (Horton and Poyser, 1974) animals with high anti-$PGF_{2\alpha}$ titres had their oestrous cycles prolonged to a greater extent than those animals with lower antibody titres. Immunisation against $PGF_{2\alpha}$ also prevented luteal regression in the sheep (Scaramuzzi, Baird, Wheeler and Land, 1973).

To summarise, the accumulated data has demonstrated that:

i) $PGF_{2\alpha}$ is present in the uterine vein at the time of

luteolysis in greater amounts than during the earlier stages of the cycle, the increase preceding luteolysis in the guinea pig, sheep, cow and pig

ii) the insertion of foreign bodies such as glass beads into the uterine lumen causes an increase in $PGF_{2\alpha}$ output from the uterus, which is followed by premature luteal regression in the guinea pig, sheep, rat and hamster

iii) indomethacin treatment prolongs luteal function in the guinea pig, rabbit and rat

(iv) immunisation against $PGF_{2\alpha}$, either active or passive, prolongs the luteal life span in the guinea pig and sheep.

Overall the findings have provided much evidence in favour of $PGF_{2\alpha}$ being the uterine luteolytic hormone.

Transfer of $PGF_{2\alpha}$ From the Uterus to the Ovary

The means by which $PGF_{2\alpha}$ travels from the uterus to instigate an effect on the corpora lutea of the ovary probably involves the vascular system. Ligation of the vascular tissue in the region of the uterus and ovaries in guinea pigs caused maintenance of the corpora lutea but ligation and removal of the oviduct had no effect on luteal regression (Bland and Donovan, 1969; Oxenreider and Day, 1967). In the sheep, bilateral ligation of the middle uterine arteries and veins caused luteal maintenance but ligation of the arteries alone were without effect (Kiracoffe, Menzies, Gier and Spies, 1966; Kiracoffe, Spies and Gier, 1973). These observations indicated that an intact uterine vein is necessary for luteal

regression to occur. Further investigations by McCracken et al. (1972) were carried out in the sheep; tritiated $PGF_{2\alpha}$ (^{3}H-$PGF_{2\alpha}$) was infused into the uterine vein at a point proximal to its confluence with the utero-ovarian vein. A small but significant increase in radioactivity was monitored in the ovarian arterial blood after 20 min. During the next 60 min this increase continued to rise and peak levels occurred approximately 20 to 30 min after termination of the infusion. The data indicated that there was a local vascular transfer of $PGF_{2\alpha}$ between the utero-ovarian vein and ovarian artery enabling $PGF_{2\alpha}$ to pass from one uterine horn to its adjacent ovary. The efficiency of transfer was estimated to be about 2%. Horton and Poyser (1972) stated that since the ability of most tissues with the exception of the lungs, liver and kidney, to metabolise prostaglandins is very low then "most of the $PGF_{2\alpha}$ reaching the ovary via the ovarian artery will eventually have to leave the ovary via the utero-ovarian vein and will mix with the fresh $PGF_{2\alpha}$ coming from the uterus. If the transfer process were 100% and metabolism were nil, $PGF_{2\alpha}$ would pass through the ovary ad infinitum. Also the amount passing through would increase steadily. In order to prevent a situation like this from arising a fairly low efficiency for transfer would seem necessary". Any $PGF_{2\alpha}$ not retained by the ovarian artery/utero-ovarian vein countercurrent mechanism will pass into the systemic circulation and be inactivated in the lungs (Ferreira and Vane, 1967). This countercurrent mechanism has been demonstrated in two species namely the sheep (McCracken et al., 1972) and the cow (Hixon and Hansel, 1974). In species such as the rabbit where the uterine

vasculature is anatomically more independent from the ovarian vasculature, a countercurrent mechanism cannot explain the transfer of $PGF_{2\alpha}$ from the uterus to the ovary (Hunter and Casida, 1967). It has been reported that the vasculature of the guinea pig, rat, hamster and pig is such as to allow a counter current mechanism of $PGF_{2\alpha}$ to exist (Del Campo and Ginther, 1972 and 1973). However Egund and Carter (1974) reported that no special arrangement for countercurrent exchange could be demonstrated in the guinea pig. To date the counter current mechanism has not been demonstrated in any of these species, or in fact how $PGF_{2\alpha}$ does reach the ovary from the uterus in a local manner.

Mechanism Of Action Of $PGF_{2\alpha}$ In Inducing Luteolysis

It was originally suggested that the potent venoconstrictor properties of $PGF_{2\alpha}$ (DuCharme, Weeks and Montgomery, 1968) on the utero-ovarian vein may result in a reduced ovarian blood flow with luteolysis ensuing as a consequence of anoxia (Pharriss, Cornette and Gutknecht, 1970). This hypothesis was not proven in practise since, in the sheep, $PGF_{2\alpha}$ infused directly into the ovary resulted in no reduction of total ovarian blood flow but there was a rapid decline in progesterone output (McCracken et al., 1970; Baird, 1974). However, $PGF_{2\alpha}$ was shown to selectively reduce blood flow to the corpus luteum in the sheep (Thorburn and Hales, 1972) without affecting total ovarian blood flow at the time of luteal regression in the oestrous cycle. These observations suggested that reduced luteal blood flow may be the cause of luteal regression. Subsequent investigations by Einer-

Jensen and McCracken (1977) demonstrated that the decrease in progesterone output from the corpus luteum preceded the reduction in luteal blood flow. Therefore the luteolytic action of $PGF_{2\alpha}$ probably resulted as a direct consequence of the hormone's effect on metabolic functions within the corpus luteum and not due to changes in blood flow. The reduced luteal blood flow occurring later was probably the result of luteolysis which caused the small luteal capillaries to become blocked with cell debris (O'Shea, Nightingale and Chamley, 1977). This blockage possibly accelerated luteal regression.

$PGF_{2\alpha}$ receptors in the corpus luteum in relation to the luteolytic activity of $PGF_{2\alpha}$ have been studied also. Specific $PGF_{2\alpha}$ receptors were found to be present in the luteal cell membrane in the sheep (Powell, Hammerstrom and Samuelsson, 1974), the rat (Wright, Luborskymoore and Behrman, 1979), the mare (Kimball and Wyngarden, 1977) and the cow (Rao, Carman and Gorman, 1978), and also in the human even though $PGF_{2\alpha}$ is not luteolytic <u>in vivo</u> in this species (Powell, Hammerstrom, Samuelsson and Sjoberg, 1974). The apparent Kd varied between 5 and 100nM among the species and in the human there was also a lower affinity receptor of apparent Kd 500nM. The low affinity receptor in the human may explain why $PGF_{2\alpha}$ has very little effect in women <u>in vivo</u> (see, Poyser 1981). In non-primates, changes in the number and/or affinity of $PGF_{2\alpha}$ receptors during the oestrous cycle may account for the resistance of the corpus luteum to the luteolytic activity of $PGF_{2\alpha}$ during the early part of the cycle. Also changes in other parameter s such as the

ability (efficacy) of the $PGF_{2\alpha}$/receptor complex to induce luteolysis may increase as the cycle progresses; such changes may be hormonally induced. The luteolytic action of $PGF_{2\alpha}$ can be divided into two categories - the decrease in progesterone output from the ovary (functional luteolysis) and the regression of the corpus luteum (structural luteolysis).

Attention by investigators has been focussed largely on the functional luteolytic processes. Progesterone production by the luteal cell is known to be stimulated by the luteinizing hormone (LH). LH binds to specific receptors in the plasma membrane of the granulosa cell and stimulates the production of cyclic-adenosine monophosphate (c-AMP) by activation of the enzyme, adenylate cyclase. The c-AMP then interacts with protein receptors to activate a protein kinase (Garren, Gill, Masui and Walton, 1971). It has been suggested that the c-AMP binds to the regulatory unit of a protein receptor to cause dissociation of this receptor moiety from the catalytic unit thereby activating the protein kinase. Should the levels of c-AMP decrease then the regulatory unit recombines with the catalytic unit to suppress protein kinase activity (Henderson and McNatty, 1975). The protein kinase phosphorylates and thereby activates enzymes like cholesterol esterase. This enzyme is responsible for conversion of the cholesterol esters stored in the lipid droplets into free cholesterol which is then converted to progesterone.

It has been proposed that one of the earliest actions of $PGF_{2\alpha}$ on the corpus luteum, occurring within 15 min, is to

prevent the LH-induced increase in c-AMP levels (Lahair, Freud and Lindner, 1976). Subsequently the reduction in c-AMP production causes cholesterol esterase to be dephosphorylated to its inactive form thereby inhibiting the availability of cholesterol for conversion to progesterone (Henderson and McNatty, 1975). A decline in cholesterol esterase activity in the rat ovary after $PGF_{2\alpha}$ treatment has been monitored (Behrman, MacDonald and Greep, 1971), though a greater effect was observed on the activity of cholesterol ester synthetase. Nevertheless overall cholesterol turnover was probably reduced leading to inhibition of progesterone synthesis.

PGE_2 - stimulated progesterone production by the ovary was not inhibited by $PGF_{2\alpha}$ *in vitro* nor *in vivo* (McNutty, Henderson and Sawers, 1975; Henderson, Scaramuzzi and Baird, 1977). $PGF_{2\alpha}$ therefore specifically antagonises the action of LH, probably by preventing the LH - induced conversion of adenosine 5'-triphosphate (ATP) into c-AMP (Wakeling and Green, 1981). It has been proposed that $PGF_{2\alpha}$, after binding to its receptor on the luteal cell, either directly or indirectly uncouples the receptor unit for LH from the catalytic unit on the adenyl cyclase enzyme, thus preventing LH from causing a rise in c-AMP levels in the luteal cell (Henderson and McNatty, 1975). Administration of c-AMP to rat luteal cells cultured *in vitro* was able to overcome the inhibition in progesterone secretion produced by $PGF_{2\alpha}$ (Thomas, Dorflinger and Behrman, 1978).

The ability of $PGF_{2\alpha}$ to uncouple the LH receptor unit from the adenyl cyclase catalytic unit was expressed only in the

intact luteal cell and may be due to $PGF_{2\alpha}$ increasing calcium influx or causing a release of intracellular bound calcium which in turn prevents activation of adenyl cyclase (Dorflinger and Behrman as reported in Behrman, 1979).

$PGF_{2\alpha}$ also significantly reduced gonadotrophin accumulation in the functional rat corpora lutea and a maximum effect was observed within 2hr; a 63% reduction in gonadotrophin uptake was observed as early as 30 min after $PGF_{2\alpha}$ treatment. Plasma progesterone levels were decreased by 47% after 30 min and by 83% after 2hr. The decrease of gonadotrophin uptake was not due to loss of LH receptors or to a decrease in the affinity of the receptor for LH. However after 8hr of $PGF_{2\alpha}$ treatment there was a 30% reduction in the number of LH receptors with 72% loss occurring after 24hr of treatment. It has been proposed that the luteolytic effect of $PGF_{2\alpha}$ is due initially to a rapid loss in the ability of the corpus luteum to respond to and to accumulate LH, although LH receptors of high affinity are still present, than to an actual loss of LH receptors thus making luteolysis irreversible (Behrman, Grinwick, Hichens and MacDonald, 1978).

A detailed analysis of the luteolytic action of $PGF_{2\alpha}$ in species other than the rat is lacking. In hysterectomised guinea pigs, $PGF_{2\alpha}$ treatment caused plasma progesterone and pregnenolone levels to decrease (Dwyer and Church, 1979a). As in the rat, the cholesterol esterase activity in the guinea pig ovary was reduced by $PGF_{2\alpha}$ treatment (Dwyer and Church, 1979b). Although cholesterol ester synthetase

activity was unaffected, ovarian steroid output was still reduced. The events described so far relate only to biochemical changes occurring during functional luteolysis. Since $PGF_{2\alpha}$ increases the rate of release of enzymes from lysosomes in luteinized rat ovaries (Weiner and Kaley, 1975), it has been suggested that these enzymes are responsible for the structural changes during luteolysis (McClellan, Abel and Niswender, 1977).

The Physiological Stimulus For Uterine $PGF_{2\alpha}$ Release

Once the role of $PGF_{2\alpha}$ as the uterine luteolysin was established, it was necessary to determine the physiological stimulus for $PGF_{2\alpha}$ output from the uterus. The cyclical pattern of release suggested that some form of hormonal control was responsible. As exogenous oestradiol induced premature luteolysis (Blatchley et al., 1972), it was proposed that perhaps endogenous oestradiol released from the developing follicles in the ovaries was the physiological stimulus for $PGF_{2\alpha}$ release from the uterus and the normal occurrence of luteal regression (Blatchley, Donovan, Horton and Poyser, 1972).

A small rise in the plasma oestradiol levels after about Day 10 of the cycle just prior to the first release of $PGF_{2\alpha}$ from the uterus has been demonstrated in the sheep (Barcikowski, Carlson, Wilson and McCracken, 1974; Cox, Thorburn, Currie and Restall, 1974), the guinea pig (Joshi, Watson and Labhsetwar, 1973), the pseudopregnant rat (Welschen, Osman, Dullaart, de Greef, Uilenbrook and de Jong, 1975) and the pig (Henricks, Guthrie and Handlin, 1972). In the cow, $PGF_{2\alpha}$

release from the uterus has been associated with increased oestradiol output from the ovary during luteolysis (Shemesh, Ayalon and Lindner, 1972; Nancarrow et al., 1973). The overall data suggest that the release of oestradiol from the ovary and the subsequent increased $PGF_{2\alpha}$ output from the uterus is related.

In ovariectomised animals oestradiol had little or no effect in stimulating $PGF_{2\alpha}$ release from the uterus. However if the ovariectomised animal was pre-treated with progesterone, oestradiol was then able to cause a much greater release of $PGF_{2\alpha}$ from the uterus as demonstrated in the guinea pig (Blatchley and Poyser, 1974) and the sheep (Caldwell, Tillson, Brock and Speroff, 1972). It was postulated, therefore, that oestradiol acting on the progesterone-primed uterus is the physiological stimulus for $PGF_{2\alpha}$ synthesis by, and release from, the uterus.

In the sheep the importance of progesterone priming of the uterus for $PGF_{2\alpha}$ release from the uterus was reflected in the observation that the small peaks of oestradiol occurring randomly in the oestrous cycle (a small peak consistently occur s at Days 3 to 4 of the cycle) are unable to stimulate $PGF_{2\alpha}$ release from the uterus (Cox et al., 1974). Progesterone priming for 7 to 9 days is probably necessary before oestradiol can induce $PGF_{2\alpha}$ release from the sheep uterus. However, other species such as the cow exhibit a small rise in plasma oestradiol levels on Days 4 to 5 of the cycle together with an increase in the level of $PGF_{2\alpha}$ in utero-ovarian venous plasma and a transient decline in plasma progesterone levels (Nancarrow et al., 1973). The overall

observations indicated that the uteri of different species exhibits different responses to oestradiol, since prolonged progesterone priming of the uterus is not always required. It is not yet clear why this occurs.

It has also been demonstrated that the joint administration of ovarian hormones to the sheep can cause progesterone under certain experimental conditions to antagonise the $PGF_{2\alpha}$ - releasing action of oestradiol on the uterus (Scaramuzzi, Baird, Boyle, Land and Wheeler, 1977). In cultured human and guinea pig endometrial tissue <u>in vitro</u>, progesterone reduced the basal $PGF_{2\alpha}$ output and, in the human, inhibited also its stimulation by oestradiol (Abel and Baird, 1980; Leaver and Seawright, 1982). The observations reported have indicated that, although progesterone priming of the uterus is necessary to obtain $PGF_{2\alpha}$ output from the uterus, too much progesterone acting for too long, results in inhibition and progesterone withdrawal is required to obtain the maximum release of $PGF_{2\alpha}$ from the uterus.

If oestradiol acting on the progesterone primed uterus is the necessary stimulus for complete luteolysis then immunisation against oestradiol should result in luteal maintenance. Passive immunisation of sheep against oestradiol was able to prevent oestrus and ovulation but not luteal regression (Fairclough, Smith and Peterson, 1976). This observation suggested that oestradiol is not the only requirement for normal luteolysis. Passive immunisation of sheep against progesterone was also unable to prevent luteolysis (Fairclough, Smith, Peterson and McGowan, 1976). These two

observations indicated that the ovarian steroids oestradiol and progesterone may not be the only stimuli for uterine $PGF_{2\alpha}$ output.

In 1906, Dale reported that intravenously injected extracts of the pituitary gland caused the uterus of the preganat cat to undergo powerful contractions (Dale, 1906). The active substance in these extracts, named oxytocin by Dale, has been implicated in the control of uterine $PGF_{2\alpha}$ synthesis and release by the uterus. Infusions of oxytocin into the uterine arterial supply of the sheep evoked a substantial increase in $PGF_{2\alpha}$ release from the uterus following administration after Day 14 of the cycle (Roberts, Barcikowski, Wilson, Skarnes and McCracken, 1975). Oxytocin treatment also stimulated $PGF_{2\alpha}$ release from the uterus of the cow (Newcomb, Booth and Rowson, 1977), the rat (Campos, Liggins and Seamark, 1980) and the rabbit (Small, Gavagan and Roberts, 1978). Since immunization of sheep against oxytocin delayed corpus luteal regression (Sheldrick, Mitchell and Flint, 1980), it has been proposed that oxytocin forms part of the physiological stimulus which increases $PGF_{2\alpha}$ release from the sheep uterus at the end of the oestrous cycle (McCracken, Schramm, Barcikowski and Wilson, 1981). Oxytocin has a negligible effect on uterine $PGF_{2\alpha}$ release in an anoestrous sheep unless the sheep has been primed with oestradiol (Sharma and Fitzpatrick, 1974). Since the relative concentrations of oxytocin receptors in the endometrium of the ovine uterus increased toward the end of the cycle (Wilson, Roberts and McCracken, 1974), it may be that oestradiol stimulates the production of the oxytocin

receptors (McCracken, 1980). The interaction of oxytocin with its receptors may then evoke PGF_{2x} synthesis and release by the uterus. *In vitro* studies using the guinea pig uterus demonstrated that oxytocin was unable to stimulate uterine $PGF_{2\alpha}$ output in this species (Poyser and Brydon, 1983). In addition oxytocin injected into guinea pigs during any part of the cycle failed to initiate premature corpus luteal regression (Donovan, 1961). The evidence suggests that oxytocin does not form part of the physiological stimulus in the guinea pig necessary for increased $PGF_{2\alpha}$ release at the end of the oestrous cycle. Therefore the requirement for oxytocin as part of the physiological stimulus for increased $PGF_{2\alpha}$ synthesis and release by the uterus varies from species to species.

Mechanism of Action of the Ovarian Hormones In Stimulating Uterine $PGF_{2\alpha}$ Output

The action of oestradiol in stimulating uterine $PGF_{2\alpha}$ production may be linked with fresh protein synthesis based on the known intracellular actions of oestradiol. Oestradiol enters a cell via a specific protein-mediated carrier process (Milgrom, Atger and Baulieu, 1977) and then becomes bound to a cytosol protein receptor (Gorski, Taft, Shyamala, Smith and Nolides, 1968). The resulting oestradiol-cytosol receptor complex is then able to attach itself to a nuclear acceptor structure. The steroid acts on the DNA within the nucleus to stimulate RNA synthesis. Small amounts of a "specific induced" protein are detectable 1hr after oestrogen administration. This protein stimulates the

production of general RNA which is detectable 2hr after administration. There is a subsequent increase in general protein levels and an accumulation of phospholipids (Mueller, Gorski and Aizawa, 1961). The intrauterine injection of actinomycin D in the guinea pig significantly prolonged luteal function and oestrous cycle length (Poyser, 1979). These results suggest that in the guinea pig the oestradiol acts on the progesterone-primed uterus to stimulate protein synthesis, by increasing DNA-dependent RNA synthesis and thereby increasing the concentration of enzymes involved in PG synthesis. Actinomycin D has been shown to prevent the increase in uterine PG synthetase levels normally observed at the end of the cycle (Poyser, 1979).

Oestrogen treatment of the ovariectomised rat was able to influence the ratio of PG products formed (Ham, Cirillo, Zanetti and Kuehl, 1975). Prior to hormone treatment, ovariectomy induced a tenfold increase in uterine tissue levels of PGE and a threefold increase in PGF levels. After oestrogen treatment there was a decrease in PGE levels and a parallel elevation in PGF levels.

In the normal cycling guinea pig, $PGF_{2\alpha}$ was the major PG synthesized by homogenates of the uterus together with lesser quantities of PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 i.e. there is a relatively specific stimulation of $PGF_{2\alpha}$ synthesising capacity toward the end of the cycle. Oestradiol had a similar effect on ovariectomised guinea pigs (Poyser, 1983a). Outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the superfused uterus of ovariectomised guinea pigs were all low (approximately 0.1 to 0.2 ng/min/uterine horn). $PGF_{2\alpha}$ was

released in greatest quantities followed by PGE_2 and then by 6-keto-$PGF_{1\alpha}$ (Poyser, 1983b). Progesterone treatment had no effect on PG output. Oestradiol treatment increased $PGF_{2\alpha}$ output sevenfold, but increased PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs 1.7-fold and 2.9-fold, respectively. Oestradiol treatment reversed the PGE_2 to 6-keto-$PGF_{1\alpha}$ ratio; progesterone priming before oestradiol treatment potentiated the stimulatory effect of oestradiol on $PGF_{2\alpha}$ release by up to twofold but had no such action on PGE_2 and 6-keto-$PGF_{1\alpha}$ release. The overall observations indicated that oestradiol has a dual action, namely increasing the uterine synthetic capacity of PGs and exerting a directional influence on the products formed.

It was subsequently noted that the output of $PGF_{2\alpha}$ from the guinea pig uterus varied 10- to 20-fold during the oestrous cycle (Blatchley et al., 1972; Earthy et al., 1975) yet there were only 2- to 3-fold fluctuations in the activity of the prostaglandin synthetase enzyme (Poyser, 1972; Wlodawer, Kindahl and Hamberg, 1976). Such results suggested that although oestradiol increased the synthetic capacity of the uterus for PGs (Poyser, 1983a), it also had an effect on other factors influencing uterine PG synthesis, some of which may control substrate availability. In the guinea pig, free arachidonic acid represented only 0.1% of the total arachidonic acid content of the uterus (Leaver and Poyser, 1981). Further analysis of uterine tissue indicated that the amount of esterified arachidonic acid available in the lipid membranes was about 1 mg/gm uterine tissue. The accumulated evidence suggested that oestradiol influenced the

availability of arachidonic acid from these lipid "stores", and the subsequent release of arachidonic acid was the rate-limiting step in prostaglandin biosynthesis. The role of progesterone in uterine $PGF_{2\alpha}$ synthesis was also considered. A period of progesterone pretreatment before oestrogen administration to the uterus resulted in maximal levels of $PGF_{2\alpha}$ in the utero-ovarian venous plasma of the guinea pig (Blatchley and Poyser, 1974), the sheep (Caldwell et al., 1972) and the mouse (Saksena and Lau, 1973). All the species studied were ovariectomized.

Since oestradiol is not totally responsible for the increase in $PGF_{2\alpha}$ output from the uterus, it is possible that progesterone aids oestradiol in its regulation of the mobilization and turnover of the prostaglandin precursor, arachidonic acid. Like oestradiol, progesterone is bound to a uterine cytosol receptor protein as demonstrated in many species including the guinea pig (Corval, Falk, Freifald and Barden, 1972), mouse and rat (Feil, Glasser, Toft and O'Malley, 1972). Progesterone has been shown to accumulate in the nucleus (Sar and Stumpf, 1974). Progesterone has been shown to specifically alter the chick oviduct gene expression to effect unique RNA transcriptions (O'Malley, McGuire, Kahler and Korlman, 1969). Such observations imply that progesterone probably has an effect on the synthesis and/or activation of the enzymes responsible for the mobilization and turnover of arachidonic acid. Consequently, Horton and Poyser (1976) stated that "both progesterone and oestrogen are necessary for the "switching on" of $PGF_{2\alpha}$ synthesis in which phospholipase A_2, cholesterol esterase or

triglyceride lipase are mobilised from their intracellular compartment(s). These in turn produce the free arachidonic acid. This is then converted to $PGF_{2\alpha}$ by the prostaglandin synthetase enzymes". The release of arachidonic acid is considered, therefore, to be the rate-limiting step in PG synthesis by the uterus (as in other tissues). Consequently, when considering how oestradiol acting on a progesterone-primed uterus stimulates $PGF_{2\alpha}$ release from the uterus for luteolysis, one must consider the factors controlling arachidonic acid turnover.

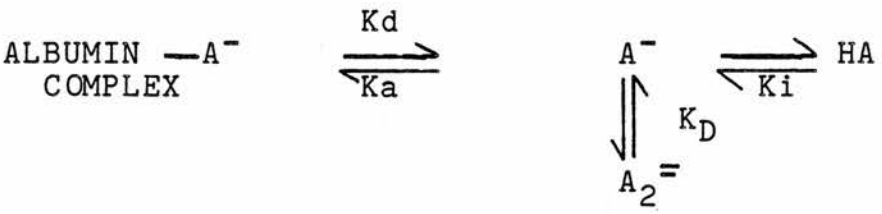
Nutritional Factors

Arachidonic acid is classified as an essential fatty acid i.e. it is required for reproduction and growth by biological systems (Burr and Burr, 1930). It is usually found in the meat and fish constituents of the diet and also in esoteric plant food such as seaweed. It may also be derived from linoleic acid via chain elongation and desaturation steps (see Gurr and James, 1971). Linoleic acid is an 18 carbon molecule containing two double bonds, one bond being six carbon atoms from the methyl terminus of the molecule i.e. 18:2 (n-6). Oxidation of linoleic acid (18:2 (n-6)) results in linolenic acid (18:3 (n-6)). Subsequent elongation and desaturation of linolenic acid results in arachidonic acid (20:4 (n-6)). The synthetase enzymes consist of tightly bound complexes of enzymes responsible for fatty acid synthesis. The lack of arachidonic acid in the body can seriously imbalance the physiological state of a subject. For example, Dyerberg and

Bang (1979) demonstrated the influence of diet at a physiological level comparing Greenland Eskimos (upholding a traditional cuisine) with a similar number of age- and sex-matched Danish controls. They showed that there was a significantly higher proportion of cis-5,8,11,14,17-eicosapentaenoic acid, which originates in Eskimo food, in the platelet lipids of the Eskimo group. Bleeding times were increased in all cases indicating that the aggregating properties of the platelets were affected by the presence of cis-5,8,11,14,17-eicosapentaenoic acid in the platelet lipids. Since the increased bleeding time discourages the formation of large blood clots it has been suggested that the difference in diets is indirectly responsible for the reduced incidence of thrombotic disorders in Greenland Eskimos (Dyerberg and Bang, 1979). Laboratory experiments have also supported the importance of a suitable dietary intake to maintain animals in a healthy, physiological condition. Willis, Comai, Kuhn and Paulsrud (1974) fed rats with dihomo-γ-linoleate, the substrate for prostaglandins of the "1" series. Since the type of PGs produced by any one cell depends on the type of fatty acid present in the lipid stores, more PGE_1 was produced by the platelets. The parallel decrease in production of TXA_2 also served to inhibit platelet aggregation. Therefore the lack of arachidonic acid in the diet can seriously affect biosynthesis of prostaglandins of the 2-series.

After digestion and intravascular lipolysis of chylomicrons or following release from adipose tissue, arachidonic acid passes readily into the blood. Previously it was assumed

that after ionization at physiological pH, the fatty acid binds immediately to albumin. However, Spector (1968) suggested that a small amount of unionised fatty acid and anionic dimers also exists in equilibrium with the i onised fatty acid, as shown below:



where, A^- = fatty acid anions

HA = unionized fatty acid

$A_2^=$ = anionic dimers

Kd = dissociation constant

Ka = association constant

Ki = ionisation constant

K_D = dimerization constant

The importance of the equilibrium of these free fatty acid forms is reflected in the fact that the existence of the anion appears to be essential for binding i.e. the bonds to albumin are not covalent. Thus, albumin has the ability to bind most of the available free fatty acid, thereby reducing the effective or unbound concentrations to levels that are not injurious to man.

It is important to note that long chain fatty acids present in blood plasma are also esterified into glycerides, phosphatides and cholesterol esters. These lipid esters are transported in combination with specific proteins, the complex being known as lipoprotein. They may act to replete the free fatty acid to albumin concentration, if the

equilibrium is disturbed (Nestel, Bezman and Havel, 1962).

Arachidonic Acid Transfer Into the Cell

Circumstantial evidence suggests that free fatty acid in unbound form crosses the cell membrane, since its rate of efflux from the plasma into the cell far exceeds that of the albumin (Havel and Frederickson, 1956; Gordon, 1960). The driving "force" that moves free fatty acids in the direction of the utilizing cell is a concentration gradient maintained because the cell can rapidly metabolize the fatty acids presented to it. Uptake of free fatty acids cannot be dependent on metabolically produced energy, for preincubation of Ehrlick Ascites tumour cells with 2,4-dinitrophenol which uncouples oxidative phosphorylation, or with cyanide which inhibits oxidative phosphorylation does not decrease the incorporation of palmitate-1-^{14}C into the cell free fatty acid pool (Spector, Steinberg and Tanaka, 1965). The rate of incorporation indicated a linear, passive mode of uptake, such as diffusion. Once within the cell, arachidonic acid is then available for prostaglandin biosynthesis. However free fatty acid levels in the uterine cells are low; in the guinea pig there is only about 1µg free arachidonic acid available per gm uterine tissue (Leaver and Poyser, 1981). Spector (1968), using prelabelled Ehrlick Ascites tumour cells, demonstrated that exogenous free fatty acid which was quickly incorporated into the cell free fatty acid pool was made available for oxidation, for release from the cell or for esterification into membrane lipid pools. The last alternative may be considered as a form of "storage container" since as much as 1mg arachidonic acid per gram

tissue is esterified in the guinea pig uterus (Leaver and Poyser, 1981).

The lipids can be classified into two types, neutral lipids and phospholipids, either one or both classes could act as the source of arachidonic acid for prostaglandin biosynthesis.

Neutral Lipids

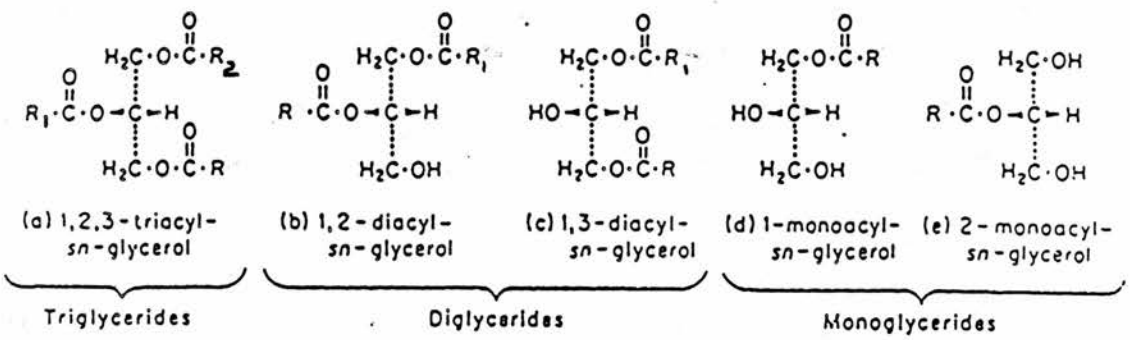
The neutral lipids are mainly composed of esters of glycerol and long chain fatty acids; the three hydroxyl groups of glycerol offer many combinations for fatty acid ester formation. The presence of one, two or three acyl groups attached to the glycerol backbone results in monoacylglycerol (monoglyceride), diacylglycerol (diglyceride) and triacylglycerol (triglyceride) respectively. The structural formulas are shown in Fig. 3. According to convention, the three carbon atoms of glycerol are identified as α, β and α_1 or 1, 2 and 3. Arachidonic acid may be esterified at any of these three carbon atom positions. The neutral lipids also include the cholesterol esters. They are formed by the addition of a fatty acid like arachidonic acid to the hydroxyl group at the carbon-3 position of cholesterol. The structures of cholesterol ester and of the parent compound cholesterol are depicted in Fig. 3.

It is important to consider the synthesis of the neutral lipids at this point in relation to the esterification of arachidonic acid.

The biosynthesis of diglycerides and triglycerides involves an inital phosphorylation of glycerol by ATP to form L-α-glycerophosphate (Bublitz and Kennedy, 1954). Before the incorporation of a fatty acid such as arachidonic acid can proceed, it is necessary to convert the fatty acid to its coenzyme A derivative. The reaction is catalyzed by an enzyme called thiokinase (acid: CoA ligase [AMP] or acyl-CoA synthetase). There are several thiokinases, each specific for a group of fatty acids with a certain range of chain lengths. A detailed study of the factors needed for phosphatidate synthesis by a "residue fraction" (principally microsomal) from guinea pig liver led Kornberg and Pricer (1953) to conclude that straight chain fatty acids with 16, 17 or 18 carbon atoms were far more effective than shorter or longer chain acids for esterification of glycerophosphate. The fatty acid reacts with ATP releasing pyrophosphate (PPi) and an intermediate called fatty-acyl AMP.

$$R.\ COOH + ATP \rightleftharpoons R.\ CO\text{-}AMP + PPi$$

where R = fatty acid chain



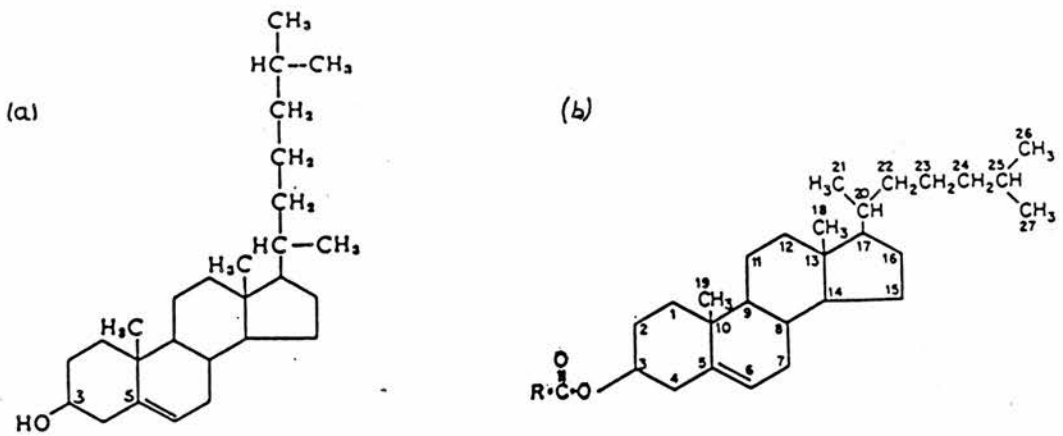


Figure 3. Structures of neutral lipids including cholesterol (a) and its ester (b), where R, R_1 and R_2 are the fatty acid carbon chains.

The intermediate then reacts with coenzyme A (CoASH) to liberate AMP.

$$R\ CO\text{-}AMP + CoA.SH \rightleftharpoons R.CO.S\text{-}CoA + AMP$$

Subsequent esterification of L-α-glycerophosphate, using the enzyme glycerol-3 phosphate acyl transferase with two coenzyme A derivatives of long chain fatty acids results in phosphatidic acid and two coenzyme A molecules (CoASH) (Kornberg and Pricer, 1953). Although phosphatidic acids are rapidly synthesized by enzyme systems <u>in vitro</u> the concentration of phosphatidic acid in mammalian tissues is very low. However their presence has been detected (Hokin and Hokin, 1958). The failure of phosphatidic acids to accumulate in tissues is due to the cleavage of the phosphate group (Pi) from phosphatidic acid by the enzyme phosphatidate phosphohydrolase to leave the 1, 2 diglyceride (Smith, Weiss and Kennedy, 1957). The addition of a final coenzyme A derivative of a long chain fatty acid to the 1, 2 diglyceride results in the synthesis of triglyceride and coenzyme A (Weiss and Kennedy, 1956). See Fig. 4. This last reaction is also catalysed by an acyltransferase.

An alternative route of formation of triglycerides involves the monoglyceride, formed by the hydrolysis of triglyceride (from the diet) to diglyceride to monoglyceride by action of the enzyme lipase which is present in the intestinal lumen (Borgstrom, 1954). The route is known as the monoglyceride pathway and involves the acylation of monoglyceride by the CoA derivative of a fatty acid using the enzyme monoglyceride acyltransferase. A second CoA derivative of a fatty acid is

then added to the resultant diglyceride to form a triglyceride (see Hübscher, 1970).

Thus,

$$\text{monoglyceride} \xrightarrow[\text{R.CO-S.CoA}]{1} \text{diglyceride} \xrightarrow[\text{R.CO-S.CoA}]{2} \text{triglyceride}$$

where 1 = monoglyceride acyltransferase

2 = diglyceride acyltransferase

The enzymes involved in triglyceride synthesis form a multi-enzyme complex called the triglyceride synthetase.

Esterification of cholesterol by a fatty acid such as arachidonic acid is brought about by three main types of enzyme. The first type of enzyme catalyses the reversible reaction:

$$\text{cholesterol} + \text{non-esterified fatty acid} \rightleftharpoons \text{cholesterol ester}$$

The enzyme ,sterol ester hydrolase, sometimes called cholesterol esterase does not require ATP or coenzyme A, and is present in the pancreas and the intestinal mucosa (see Goodman, 1965).

The second enzyme, acyl-CoA:cholesterol-O-acyl transferase is found in liver and adrenal gland. Unlike sterol ester hydrolase, this enzyme requires ATP and coenzyme A to catalyze:

$$\text{Acyl-CoA} + \text{cholesterol} \longrightarrow \text{cholesterol ester}$$

(Mukherjee, Kunitake and Alfin-Slater, 1958)

The third enzyme, 1,2-diacylglycerylphosphoryl-choline:cholesterol-O-acyl transferase is present in the plasma (Glomset, 1962). It has the ability to transfer fatty acids from phosphatidyl choline to cholesterol.

The reactions stated above indicate that there are many routes by which arachidonic acid is incorporated into the neutral lipids. The contribution of arachidonic acid from the neutral lipid stores for prostaglandin synthesis by and release from various tissues has been difficult to assess. Lipases are responsible for hydrolysing the ester bonds of triglycerides and esterases act on cholesterol ester to release arachidonic acid (see Gurr and James, 1971). In tissues where the mass of neutral lipids matches or exceeds that of phospholipids, such as adrenal cortex or adipose tissue, it is possible that there is a significant contribution of arachidonic acid contained within neutral lipids to the free arachidonate pool (Christ and Nugteren, 1970; Vahouny, Chamberbhan, Hodges and Treadwell, 1978). On the other hand, in rabbit polymorphonuclear leucocytes, it is not possible to determine to what extent the neutral lipids contributed to arachidonate release because even a small (unquantifiable) phospholipid breakdown resulting in arachidonate release would have exceeded the arachidonate loss from neutral lipids (Kaplan-Harris and Elsbach, 1980).

It is therefore necessary to consider the pathways by which arachidonic acid is incorporated into the phospholipids.

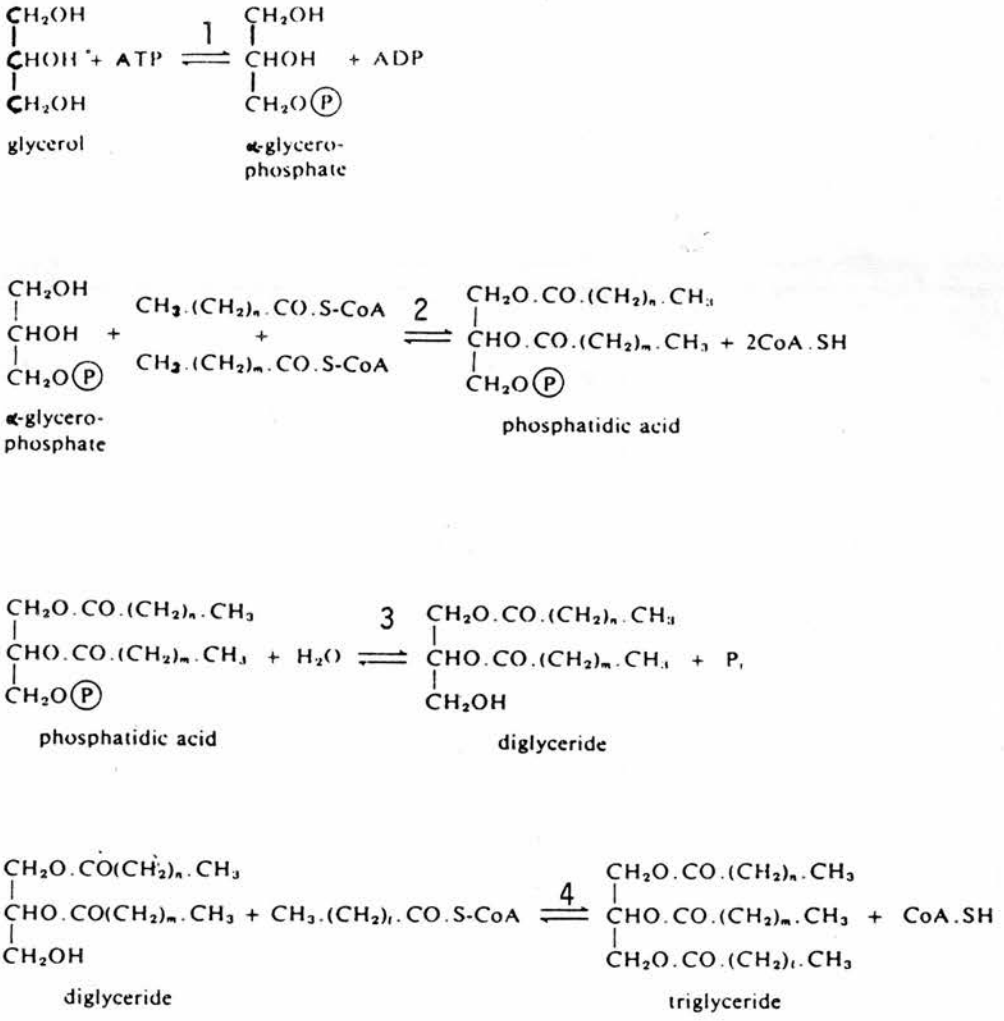


Figure 4. Biosynthetic steps leading to the formation of diglycerides and triglycerides

where 1 = reaction catalysed by glycerokinase
2 =reaction catalysed byglycerol-3phosphate:acyl transferase
3 = reactioncatalysed by phosphatidate phosphohydrolase
4 = reaction catalysed by acyltransferase

Phospholipids

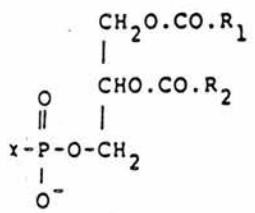
The major phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (Sph) (see Fig. 5). All the compounds have a 3-carbon "backbone" structure which is a derivative of glycerol. According to convention the three carbon atoms are identified as α,β and α_1, or 1, 2 and 3. The glyceryl esters of two fatty acid ester molecules occur at the 1- and 2-carbon positions, and esterified at the 3-carbon position is a phosphate group. The phosphate group in turn is joined by an ester linkage to a nitrogenous base such as choline or ethanolamine, from which the name of the phospholipid is derived. Unsaturated fatty acids like arachidonic acid are preferentially esterified at the 2-carbon position (Lands and Merk 1, 1963).

In sphingomyelin, a long chain alcohol called sphingosine is bound by an amide linkage to a long chain fatty acid and by ester linkage to phosphorylcholine, (see Fig. 5).

Studies of the biosynthesis of phospholipids have usually involved the administration of isotopically labelled precursors to define the interrelationships between the pathways; these are summarised below:

a) Phosphatidylcholine may be synthesised in two ways. The first pathway involves an initial phosphorylation of choline (as the free base) by adenosine triphosphate (ATP) to produce phosphorylcholine and adenosine diphosphate (ADP) (Wittenberg and Kornberg, 1953). Phosphorylcholine is then

Phosphoglycerides

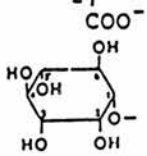


x = OH, Phosphatidic acid

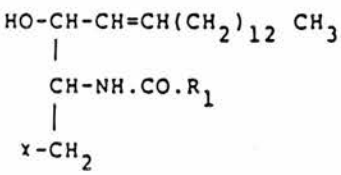
x = $OCH_2CH_2.\overset{+}{N}(CH_3)_3$, Phosphatidylcholine

x = $OCH_2CH_2.\overset{+}{N}H_3$, Phosphatidylethanolamine

x = $OCH_2\underset{\underset{COO^-}{|}}{CH}.\overset{+}{N}H_3$, Phosphatidylserine

x =  Phosphatidylinositol

Sphingolipids



x = $O\text{-}\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}\text{-}OCH_2CH_2\overset{+}{N}(CH_3)_3$, Sphingomyelin

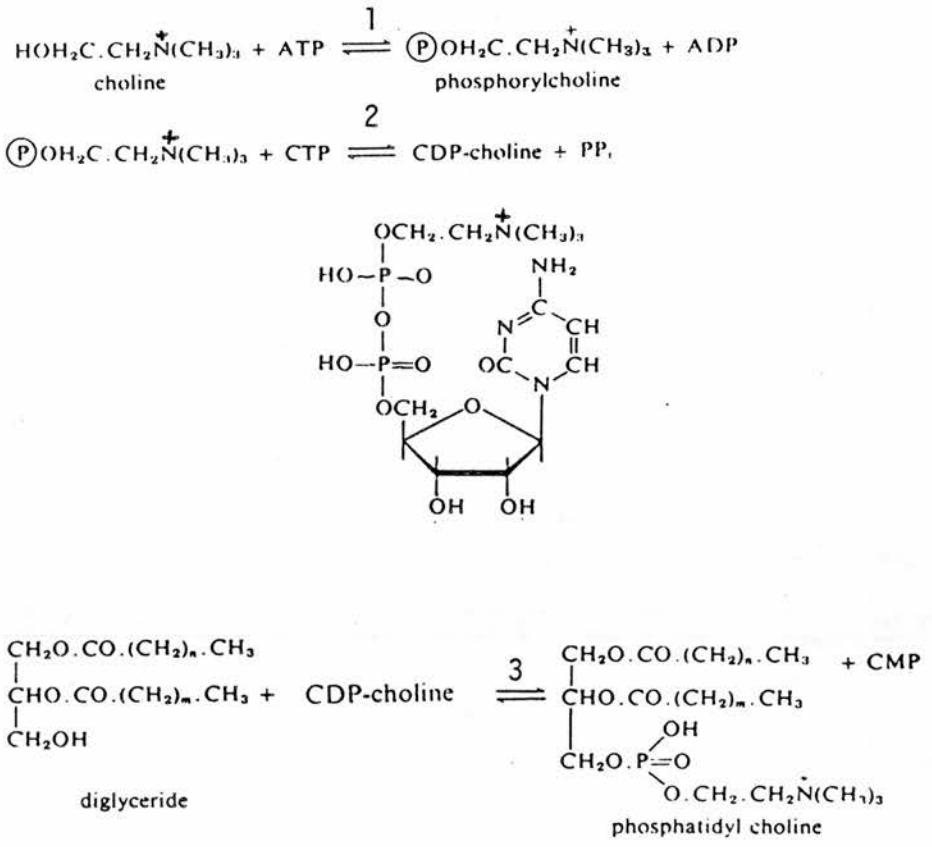
Figure 5 Structures of phospholipids + sphingomyelin, where R_1 and R_2 are the fatty acid carbon chains.

"activated" in a reaction with cytidine-5'-triphosphate (CTP). The resultant products are cytidine diphosphate-choline (CDP-choline) and pyrophosphate (PPi) (Kennedy and Weiss, 1956). The enzyme, phosphorylcholine-cytidyl transferase is completely specific for cytidine nucleotides. The phosphorylcholine moiety of CDP-choline is then transferred to the vacant primary hydroxyl group of a diglyceride, a step catalysed by the enzyme phosphorylcholine-glyceride transferase (Weiss, Smith and Kennedy, 1958). Phosphatidylcholine is then formed together with the byproduct cytidine monophosphate (CMP). The diglyceride involved in this reaction is synthesized as described previously for triglyceride synthesis.

The second pathway for phosphatidylcholine is catalyzed by two methyltransferases and involves the stepwise methylation of phosphatidylethanolamine with 3 moles of S-adenosyl-methionine as the methyl donor (Bremer and Greenberg, 1961). See Fig. 6.

b) The enzymic synthesis of phosphatidylethanolamine is essentially analagous to the formation of phosphatidyl-choline, except that phosphorylethanolamine and cytidine diphosphate ethanolamine are the intermediates involved (Kennedy and Weiss, 1956). The syntheses of cytidine diphosphate ethanolamine (CDP-ethanolamine) and phosphatidyl-ethanolamine in these reactions are catalysed by enzymes different from those involved in the analagous synthesis of CDP-choline and phosphatidylcholine.

a)



b)

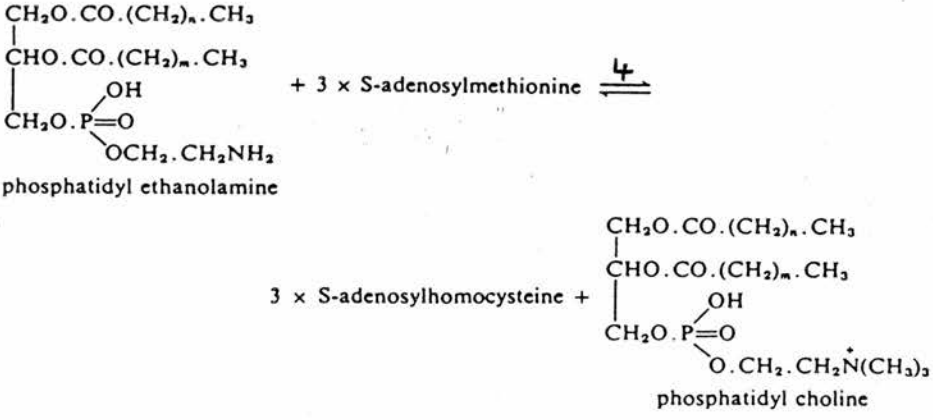


Figure 6 Summary of pathways involved in the synthesis of phosphatidyl choline. Pathway a) outlines de novo synthesis of phosphatidylcholine from choline and diglyceride. Pathway b) outlines the synthesis of phosphatidylcholine from the stepwise methylation of phosphatidylethanolamine with S-adenosylmethionine

where :

1 = reaction catalysed by phosphokinase

2=reactioncatalysedbyphosphorylcholine-cytidyl transferase

3 = reaction catalysed by phosphorylcholine-glyceride transferase

4 = reaction catalysed by two methyltransferases

Decarboxylation of phosphatidylserine is also a means by which phosphatidylethanolamine is synthesized. To date, the importance of this pathway has not been assessed, see Fig. 7.

c) Unlike phosphatidylcholine and phosphatidylethanolamine, phosphatidylinositol synthesis involves the activation of the diglyceride rather than the nitrogenous base. The initial step in the reaction involves phosphatidic acid and cytidine triphosphate (CTP) to form cytidine diphosphate-diglyceride (CDP-diglyceride) and pyrophosphate (PPi). The phosphatidic acid involved in this reaction is synthesized as previously described for triglyceride synthesis. The enzyme which catalyses this reaction is CTP-phosphatidate cytidyltransferase. Addition of inositol to the CDP-diglyceride completes the synthesis of this phosphoinositide (Possmayer and Strickland, 1967). The enzyme responsible for catalysing this reaction is CMP-phosphatidate:inositol phosphatidate transferase.

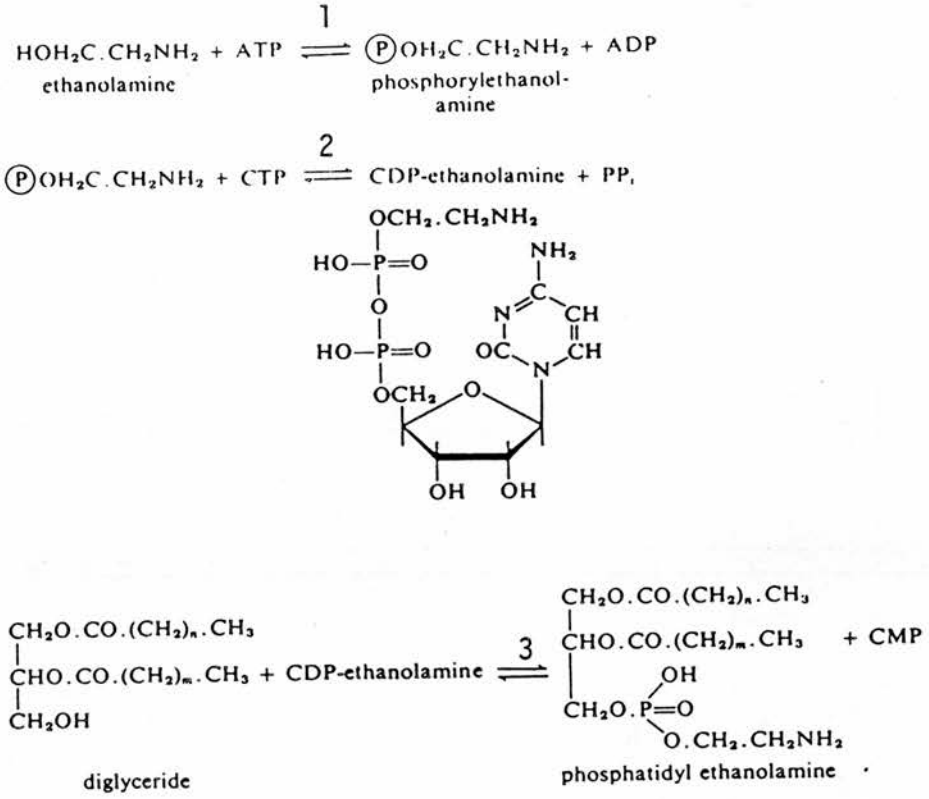
Therefore,

$$\text{phosphatidic acid} + \text{CTP} \rightleftharpoons \text{CDP - diglyceride} + \text{PPi}$$

$$\text{CDP - diglyceride} + \text{inositol} \rightleftharpoons \text{CMP} + \text{phosphatidylinositol}$$

d) Like phosphatidylinositol, the synthesis of phosphatidylserine involves the activation of the diglyceride rather than the nitrogenous base. The initial step is catalysed by CTP-phosphatidate cytidyltransferase and involves the reaction of phosphatidic acid and cytidine triphosphate (CTP) to form cytidine diphosphate-diglyceride (CDP-diglyceride) and pyrophosphate (PPi). The phosphatidic acid is synthesized as previously described for triglyceride synthesis.

a)



b)

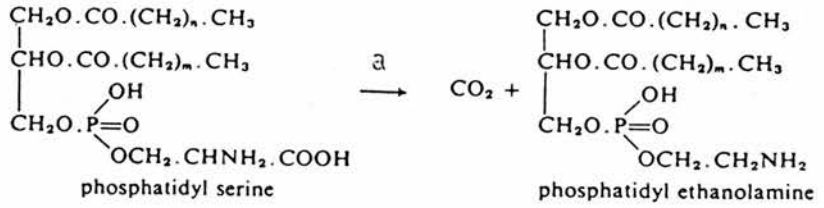


Figure 7 Summary of biosynthetic pathways involved in the synthesis of phosphatidylethanolamine. Pathway (a) outlines the de novo synthesis of phosphatidylethanolamine from ethanolamine and diglyceride. Pathway (b) outlines the synthesis of phosphatidylethanolamine from phosphatidylserine.

where :
1 =reaction catalysed by phosphatidylkinase
2 =reaction catalysed by phosphoryl-ethanolamine transferase
3=reaction catalysed by phosphoryl ethanolamine-glyceride transferase
a =reaction catalysed by decarboxylase

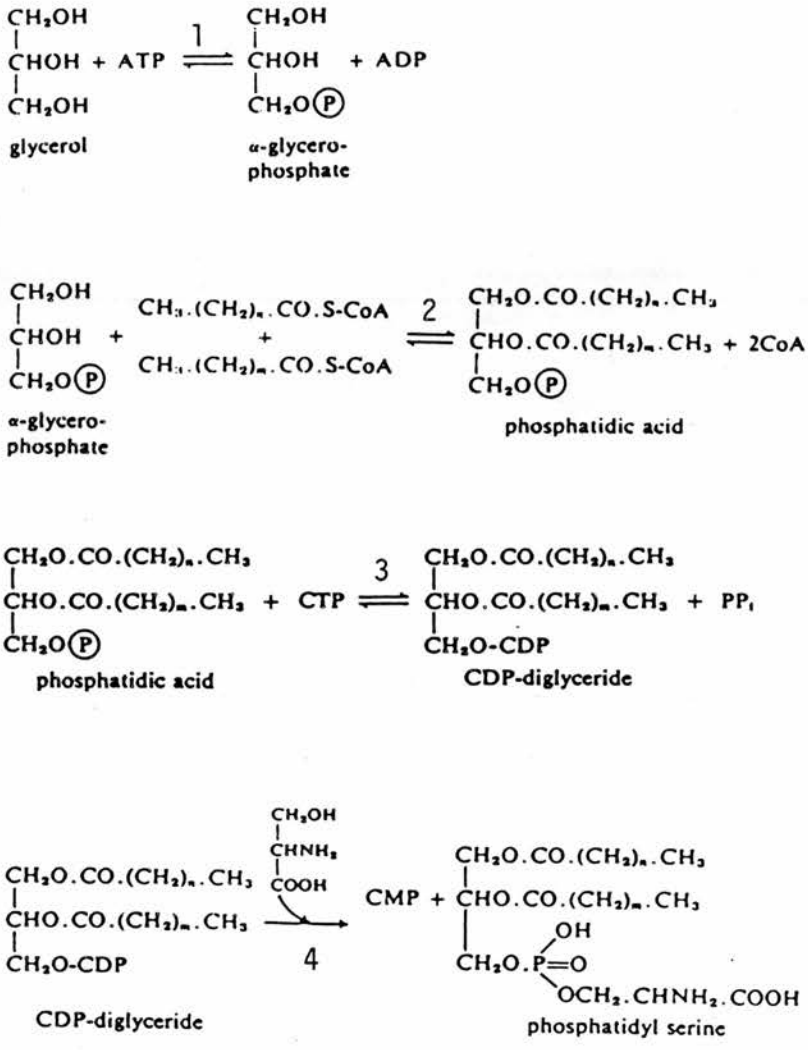


Figure 8 The de novo synthesis of phosphatidylserine where :

1 =reaction catalysed by glycerokinase
2 =reaction catalysed by glycerol-3-phosphate:acyl transferase
3 =reaction catalysed by CTP-phosphatidate:cytidyl transferase
4 =reaction catalysed by CMP-phosphatidate:serine phosphatidate transferase

Addition of serine to CDP-diglyceride results in the synthesis of phosphatidylserine. The enzyme responsible for the reaction is CMP-phosphatidate:serine phosphatidate transferase. See Fig. 8.

e) Sphingomyelin biosynthesis involves the nitrogenous base choline. Choline reacts with cytidine triphosphate (CTP) as described previously in phosphatidylcholine synthesis to form cytidine diphosphate-choline (CDP-choline). Sphingosine then combines with CDP-choline to form sphingosylphosphoryl-choline and cytidine monophosphate (CMP). Sphingomyelin is subsequently formed by the acylation of sphingosylphosphoryl-choline by acyl-S.CoA. Coenzyme (CoASH) is also formed as a byproduct.

Sphingosine + CDP-choline $\longrightarrow$ sphingosylphosphorylcholine + CMP

Sphingosylphosphorylcholine + acyl-S.CoA $\longrightarrow$ Sphingomyelin + CoASH

An alternative pathway for the synthesis of sphingomyelin involves the reaction catalysed by phosphoryl choline-ceramide transferase between N-acyl sphingosine (ceramide) and CDP-choline. Cytidine monophosphate (CMP) is formed as well as sphingomyelin (Sribney and Kennedy, 1957).

N-acyl-sphingosine + CDP-choline $\longrightarrow$ sphingomyelin +CMP

The relative importance of these two pathways <u>in vivo</u> has not been established.

Fatty acids such as arachidonic acid are incorporated into diglycerides. Since diglycerides are also essential intermediates for the synthesis of phosphatidylcholine,

phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, it is obvious that arachidonic acid may be incorporated into these phospholipids at this stage. Arachidonic acid may also be linked to sphingomyelin (see Fig. 5) via sphingosine by an amide linkage. However the pathways described here are not the only routes by which fatty acids, such as arachidonic acid, are incorporated into the phospholipids. An alternative means of incorporation can occur after de novo synthesis of phospholipid has been completed. This route involves the formation of a lysophospholipid by removing one fatty acid moiety of a phospholipid. This reaction can be catalysed by a phospholipase. Before the arachidonic acid can be incorporated into the lysophospholipid, it must be converted to the coenzyme A derivative, as previously described in the synthesis of triglycerides. The enzyme acyl Co.A:lysophosphatide acyltransferase (Lands and Merk 1, 1963) catalyses the addition of arachidonyl CoA to the lysophospholipid. In addition Ferber and Trotter (1981), using homogenates of murine thymocytes, have shown that a CoA-dependent transfer of fatty acyl chains between phospholipids can also provide a means of achieving turnover of phospholipid acyl chains.

Investigation into acyl CoA:1-acyl-sn-glycero-3-phosphocholine acyltransferase extracted from rat liver microsomes demonstrated the enzyme's maximal activity with arachidonyl CoA at pH 8 (Hasegawa-Sakai and Ohno, 1980) i.e. esterification by this pathway is increasingly favoured with more unsaturated fatty acids.

Figure 9 Deacylation and Reacylation of Phospholipids

FFA + acyl CoA $\longrightarrow$ FA-acyl CoA

FA - acyl CoA + lysophospholipid $\underset{2}{\overset{1}{\rightleftharpoons}}$ phospholipid

where FFA = free fatty acid

FA = fatty acid

1 = acyltransferase

2 = phospholipase

The reaction in Fig. 9 may be of importance for the incorporation of arachidonic acid into phospholipids.

Mobilization of Arachidonic Acid From Phospholipids

It seems reasonable to consider the phospholipases (the phospholipid degrading enzymes) as the enzymes responsible for the release of arachidonic acid from phospholipids. Initial evidence for the involvement of these enzymes came from the perfusion of guinea pig lungs or frog intestine with phospholipase A. These perfusions led to a rapid release of large quantities of prostaglandins in both tissues (Vogt, Meyer, Kunze, Luft and Babilli, 1969; Bartels, Kunze, Vogt and Wille, 1970). Since prostaglandins could also be generated by the infusion of arachidonic acid through frog intestine (Bartels et al., 1970) or lungs (Palmer, Piper and Vane, 1973), it was possible to connect phospholipase A activity with arachidonic acid release and prostaglandin synthesis.

Phospholipase Enzymes

a) Phospholipase A is readily available from certain snake venoms. Enzyme activity was investigated by de Haas, Daemen and Van Deenan (1962) who prepared "mixed acid" phosphatidylcholines and ethanolamines so that the 1- and 2-position fatty acids were specified exactly. Hydrolysis of these lipids with phospholipase A demonstrated the cleavage action at the 2 position of 3-phosphoglycerides yielding a 1-monoacyl analogue. Further studies with synthetic analogues of the substrate were able to pinpoint the precise structural and stereochemical features necessary for the enzyme to hydrolyse a fatty ester bond (Van Deenan and Haas, 1963). The enzyme seems to require the presence of three points of attachment: the hydroxyl function of the phosphoryl moiety (calcium ions may act as a "linking bridge" between the enzyme and substrate at this point), the carbon-2 fatty acid ester linkage and the oxygen alkyl function connected to the carbon-1 atom.

Conclusive pharmacological evidence was provided by Flower and Blackwell (1976) who labelled phosphatidylcholine of guinea pig spleen slices with [1-^{14}C] arachidonic acid chiefly in the carbon-2 position. Cellular damage caused by homogenisation, mechanical stimulation or anaphylactic shock resulted in an increase in free substrate levels and an increase in the biosynthesis of prostaglandins. Mepacrine, an antimalarial drug inhibited the release of arachidonic acid by blocking phospholipase action at the carbon-2 position. This hydrolytic enzyme is now denoted as phospholipase A_2 to distinguish it from the other members of

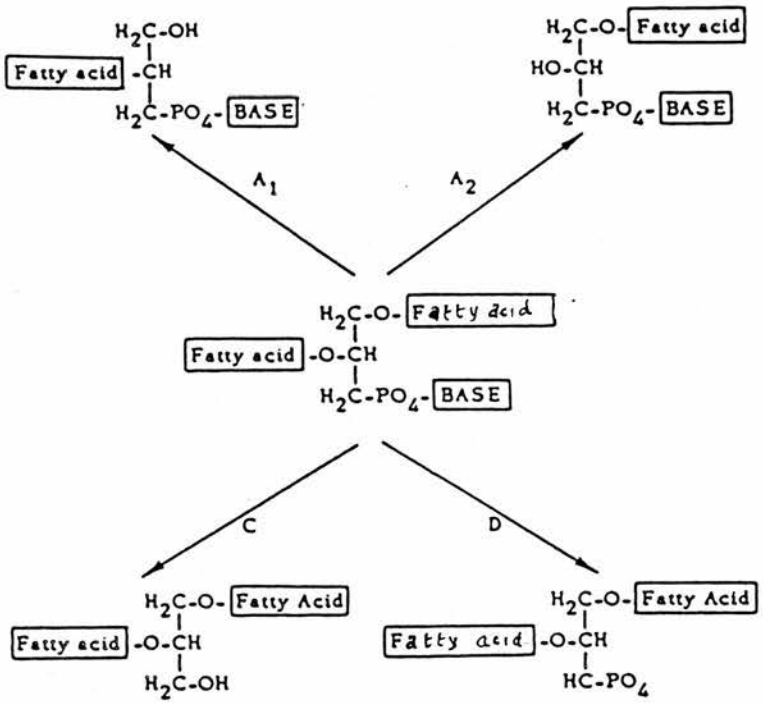


Figure 10 Simplified diagram showing the points of attack of the various phospholipase enzymes

where A_1 = phospholipase A_1
A_2 = phospholipase A_2
C = phospholipase C
D = phospholipase D

the phospholipase family, such as phospholipase A_1 phospholipase D and phospholipase C. The four types of phospholipases and their points of attack are illustrated in Fig. 10.

Phospholipase A_2 hydrolyses the carbon-2 fatty acid from the glycerol backbone leaving a 1-acyl lysophosphatide (as described above). Since unsaturated fatty acids (including arachidonic acid) are found predominantly in the 2-position of phospholipids, it is clear that phospholipase A_2 is important for the release of arachidonic acid from phospholipids. It acts with equal facility upon phosphatidylcholine with oleic or arachidonic acid, i.e. there is no marked preference to either acid. The enzyme shows no marked preference for phosphatidylethanolamine over phosphatidylcholine (Rittenhouse-Simmons and Deykin, 1981). Conditions for optimal phospholipase A_2 activity vary from tissue to tissue e.g. using human platelet homogenates and membranes, the enzyme's maximum hydrolysis leading to release of arachidonic acid occurs at pH 9.5 and is dependent upon 10mM calcium ions (Derksen and Cohen, 1975; Jesse and Cohen, 1976). However in guinea pig endometrium, phospholipase A_2 activity is maximal at a pH of 8; the membrane-bound enzyme requires 5mM calcium ions whilst the soluble enzyme requires 2mM calcium ions (Downing and Poyser, 1983).

b) Phospholipase A_1 hydrolyses the fatty acids at the carbon-1 position giving a 2-acyl lysophosphatide. These lysophosphatides are generally cytotoxic causing haemolysis in red blood cell suspension and membrane damage to other cells or organelles. They are usually quickly disposed of

in the cell either by lysophospholipases or by reacylation to phosphatides (Lands and Merk 1, 1963).

Since phospholipase A_1 only releases fatty acids from the carbon-1 position, its activity is not important for the release of arachidonic acid, which is usually found esterified at the carbon-2 position of a phospholipid.

c) Phospholipase D acts by removing the base to leave phosphatidic acid. Since it has only been detected in plants it is of little consequence for arachidonic acid release and PG synthesis in animals (see Flower, 1981).

d) Phospholipase C (sometimes known as phosphatidyl inositol phosphodiesterase) acts by cleaving the phosphate group from phosphatidylinositol leaving the diglyceride molecule intact (Rittenhouse-Simmons, 1979). It is present in the supernatant fraction of platelets that have been sonicated and sedimented at 100,000g for 60 min. Optimal activity is observed near pH 7 in the presence of 5mM calcium ions (Rittenhouse-Simmons, 1979). Half maximal enzyme activity can be observed in the platelet cytosol in the presence of calcium concentrations as low as 5 to 10μM, (Mauco, Chap and Douste-Blazy, 1979). This is about one to two orders of magnitude more sensitive than platelet phospholipase A_2 whose half maximal enzyme activity in the presence of calcium is 0.25 to 0.3mM (Jesse and Franson, 1979).

Evidence for the specific activity of phospholipase C on phosphatidylinositol has been mainly described in

thrombin - or collagen-stimulated platelets. Degradation of phosphatidylinositol results in the generation of 1, 2 diacylglycerol as well as small quantities of myo-inositol 1:2 cyclic phosphate and myo-inositol 1-phosphate (Rittenhouse-Simmons, 1978; 1979).

The amount of diglyceride occurring 15 sec after thrombin addition may be as much as 30 times greater than basal levels. However, this elevation is transitory and within 2 min diglyceride is at the low levels that characterize the resting state. It is clear that the diglyceride is metabolized further.

Two hypotheses exist at present to explain the next stage of diglyceride metabolism. The first, put forward by Call and Rubert (1973) and Lapetina and Cuatrecasas (1979) indicates the presence of a diglyceride kinase enzyme which is able to phosphorylate diglycerides. The resultant phosphatidic acid yielded is thought to constitute the "primary response" to stimulation and appears to precede the liberation of arachidonic acid from the various phospholipids. The mechanism by which this latter phase is brought about is not clear, but it is believed that phosphatidate and its metabolite lysophosphatidate have the properties of a calcium ionophore (Lapetina, 1982). An increase in the concentration of free, intracellular calcium could then activate phospholipase A_2 and result in arachidonic acid liberation from phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Lapetina, Billah and Cuatrecasas, 1981). Phosphatidic acid may be removed by combining with cytidine triphosphate to form cytidine diphosphate

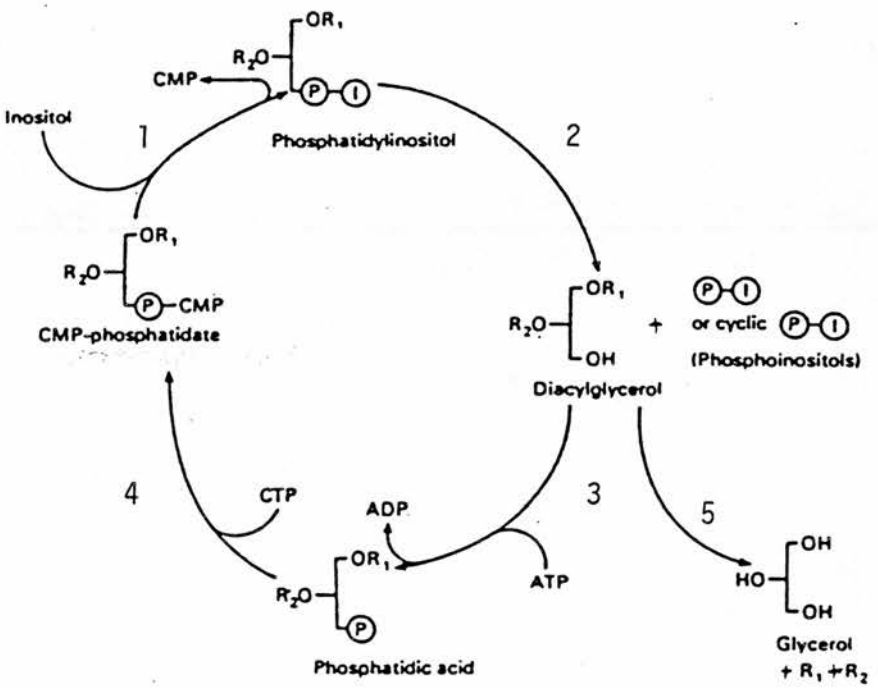


Figure 11 Phosphatidylinositol (PI) Cycle

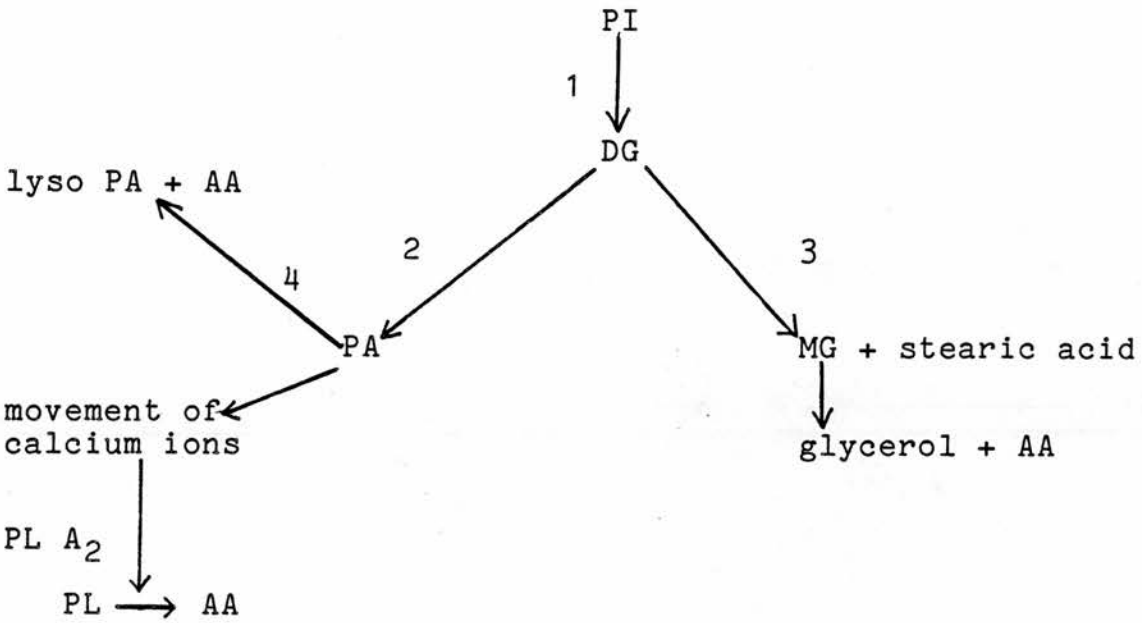
The turnover of phosphatidylinositol is represented diagramatically, thus

the circled P = phosphate group
the circled I = inositol
R_1 + R_2 = fatty acid chains
1 = reaction catalysed by CMP-phosphatidate:inositol phosphatidate transferase
2= reaction catalysed by phosphatidylinositol phosphodiesterase (phospholipase C)
3 = reaction catalysed by diglyceride kinase
4 = reaction catalysed by CTP-phosphatidate cytidyltransferase
5 = reaction catalysed by diglyceride lipase and monoglyceride lipase

diglyceride and pyrophosphate. The "activated" cytidine diphosphate diglyceride is then once again available for phosphatidylinositol synthesis. The continuous turnover of phosphatidylinositol (the PI cycle) is hypothesized as being the key factor for arachidonate release in platelets (Lapetina, 1982). (See Fig. 11.) Another view which is under investigation suggests that a phospholipase exists which is capable of hydrolyzing fatty acids specifically from phosphatidic acid (Billah, Lapetina and Cuatrecasas, 1981). The second theory, proposed by Bell, Kennerly, Stanford and Majerus (1979), involves a diglyceride-specific lipase which degrades diglyceride to monoacylglycerol and stearic acid. The enzyme is membrane bound and shows enhanced activity at neutral pH in the presence of calcium ions and sulphydryl-reducing agents. Release of arachidonic acid from 2-arachidonyl monoglyceride requires the action of monoglyceride lipase (Chau and Tai, 1981). It has been suggested that diglyceride lipase activity is very low in platelets. However it has been demonstrated in human platelets that indomethacin (25 to 50µg/ml) blocked the activity of diglyceride lipase at concentrations that do not affect diglyceride kinase or phospholipase C (Rittenhouse-Simmons, 1980). Increased amounts of diglyceride were formed suggesting that diglyceride lipase activity was important in platelets for the release of arachidonic acid.

Such evidence implicates both pathways in diglyceride metabolism within the platelet. The pathways are summarised in Fig. 12. The two pathways described for the release of arachidonic acid from platelet membrane phospholipids as a

Figure 12 Diagram To Summarise Suggested Pathways For Phosphatidylinositol Metabolism and Arachidonic Acid Release From Phospholipids



where

PI	=	phosphatidylinositol
DG	=	1, 2 diglyceride
PA	=	phosphatidic acid
PL	=	phospholipid
AA	=	arachidonic acid
MG	=	monoglyceride
1	=	phospholipase C
2	=	diglyceride kinase
3	=	diglyceride lipase
4	=	PA specific phospholipase A_2

consequence of activation were recently monitored by Imai, Yano, Kameyama and Nozawa (1982). Their results revealed that although phosphatidylinositol provided some arachidonic acid by phospholipase C activation, the pathway involving phospholipase A_2 was of more importance for providing arachidonic acid for prostanoid synthesis by, and release, from thrombin activated human platelets.

Lysophospholipase

Lysophospholipase (lysophospholipid acyl hydrolase) is another fatty acid releasing enzyme. The enzyme removes the fatty acid at the carbon-1 position from a 1-monoacyl-lysophosphatide to form glycerol-3-phosphate. Since arachidonic acid is not usually found esterified at the carbon-1 position, the activity of the enzyme is not important for the release of arachidonic acid.

Sulpholysis

The liberation of arachidonic acid from the probable main sources (phosphatidylethanolamine, phosphatidylcholine or phosphatidylinositol) may not take place by direct hydrolytic action of phospholipase A_2, but by a CoA-mediated transfer of fatty acid from a donor phospholipid to another phospholipid, followed by hydrolysis of the acceptor lipid; this is known as sulpholysis. The pathway is catalysed by acyl-CoA:lysophosphatide acyltransferase. The same enzyme is involved in the acylation of a lysophospholipid (Lands and Merk 1, 1963). *In vitro* the enzyme exhibits some specificity for arachidonate (Irvine and Dawson, 1979;

Trotter and Ferber, 1981).

$$\text{Thus,}\quad \underset{\text{phospholipid}}{\text{PL}} + \underset{\text{Coenzyme A}}{\text{CoA-SH}} \rightleftharpoons \text{Lyso PL} + \text{acyl-CoA}$$

$$\text{Acyl-CoA} + \underset{\text{lyso phospholipid 1}}{\text{Lyso PL}_1} \rightleftharpoons \text{PL}_1 + \text{CoA-SH}$$

Thus, the availability of arachidonic acid for prostaglandin synthesis may be indirectly brought about by influencing the fatty acid pattern of the phospholipids. It is possible that more arachidonic acid is esterified into one particular phospholipid which is more likely to undergo hydrolysis than any other phospholipid source.

It is clear that the mechanisms controlling arachidonic acid turnover in tissues are complex but nevertheless it is important to study these processes in relation to PG synthesis particularly in tissues where PG synthesis is necessary for normal physiological functions.

Experimental Aims

The overall evidence from the literature indicates that $PGF_{2\alpha}$ is the uterine luteolytic hormone in many non-primate, mammalian species including the guinea pig. The release of arachidonic acid from some bound source is necessary for $PGF_{2\alpha}$ synthesis as it is the rate-limiting step in PG synthesis by tissues including the guinea pig uterus (Leaver and Seawright, 1982). The work presented in this thesis has investigated some of the mechanisms involved in controlling arachidonic acid uptake into, and release from, the various

lipid classes in the guinea pig uterus in relation to endometrial $PGF_{2\alpha}$ production and luteolysis.

SECTION 1. MATERIALS AND GENERAL METHODS

The work presented in this thesis has involved a radiolabelling technique to monitor the uptake of the prostaglandin precursor, arachidonic acid, as it passes into the endometrial cell and becomes incorporated by esterification into the lipid components of the cell. Subsequently, the basal release and the effects of the steroid hormones, oestradiol and progesterone, and the calcium ionophore A23187 on the release of the unsaturated fatty acid from the various lipids have been measured. Some of the pathways involved in the incorporation of arachidonic acid into the phospholipid components of the cell have also been examined. Methods of analysis have involved the isolation and purification of neutral lipids and phospholipids using silicic acid column chromatography and thin layer chromatography, and the detection and measurement of radioactivity by liquid scintillation counting. The experimental work has been divided into several sections but, as many of the analytical procedures are common to each section, the details of such procedures are described in this section.

a) List of Materials

Solvents

Absolute ethanol	J. Boroughs Ltd., London
Acetic acid, glacial (analar grade)	B.D.H. Chemicals Ltd., Poole
Acetone	Mackay and Lynn, Edinburgh
Chloroform (analar grade)	B.D.H. Chemicals Ltd., Poole
Diethyl ether (analar grade)	B.D.H.Chemicals Ltd., Poole

Methanol (analar grade)	Fisons Scientific Apparatus, Loughborough
Methyl acetate (analar grade)	B.D.H. Chemicals Ltd., Poole
Petroleum spirit (analar grade) (b. pt 40° - 60° C)	B.D.H. Chemicals Ltd., Poole
Propan-1-ol (analar grade)	B.D.H. Chemicals Ltd., Poole
Toluene (analar grade)	Fisons Scientific Apparatus, Loughborough

Radioactive Compounds

[5,6,8,9,11,12,14,15-^{3}H] arachidonic acid
(Sp. act. 120-131 Ci/mmol)
Amersham International Ltd., Cardiff

6-keto[5,8,9,11,12,14,15(n) - ^{3}H] prostaglandin $F_{1\alpha}$
(Sp. act. 150 Ci/mmol)
Amersham International Ltd., Cardiff

[Methyl - ^{3}H] choline chloride
(Sp. act. 77 Ci / mmol)
Amersham International Ltd., Cardiff

S-adenosyl-L-[methyl-^{3}H] methionine
(Sp. act. 60-68 Ci / mmol)
Amersham International Ltd., Cardiff

[9,10(n) - ^{3}H] oleic acid
(Sp. act. 8.2 Ci / mmol)
Amersham International Ltd., Cardiff

L-α-phosphatidyl[methyl - ^{14}C] choline,dipalmitoyl
(Sp. act. 80 mCi / mmol)
Amersham International Ltd., Cardiff

5,6,8,11,12,14,15(n) - ^{3}H prostaglandin E_2

(Sp. act. 160 Ci / mmol)

Amersham International Ltd., Cardiff

5,6,8,9,11,12,14,15(n) - ^{3}H prostaglandin $F_{2\alpha}$

(Sp. act. 180 Ci / mmol)

Amersham International Ltd., Cardiff

Other Chemicals and Materials

Ammonium molybdate	B.D.H. Chemicals Ltd., Poole
Amphotericin B	Flow Labratories, Irvine
Arachidonic acid (99% pure)	Sigma Chemical Co., London
Bovine serum albumin (fatty acid free)	Sigma Chemical Co., London
Cholesterol oleate	Sigma Chemical Co., London
Diglyceride standard (33.33% dipalmitin, 33.33% diolein 33.33% distearin)	Sigma Chemical Co., London
2,5 - diphenyloxazole	B.D.H. Chemicals Ltd., Poole
Glutamine	Flow Laboratories, Irvine
Iodine	Sigma Chemical Co., London
Kanamycin	Flow Laboratories, Irvine
Medium 199 (plus Earles salts)	Flow Laboratories, Irvine
Mercury	B.D.H. Chemicals Ltd., Poole
Monoglyceride standard (33.33% monopalmitin, 33.33% monostearin, 33.33% monolein)	Sigma Chemical Co., London
17-β Oestradiol	Sigma Chemical Co., London
Oleic Acid	Sigma Chemical Co., London
Phenolphthalein	BDH Chemicals Ltd., Poole

L-α-phosphatidylcholine Type V11 E	Sigma Chemical Co., London
L-α-phosphatidyl-N, N-dimethyl ethanolamine, dipalmitoyl	Sigma Chemical Co., London
L-α-phosphatidylethanolamine Type III	Sigma Chemical Co., London
L-α-phosphatidylinositol Grade III	Sigma Chemical Co., London
L-α-phosphatidylinositol 4,5-diphosphate (triphosphoinositide)	Sigma Chemical Co., London
L-α-phosphatidylinositol 4-monophosphate (diphosphoinositide)	Sigma Chemical Co., London
L-α-phosphatidyl-N-monomethyl ethanolamine, dipalmitoyl	Sigma Chemical Co., London
L-α-phosphatidyl-L-serine	Sigma Chemical Co., London
Potassium oxalate	BDH Chemicals, Poole
Progesterone	Sigma Chemical Co., London
Sphingomyelin	Sigma Chemical Co., London
Triglyceride standard (25% tristearin, 25% trilinolein 25% triolein , 25% tripalmitin)	Sigma Chemical Co., London
Calcium Ionophore A23187	Calbiochem-Behring Corp., San Diego
Zinsser disposable plastic scintillation vials	Mackay and Lynn, Edinburgh

Thin-layer chromatography plates (silica gel, 0.25mm thick)	Merck A.G., Darmstadt
Unisil silicic acid (100 to 200 mesh)	Clarkson Chemical Co., Inc. Williamsport
Vented petri dishes, 5cm diameter	Sterilin Ltd., Teddington

b) Preparation of Chemicals and Solutions

i) Sodium Salts of Tritiated Arachidonic Acid And Tritiated Oleic Acid

Sufficient of the radioactive fatty acid for each investigation was dissolved in ethanol (5ml) which contained phenolphthalein as indicator. Ethanolic sodium hydroxide (0.1M) was slowly added until the solution just changed colour from clear to purple. The solution was then evaporated to dryness at 35°C on a rotary evaporator and was dissolved in an appropriate volume of culture medium. Preparation of the sodium salts in this manner ensured that the fatty acids readily and completely dissolved in the aqueous media for uptake into the tissue.

ii) Scintillation Fluid

Scintillation fluid was made up as required using the following chemicals: 10.5g 2,5-diphenyloxazole (PPO)

2.5 l toluene

iii) Supplemented Culture Medium 199

The Medium 199 used in tissue culture has as a basis a

synthetic mixture of inorganic salts known as a physiological or balanced salt solution (see Table 1.1). The functions of this salt solution in the medium are:

1. to maintain the pH
2. to maintain the osmotic pressure
3. to provide a source of energy

The buffer utilised in the tissue culture medium is sodium bicarbonate (2200mg present in every litre of medium). Sodium bicarbonate is also a nutritional requirement.

Medium 199 does not contain glutamine, an essential factor for stromal viability and good epithelial preservation, which is only stable below 0°C (see Kaufman, Adamec, Walton, Carney, Melin, Genta, Mass, Dorman, Rodgers, Photopulos, Powell and Grisham, 1980). The tissue culture medium is stored at 5°C and glutamine is then added when required. Since the medium may also be susceptible to bacterial growth, the culture medium was supplemented with antibiotics (Abel and Baird, 1980; Leaver and Seawright, 1982). Supplemented tissue culture medium was made up under sterile conditions in the following manner:

To Medium 199 (500ml) was added glutamine (200mM; 4ml) kanamycin (5000 µg/ml; 3ml) and amphotericin B (250 µg/ml; 3ml). After mixing well, the supplemented culture medium was dispersed into 20ml storage bottles and stored at -20°C until required.

Medium 199

Ingredient	199 with Earle's Salts mg/litre
L-Alanine	25.00
L-Arginine HCl	70.00
L-Aspartic acid	30.00
L-Cysteine HCl	0.0987
L-Cystine, disodium salt	23.66
L-Glutamic acid	66.82
L-Glutamine	100.0
Glutathione	0.05
Glycine	50.00
L-Histidine HCl H_2O	21.88
L-Hydroxyproline	10.00
L-Isoleucine	20.00
L-Leucine	60.00
L-Lysine HCl	70.00
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	40.00
L-Serine	25.00
L-Threonine	30.00
L-Tryptophan	10.00
L-Tyrosine	40.00
L-Valine	25.00
L-Ascorbic acid	0.05
Biotin	0.01
Calciferol	0.10
D-Calcium pantothenate	0.01
Choline chloride	0.50
Folic acid	0.01
i-Inositol	0.05
Menaphthone sodium bisulphite trihydrate	0.019
Nicotinic acid	0.025
Nicotinamide	0.025
p-Aminobenzoic acid	0.05
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.01
Thiamin HCl	0.01
DL- Tocopherol phosphate, disodium salt	0.01
Vitamin A acetate	0.1147

Ingredient	199 with Earle's Salts mg/litre
$CaCl_2$ $2H_2O$	264.9
$Fe(NO_3)_3$ $9H_2O$	0.10
KCl	400.0
KH_2PO_4	
$MgSO_4$ $7H_2O$	200.0
NaCl	6800
$NaHCO_3$	2200
NaH_2PO_4 $.2H_2O$	158.3
Na_2HPO_4	
Adenine sulphate	10.00
5'-AMP	0.20
ATP, disodium salt	10.00
Cholesterol	0.20
2-Deoxyribose	0.50
Glucose	1000
Guanine HCl	0.30
Hypoxanthine	0.30
Ribose	0.50
Sodium acetate	36.71
Sodium phenol red	17.00
Thymine	0.30
Tween 80	5.00
Uracil	0.30
Xanthine	0.30

Table 1.1

The individual ingredients of the tissue culture Medium 199

c) Animals Used

Virgin guinea pigs weighing between 500 and 1000 g were examined daily and a vaginal smear was taken when the vaginal membrane was open. The first day of the oestrous cycle was taken as the day before the post-ovulatory influx of leucocytes when cornification was maximal (Nicol and Snell, 1954). All animals had exhibited at least two normal cycles immediately before being used.

Each guinea pig was killed by stunning and incising the neck either on Day 7 or Day 15 of the cycle, days of low and high $PGF_{2\alpha}$ output from the uterus respectively (Blatchley et al., 1972). The uterus was rapidly removed and placed in culture medium in preparation for culture by a method based on that of Baker and Neal (1969)

Histological Studies

Since a number of parameters are involved in tissue culture, it has been necessary to determine the quality of tissue maintenance before and after experimental conditions by morphologic evaluation using light microscopy. Slides were prepared by courtesy of Dr. W. Blyth and Mrs Susan Bartlett of the Botany Department, Edinburgh University.

Endometrial tissue was obtained from a guinea pig on Day 15 of the oestrous cycle. Uncultured tissue and specimens of tissue incubated for 72 hours (under conditions to be described later) were fixed in formalin for 24 hours. The tissue was then dehydrated through graded concentrations of ethanol (from 70 to 100%) over a period of two days (see

Table 1.2.) After being embedded in wax, the tissues were blocked, sectioned and stained with hematoxylin and eosin (Boutsellis, De Neef, Ullery and George, 1963).

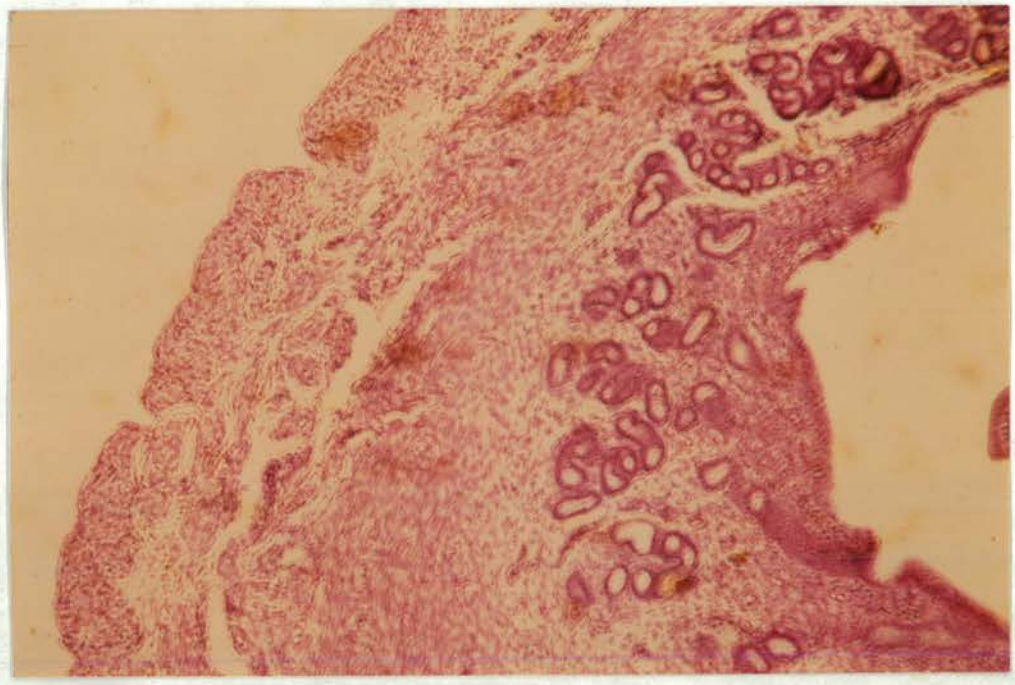
Table 1.2

Dehydration and Embedding Procedures

Formalin	- 24hr
70% alcohol	- 29hr
85% alcohol	- 1hr
95% alcohol	- 2hr
95% alcohol	- 2hr
Absolute alcohol	- overnight
Absolute alcohol	- 1hr
Absolute alcohol	- 1hr
Chloroform	- 1.5hr
Chloroform	- 1.5hr
Wax	- 2hr
Wax	- 1hr
Wax	- solidify overnight
Wax	- Warm vacuum 2hr

Tissues now blocked and ready for sectioning

A.



B.

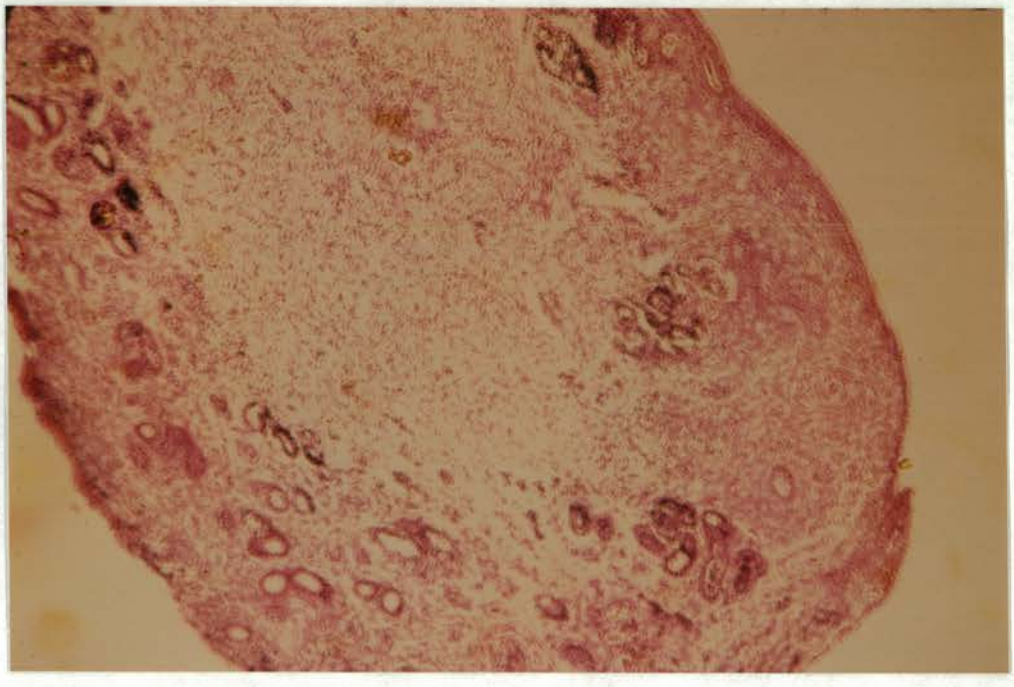


Figure 1.1

Photograph A is taken from a slide preparation of uncultured Day 15 endometrial tissue
Photograph B is taken from a slide preparation of Day 15 endometrial tissue cultured for 72 hr.

Histologic appearance of uncultured tissue was compared with that obtained from endometrial tissue cultured for 72 hours. Tissue was considered viable if the nuclear and cell membranes appeared intact and the nucleus stained well with the basic dye, haematoxylin. Loose cellular stroma surrounding glands composed of cuboidal to columnar epithelium were also observed, confirming that the tissue culture system was functioning adequately (see Fig. 1.1). Also there were no obvious differences between tissue before and after culture; therefore the tissue culture procedure is adequate for normal maintenance and survival of endometrium.

d) Tissue Culture Technique

i) Preparation of tissue

When the endometrium was required for tissue culture, it was necessary to dissect it from the myometrium by cutting away 1mm by 2mm portions of endometrium under sterile conditions. This technique produced greater than 85% separation of uterine tissues (Leaver and Poyser, 1981). The myometrium was discarded and approximately 20mg endometrial tissue was placed onto sterile lens paper which lay across a sterile, stainless steel grid or cradle in a vented petri dish (Fig. 1.2) The dish contained 4ml supplemented Medium 199 together with other agents, according to the nature of the experiment. This arrangement enabled the tissue to remain above the culture medium but still allowed the medium to be readily available to the endometrial tissue by capillary action through the lens paper. The number of dishes prepared again depended on the nature of the experiment.

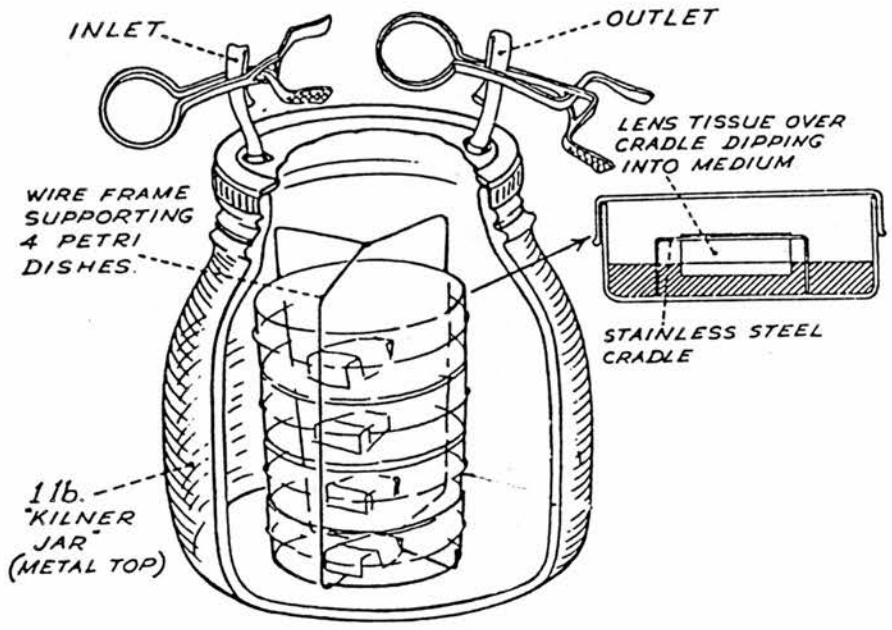


Figure 1.2

The above diagram illustrates the arrangements for tissue culture. The endometrial tissue was placed onto the lens tissue which lay across a stainless steel grid or cradle in a vented petri dish containing culture medium. Four petri dishes supported by a wire frame could be placed in the modified "Kilner jar" for incubation.

ii) Conditions Required for Tissue Culture

Groups of 4 petri dishes were placed in racks and then into modified Kilner jars to be incubated at 37°C in the presence of a 1:1 mixture of air/carbon dioxide (95%:5%) and oxygen / carbon dioxide (95%:5%) at a pressure of 10 lb/in^2 for periods of time as required. The presence of a small amount of carbon dioxide in the atmosphere is necessary to set up a CO_2/HCO_3^- buffer situation. If this procedure is not carried out, the pH increases rapidly at the normal temperature of incubation. The resultant red to purple colour change in the medium then indicates that the pH has risen and cell growth may be inhibited.

iii) Use of Bovine Serum Albumin

In the literature, experiments investigating the stimulated release of arachidonic acid from prelabelled lipids have been unsuccessful. For example cultured rabbit alveolar macrophages were prelabelled with ^{14}C-arachidonic acid (Hsueh, Desai, Gonzalez-Crussi, Lamb and Chu, 1981). Stimulation of the macrophages with various phagocytic and non-phagocytic stimuli resulted in prostaglandin release. However, only a small amount of arachidonic acid could be detected in the medium. It was suggested that free arachidonic acid which passed out of the cell was immediately taken back up again and reincorporated into the phospholipids. To avoid this problem, fatty acid free bovine serum albumin, a protein with a molecular weight of about 70,000, was used to "trap" the released fatty acid; a more accurate assessment of the effects of the stimuli was then observed.

Since an immediate re-uptake and reincorporation of radioactive arachidonic acid could have occurred in the release experiments to be described in Section 4, it was decided that the addition of 0.1% bovine serum albumin to the culture medium would be advantageous in this respect.

e) Silicic Acid Column Chromatography

The separation of phospholipid and neutral lipids into individual molecular species is a complex multistep procedure. The initial step has to allow quantitative isolation in amounts large enough for further analysis. It was therefore necessary to utilise silicic acid column chromatography to produce group separation of the neutral lipids from the phospholipids.

i) Preparation

Preparation involved the use of glass columns (1cm x 10cm). About 0.5 cm of fine white sand was placed initially into each column so that none of the smaller particles of the Unisil silica gel would damage the scinter. After filling the columns with 20ml diethyl ether, any air bubbles in the sand were removed by shaking. About 3 to 3.5g silica gel in diethyl ether was transferred to each column by using a Pasteur pipette. Excess ether was then removed via the tap outlet. Before use, each column was washed with 100ml diethyl ether.

iii) Elution of Neutral Lipids, Phospholipids and Prostaglandins Methodology

Three silicic acid columns, labelled A, B and C, were set up

and each was washed with 100ml diethyl ether. 1μCi Tritiated arachidonic acid (used to represent the movement of neutral lipids) was applied to column A. 0.1μCi ^{14}C-Phosphatidylcholine (used to represent the movement of phospholipids) was applied to column B. 0.1μCi of each of the following prostaglandins, [^{3}H]PGF$_{2\alpha}$, [^{3}H]PGE$_{2\alpha}$ and [^{3}H]6-keto PGF$_{1\alpha}$ were applied to column C. The columns then underwent stepwise elution using the following solvent mixtures.

TABLE 1.3

Solvent mixtures used to elute silicic acid columns

Volume (ml)	Methanol:Diethyl ether Ratio
50	0:100
50	0.5:99.5
50	1:99
50	2:98
50	4:96
50	10:90
50	20:80
50	50:50
50	100:0

Results and Conclusion

The results in Table 1.4 indicated that the initial 100ml of solvent was enough to elute 99% of the tritiated arachidonic acid from column A. All tritiated prostaglandins were removed by the solvent mixture of 20% methanol:80% diethyl ether from column C. Phosphatidylcholine was eluted by 100% methanol from column B.

TABLE 1.4

Radioactivity eluted by varying solvent mixtures.

% MeOHin Diethyl Ether	DPM[^{3}H] prostaglandin	DPM[^{14}C] phosphatidyl-choline	DPM[^{3}H] arachidonic acid
0	1995	2839	1394637
0.5	7232	137	76972
1	5120	0	2313
2	29508	0	1120
4	4374604	0	325
10	19815	0	0
20	692	0	0
50	6566	578	0
100	316	55787	0

Therefore, for silicic acid column chromatography it was decided to use :-

1) 100ml diethyl ether to elute neutral lipids
2) 20ml methanol in 80ml diethyl ether to elute prostaglandins
3) 100ml methanol to elute phospholipids

iii) Recovery from Columns

Before samples for analysis(derived from endometrial tissue by methods to be described later) underwent silicic acid column chromatography, each sample was placed in a known volume (5ml) of chloroform and methanol (2:1) and a 100µl aliquot portion was taken for liquid scintillation counting. Each sample was then applied to the top of a silicic acid column and eluted with, in succession, 100ml diethyl ether (which elutes neutral lipids including free arachidonic

acid), 20ml methanol in 80ml diethyl ether (which elutes cyclooxygenase and probably lipoxygenase products), and 100ml methanol (which elutes phospholipids).

After each fraction was collected from the column, the solvent was removed by a rotary evaporator at 35°C and the sample was resuspended in 5ml chloroform and methanol (2:1). Again, 100µl portions of each fraction obtained from the column were taken for liquid scintillation counting.

The radioactivity present in the sample before and after column chromatography may then be compared. The results showed that, in all experiments to be described later, 80% or more of the radioactivity was recovered. All subsequent results are corrected for recovery.

f) Thin-layer Chromatography (tlc)

It was decided that tlc would be the best means for the further separation of neutral lipids and phospholipids. The thin layer of powdered absorbent can cause the solvent to flow rapidly giving an exceptionally fast separation. Also the fineness of the powder cuts down the interstitial volume between the granules and this minimises diffusion and gives rise to very sharp spots. Adsorption chromatography in the tlc form is extremely useful for another reason ; adsorption and desorption take place well in non-polar media and lipids are therefore better separated in such systems.

i) Preparation

Precoated glass tlc plates (silica gel thickness 0.25mm, 5cm by 20cm) were used rather than "home - made" plates, as they gave very reproducible separations. The baseline was drawn

2cm from the bottom of each plate, to indicate the starting point of the sample. A line was then scored 3cm from the top of each plate to indicate the point which the solvent has to reach.

ii) Application of a Sample

After drying down in a rotary evaporator at 35°C, the sample was resuspended in 200µl chloroform and methanol (2:1) and "streaked" onto the tlc plate by using a micro-syringe. Each sample was applied to individual tlc plates and care had to be taken that no material was near the edge of the plate. If this had been allowed to occur, then the sample at the edge of the plate would have developed faster than the sample applied to the middle of the plate, and a curved profile of separation of the different lipids would result.

Standards of phospholipids and neutral lipids were spotted onto separate tlc plates and developed at the same time as the samples. Solvent tanks, which were lined with white absorbent paper and which contained approximately 100ml of an appropriate solvent mixture, were used to develop ten 5cm by 20cm plates simultaneously.

iii) Choice of Solvents

Neutral lipids were separated into individual lipid classes by tlc using a solvent system consisting of petroleum spirit (b.pt. 40° - 60°C), diethyl ether and acetic acid (75:25:1 by volume). Standards (25µg of each) for triglyceride, diglyceride, monoglyceride, cholesterol ester and arachidonic acid were also developed. Plates were usually fully developed within 1hr, and allowed to air dry for 20min (Fig. 1.3).

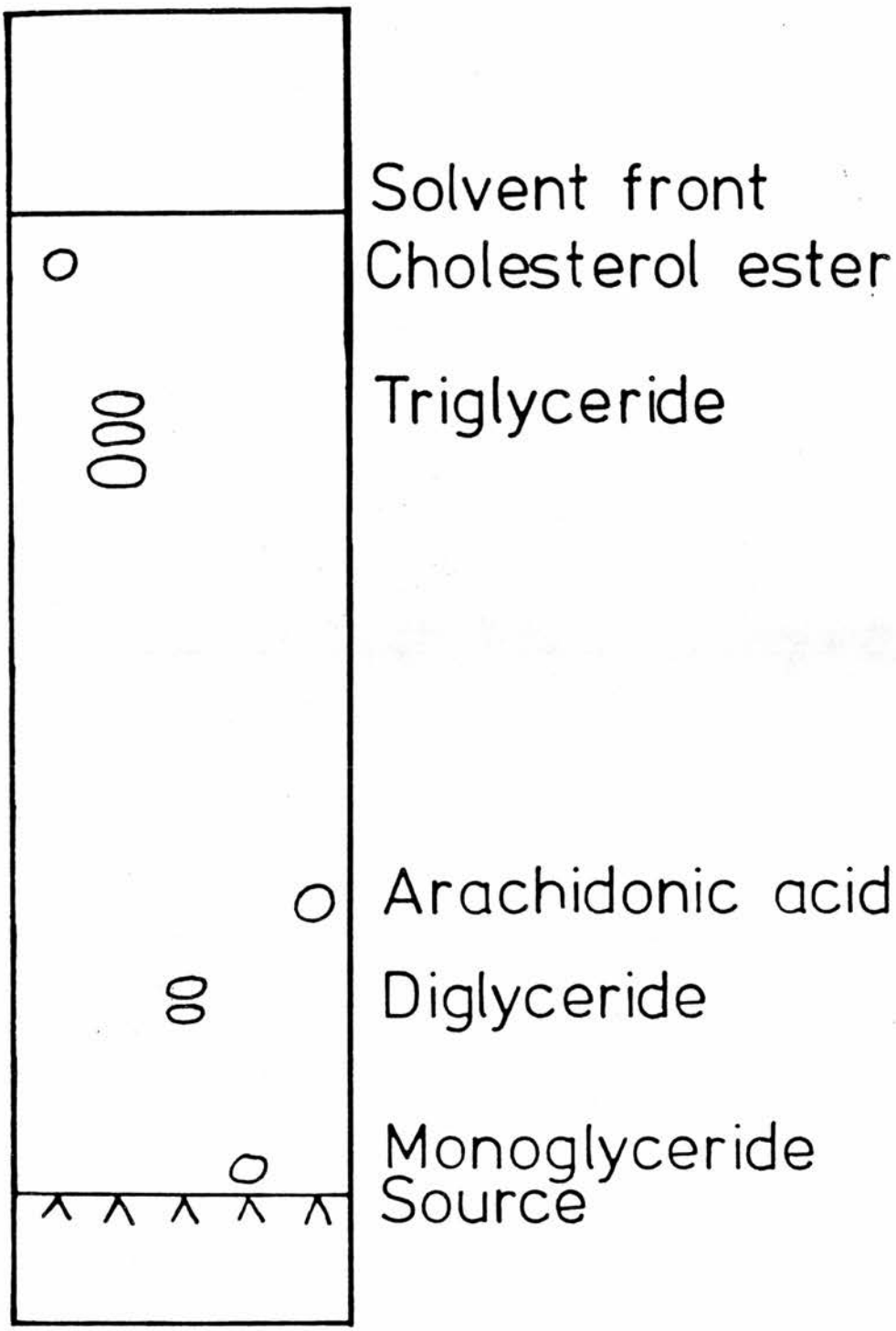


Figure 1.3

The pattern of neutral lipid distribution after development in a solvent system of petroleum spirit (b. pt. 40° - 60° C), diethyl ether and acetic acid (75:25:1 by volume) is illustrated above. The calculated R_f value of

cholesterol ester	is	0.94 - 0.96
triglyceride	is	0.72 - 0.82
arachidonic acid	is	0.28 - 0.32
diglyceride	is	0.17 - 0.23
monoglyceride	is	0.01 - 0.04

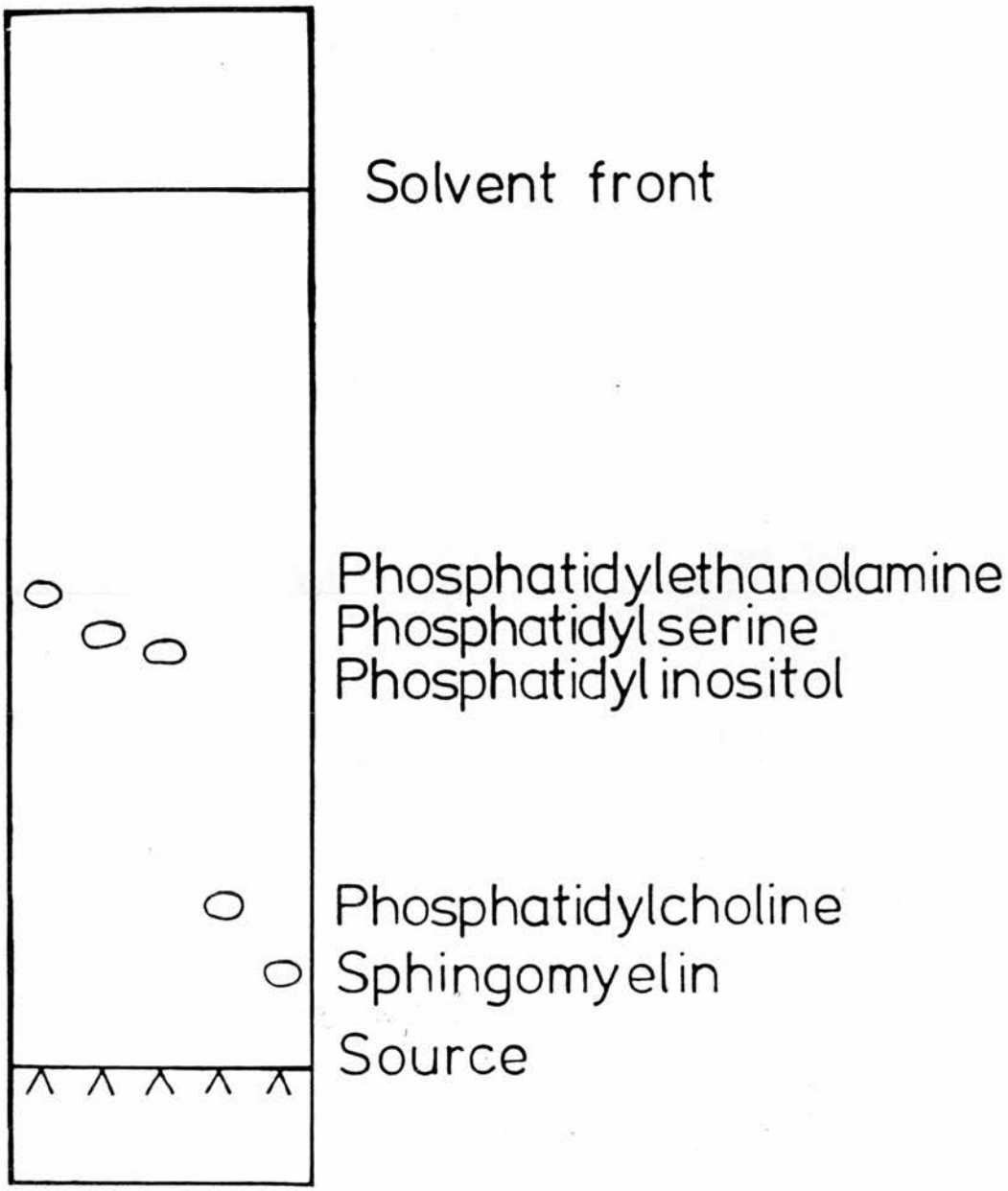


Figure 1.4

The pattern of phospholipid distribution after development in a solvent system consisting of methyl acetate, propan-1-ol, chloroform, methanol and 0.25% aqueous solution of potassium chloride (25:25:25:10:9 by volume).

The calculated R_f value of

phosphatidylethanolamine	is	0.52 - 0.56
phosphatidylserine	is	0.47 - 0.50
phosphatidylinositol	is	0.46 - 0.48
phosphatidylcholine	is	0.16 - 0.20
sphingomyelin	is	0.09 - 0.12

Phospholipids were separated by tlc using a solvent system consisting of methyl acetate, propan-1-ol, chloroform, methanol and 0.25% aqueous solution of potassium chloride (25:25:25:10:9 by volume). Standards (25μg of each) of phosphatidylcholine, phosphatidylethanolamine, phosphatidyl-inositol, phosphatidylserine and sphingomyelin were developed with the samples. After development for about 2hr, the plates were left to air dry for 20min (Fig. 1.4).

iv) Detection of Lipids on the Plates

Iodine vapour was used for the detection of the standard lipids, as this is the simplest technique. When developed and dried, the plates on which the standards had been spotted, were placed into a tlc tank containing a few iodine crystals. Neutral lipids were visualized after 5 min and phospholipids were detected after about 1hr exposure to iodine. The brown hue evaporated spontaneously after a few minutes.

The plates on which the neutral lipid samples had been streaked were then marked with 15 lanes 1 cm wide. Each lane was scraped into a separate scintillation vial, containing 1ml diethyl ether to aid elution of neutral lipids from the silica gel.

The plates on which the phospholipid samples had been streaked were then marked (from the baseline) with 16 lanes 0.5 cm wide followed by 7 lanes 1 cm wide i.e. wider lanes were marked nearer the solvent front. Each lane was scraped into a separate scintillation vial, containing 0.5ml methanol to aid elution of phospholipids from the silica gel.

Scintillation fluid (10ml) was added to each scintillation vial, and the lids were labelled and screwed down tightly. Each vial was shaken severely. The R_f value of each standard lipid was calculated using the formula

$$\frac{\text{distance lipid moved (mm)}}{\text{distance solvent front travelled (mm)}}$$

The resultant R_f values for the standard lipids were then compared with the R_f values of the sample lipids to determine the precise nature of the sample lipids.

v) Recovery of Compounds from the Plates

A comparison of results before and after tlc of neutral lipids indicated that between 50% and 90% of compounds were removed from the plates. In the case of phospholipids, the recovery of radioactivity from the tlc plates ranged from 60% to 90%. All subsequent results have been corrected for recovery.

g) Liquid Scintillation Counting

After addition of 10ml scintillation fluid to each vial, samples were counted for 4 min using a Phillips PW4540 liquid scintillation analyser. This gave a value of counts per minute (cpm) for each sample.

The greatest disadvantage of liquid scintillation counting is quenching, which occurs when the maximum photon yield for a given radioactive source is interfered with at any point. Quenching can be due to any one of three reasons. Optical quenching occurs if dirty scintillation vials are used. Dirty vials interfere with light being emitted by the sample

before it reaches the photomultiplier. Colour quenching occurs if the sample is coloured and results in some of the light being absorbed before it leaves the sample bottle. These types of quenching can easily be corrected for by using clean vials and by adding a drop of hydrogen peroxide, respectively. However, no problems in counting were encountered in these ways.

The most difficult form of quenching to correct for is chemical quenching which occurs if anything in the sample interferes with the transfer of energy from the solvent to the photomultiplier. Unless it is certain that all samples are chemically identical except for radioactive content, it is not sufficiently accurate to compare the counts (cpm) obtained by liquid scintillation counting. Instead, by using an appropriate means of standarization, the counting efficiency of every sample can be determined and the cpm recorded must subsequently be converted to disintegrations per minute (DPM). Efficiency is defined as the ratio of the observed cpm to true DPM.

To determine the efficiency of counting, the scintillation counter machine constants were first determined by constructing a quench curve where a calibrated tritium standard (^{3}H hexadecane) was used to provide a known number of DPM, and its cpm were determined at different levels of quenching. Chloroform was used as the quenching agent, and the protocol is shown in Table 1.5.

TABLE 1.5

Quench Curve Protocol

Vial No.	µL Chloroform added	DPM added
1	-	31000
2	5	31000
3	10	31000
4	20	31000
5	50	31000
6	100	31000
7	200	31000
8	400	31000
9	600	31000
10	500	31000
11	1000	31000
12	-	-

The quench curve is plotted by measuring the efficiency of counting, cpm/DPM (y axis) against the ratio (x axis). Sample channel ratio was used. The quench curve is described by the equation $y = k_0 + k_1x + k_2x^2$ where k_0, k_1 and k_2 are constants. It was necessary to check and if necessary recalculate the machine constants every year, just after the liquid scintillation counter was serviced. Typical quench curves and the values of k_0, k_1, and k_2 are given in Fig. 1.5.

These constants are used to determine the efficiency of counting in each sample so that DPM could be calculated.

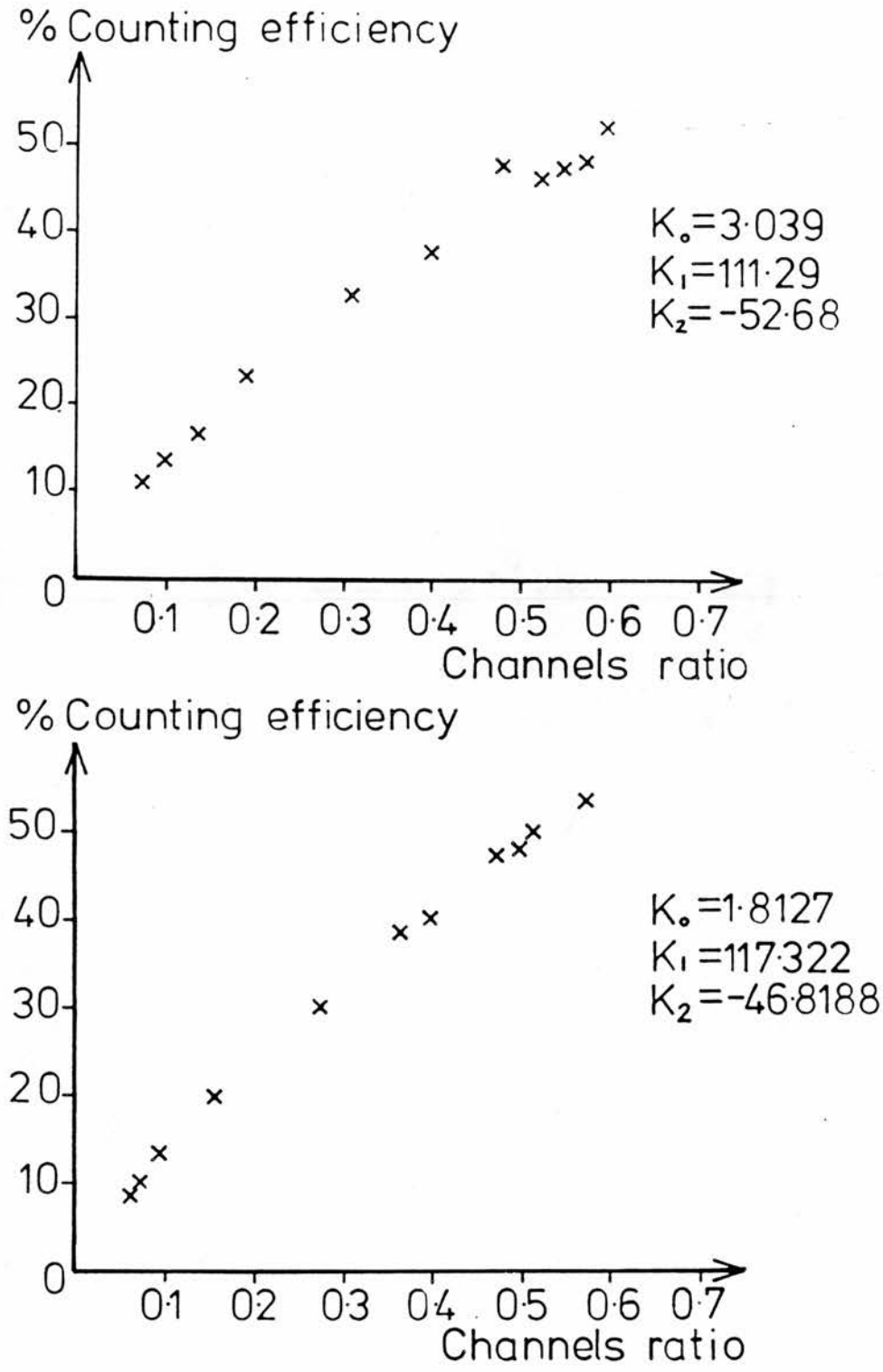


Figure 1.5

Plotting percentage counting efficiency versus sample channels ratio resulted in two typical quench curves. The machine constants derived from the two curves were used to convert cpm to DPM.

h) Statistical Tests

Appropriate data was analysed by Student's t-test or Duncan's multiple range test. Where the variances of the groups were unequal (as analysed by the variance ratio 'F' test), then data was analysed by a modified t-test (see Steel and Torrie, 1980).

SECTION 2. INCORPORATION OF UNSATURATED FATTY ACIDS IN GUINEA PIG ENDOMETRIUM

Introduction

Free arachidonic acid is known to be present in low amounts within tissues (Kunze and Vogt, 1971) including the guinea pig uterus (Leaver and Poyser, 1981). Prolonged synthesis of prostaglandins *in vivo* would tend to quickly deplete the available substrate (free arachidonic acid) and would then have to draw upon the reservoir of arachidonic acid bound in ester form to the neutral lipids and phospholipids (Van Dorp, Beerthuis, Nugteren and Vonkeman, 1964; Lands and Samuelsson, 1968). The arachidonic acid and other fatty acids released from one or more of these reservoirs must be replenished by utilizing uptake processes. The fatty acids may be recycled or fresh supplies derived from dietary sources or from precursor free fatty acids (as summarised in the General Introduction). It is important therefore to monitor the incorporation of fatty acids into these lipid reservoirs. The experiments reported in this section were designed to answer the following points :-

a) Which lipids incorporated arachidonic acid ?

b) The extent to which arachidonic acid was incorporated into each lipid.

c) To determine whether there was any difference in incorporation of arachidonic acid into lipids at different stages of the oestrous cycle.

d) To determine whether a similar pattern of incorporation was observed using other unsaturated fatty acids.

e) To determine whether there was any difference in incorporation of other unsaturated fatty acids into lipids throughout the oestrous cycle.

The experiments employed tritiated arachidonic acid (^{3}H-AA) to monitor incorporation of this fatty acid into the various lipids. Measurements were made on Day 7 and Day 15 of the oestrous cycle as these are respectively days of low and high $PGF_{2\alpha}$ synthesis by, and release from, the guinea pig uterus (Blatchley et al., 1972; Poyser, 1972; Earthy et al., 1975; Wlodawer et al., 1976). The uptake of other unsaturated fatty acids was monitored by using tritiated oleic acid (^{3}H-OA), a molecule composed of 18 carbon atoms and one double bond.

Methods

Experiment 1. Arachidonic Acid Incorporation

Ten guinea pigs were killed by stunning and incising the neck: five were on Day 7 and five were on Day 15 of the oestrous cycle. In each case the uterus was rapidly removed and was placed in culture medium, where the endometrium was subsequently dissected from the myometrium by cutting away 1mm by 2mm portions of endometrium. The myometrium was discarded. Approximately 20mg endometrial tissue was placed onto sterile lens paper which lay across a sterile, stainless steel grid in a vented petri dish. The dish contained 4ml supplemented Medium 199 and 10μCi ^{3}H-AA (as the sodium salt). Six petri dishes containing endometrial tissue were prepared from the uterus of each guinea pig. The tissue was then cultured for 3, 9 or 24 hr by a method based on that of Baker

and Neal (1969) previously described in Section 1(d). Two samples were cultured for each time period. Following incubation, the tissue from each dish was washed in fresh medium, blotted dry, weighed and homogenised in 15ml chloroform and methanol (2:1). Each homogenate was subjected to silicic acid column chromatography to separate the neutral lipids and arachidonic acid products from the phospholipids as described in Section 1 (e). The individual components of the neutral lipids were resolved by thin-layer chromatography as described in Section 1(f). Lanes were marked on each tlc plate as described in Section 1(f) and subsequently each lane was measured for radioactivity by liquid scintillation counting as described in Section 1 (g). The excess solvent in the second fraction from the silicic acid columns was removed under vacuum using a rotary evaporator at 35°C. The radioactivity in the arachidonate products was measured by liquid scintillation counting.

Experiment 2. Oleic Acid Incorporation

Twelve guinea pigs were killed by stunning and incising the neck, six being on Day 7 and six being on Day 15 of the oestrous cycle. Experiment 1 was repeated but using 10μCi ^{3}H-OA (as the sodium salt) instead of ^{3}H-AA in each petri dish. Duplicate samples of endometrium from each guinea pig were cultured for 3, 9 or 24 hr as described in Section 1(d). Following incubation, the tissue from each dish was washed in fresh medium, blotted dry, weighed and homogenised in 15ml chloroform and methanol (2:1). The resultant homogenate was then subjected to silicic acid column chromatography as

previously described in Section 1(e). One of the duplicate samples from four guinea pigs and both duplicate samples from two guinea pigs on each day were further analysed by tlc as previously described in Section 1(f). Lanes were marked as described in Section 1(f) and subsequently each lane was measured for radioactivity by liquid scintillation counting as described in Section 1(g).

Statistical Analysis

All data was analysed by the Student's unpaired "t" test.

Results

Experiment 1. Arachidonic Acid Incorporation

The total amount of ^{3}H-AA taken up into both Day 7 and Day 15 guinea pig endometrial tissue increased with time and there was 2 to 3 times more radioactivity incorporated after 24 hr than after 3 hr (Fig. 2.1). Day 15 tissue incorporated significantly more ^{3}H-AA than Day 7 tissue ($P<0.05$) at 3 hr only (represented as 'total' in Fig. 2.1). At 24 hr there was no significant difference in incorporation of total ^{3}H-AA between Day 7 and Day 15 tissue. Data obtained after silicic acid column chromatography analysis indicated that both the neutral lipid and phospholipid fractions incorporated ^{3}H-AA over the 24 hr time period (Fig.2.1). In Day 7 tissue there was no significant difference in incorporation of ^{3}H-AA between the neutral lipid and phospholipid fractions. A similar finding was also observed in Day 15 tissue. Although no significant differences were found, it was interesting to note that more ^{3}H-AA was incorporated into neutral lipids than into phospholipids at

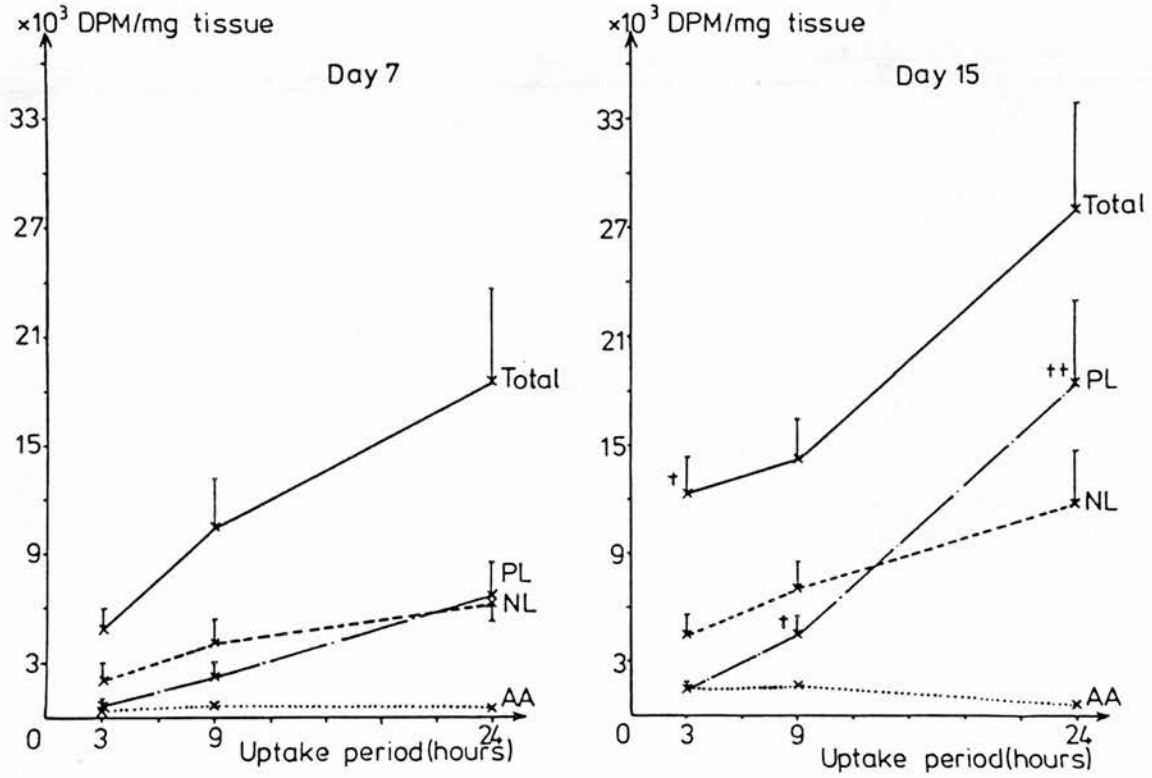


Figure 2.1 Mean (± s.e.m., n=10) total uptake of ^{3}H-AA into Day 7 and Day 15 guinea pig endometrium during a 24 hr culture period, and its distribution among phospholipids (PL), neutral lipids (NL) and free ^{3}H-AA (standard errors for AA are too small to include).

† ($P < 0.05$), †† ($P < 0.01$) Significantly higher than corresponding Day 7 value

3 hr on both Days 7 and 15. By 24 hr however there was no difference in the incorporation of ^{3}H-AA between the two lipid fractions on Day 7, and more ^{3}H-AA was incorporated into phospholipids than into neutral lipids on Day 15.

A further comparison of the data indicated that the uptake of ^{3}H-AA into phospholipids was significantly greater on Day 15 than on Day 7 at 9 hr ($P<0.05$) and 24 hr ($P<0.01$) respectively. There were no significant differences in the uptake of ^{3}H-AA into neutral lipids between Day 15 and Day 7 tissue at any of the times studied, although incorporation at 24hr tended to be higher on Day 15 than on Day 7. There were relatively low levels of free ^{3}H-AA in endometrial tissue at 3, 9 and 24 hr on both Day 7 and Day 15. Very little radioactivity (between 1.3 to 3.9 x 10^{3} DPM) was found in the second fraction eluted from the silicic acid columns indicating that very little ^{3}H-AA was converted to cyclo-oxygenase and possibly lipoxygenase products (for clarity results were omitted from the graphs).

Data obtained after further separation by thin-layer chromatography indicated that the incorporation of ^{3}H-AA into the different neutral lipid classes was in the order of monoglyceride > diglyceride > triglyceride > cholesterol ester at 3 hr and triglyceride > monoglyceride = diglyceride > cholesterol ester at 24 hr, on both Day 7 and Day 15 (Fig.2.2). There was a significantly higher incorporation ($P < 0.05$) of ^{3}H-AA into monoglycerides and diglycerides at 3 hr and 9 hr on Day 15 than on Day 7. At 24 hr, more ^{3}H-AA seemed to be incorporated into triglycerides, monoglycerides and diglycerides on Day 15 than on Day 7, but none of the

differences between the two days were of statistical significance.

Incorporation of ^{3}H-AA into phospholipids was in the order of phosphatidylcholine > phosphatidylethanolamine> phosphatidylserine+phosphatidylinositol > sphingomyelin at 3, 9 and 24 hr on both Day 7 and Day 15 (Fig.2.2). There was significantly greater incorporation ($P<0.05$) of ^{3}H-AA into phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine+phosphatidylinositol at 24 hr on Day 15 than on Day 7.

Experiment 2. Oleic Acid Incorporation

The total amount of ^{3}H-OA taken up into Day 7 and Day 15 guinea pig endometrial tissue increased with time and there was twice as much radioactivity incorporated after 24 hr than after 3 hr (Fig. 2.3). Day 15 tissue incorporated significantly less ($P<0.05$) ^{3}H-OA than Day 7 tissue at 3 hr and 24 hr.

Data obtained after silicic acid column chromatography analysis indicated that both the neutral lipid and phospholipid fraction incorporated ^{3}H-OA over the time period observed (Fig. 2.3). More ^{3}H-OA was incorporated into neutral lipids than into phospholipids in both Day 7 and Day 15 tissue. Incorporation of ^{3}H-OA into neutral lipids at 3 hr and into phospholipids at 24 hr was significantly lower ($P<0.05$) on Day 15 than on Day 7 (Fig. 2.3). There was a relatively high level of free ^{3}H-OA in both Day 7 and Day 15 tissue at the three time periods studied. Very little

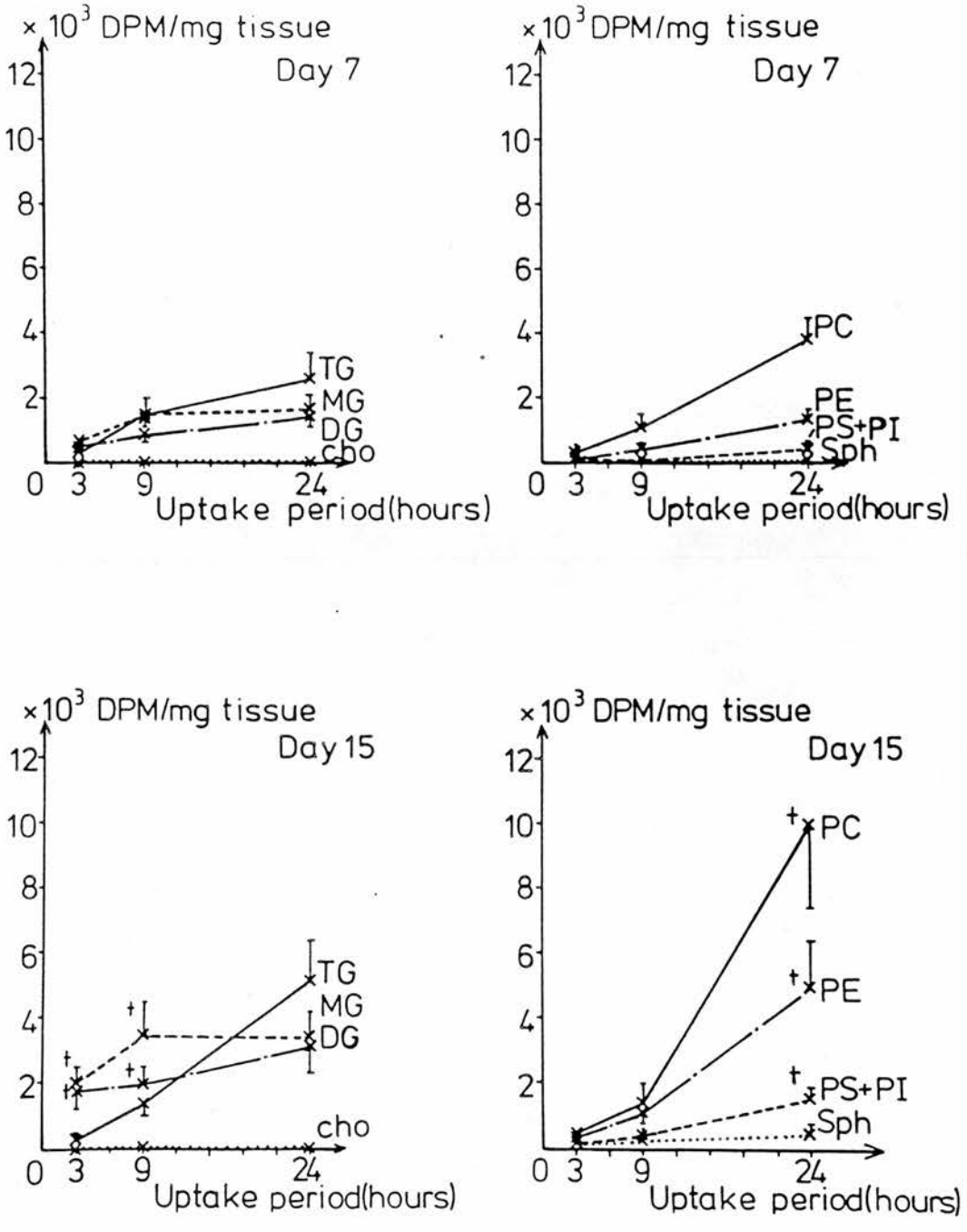


Figure 2.2 Mean (± s.e.m., n=10) uptake of ^{3}H-AA into triglyceride (TG), monoglyceride (MG), diglyceride (DG), cholesterol ester (cho), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine + phosphatidylinositol (PS + PI) and sphingomyelin (Sph) in Day 7 and Day 15 guinea pig endometrium during a 24 hr culture period (Standard errors for cho, and for Sph on Day 7 are too small to include).

† Significantly higher ($P < 0.05$) than corresponding Day 7 value.

radioactivity (between 0.1 to 1.6 x10^3 DPM) was found in the second fraction eluted from the silicic acid columns indicating that, as expected, very little ^{3}H-OA was converted to cyclooxygenase and lipoxygenase products (for clarity results were omitted from the graphs).

Data obtained after further separation by thin-layer chromatography indicated that neutral lipids and phospholipids incorporated ^{3}H-OA over the time period observed (Fig. 2.4). Of the ^{3}H-OA incorporated into neutral lipids, generally more ^{3}H-OA was incorporated into triglycerides and diglycerides than into monoglycerides at 3, 9 and 24hr on both days. There was a significantly greater ($P<0.05$) incorporation of ^{3}H-OA into monoglycerides on Day 7 than on Day 15 at 24hr only. Incorporation of ^{3}H-OA into cholesterol ester was low at all time periods studied although uptake on Day 7 was generally higher than on Day 15 (significantly different ($P<0.05$) at 24hr).

Incorporation of ^{3}H-OA into phospholipids was in the order of phosphatidylcholine > phosphatidylethanolamine > phosphatidylserine + phosphatidylinositol at 3, 9 and 24 hr on both Day 7 and Day 15 (Fig.2.4). However, at 24 hr it was noted that there was a significantly greater ($P<0.05$) incorporation of ^{3}H-OA into phosphatidylethanolamine and phosphatidylserine + phosphatidylinositol on Day 7 than on Day 15. The incorporation of ^{3}H-OA into phosphatidylcholine was similar on both days. The incorporation of ^{3}H-OA into sphingomyelin was so low that it was not feasible to mark the data on the graphs.

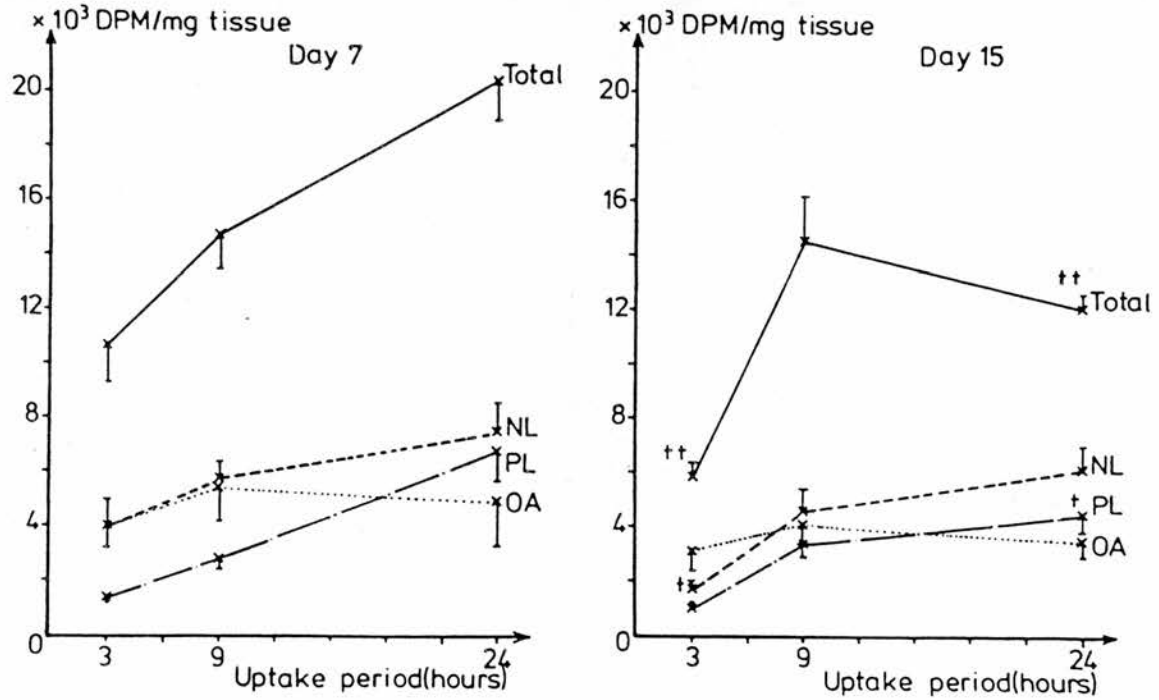


Figure 2.3 Mean (± s.e.m., n=12) total uptake of ^{3}H-OA into Day 7 and Day 15 guinea pig endometrium during a 24 hr culture period, and its distribution among phospholipids (PL), neutral lipids (NL) and free ^{3}H-OA.

† ($P < 0.05$) †† ($P < 0.01$) Significantly lower than corresponding Day 7 value.

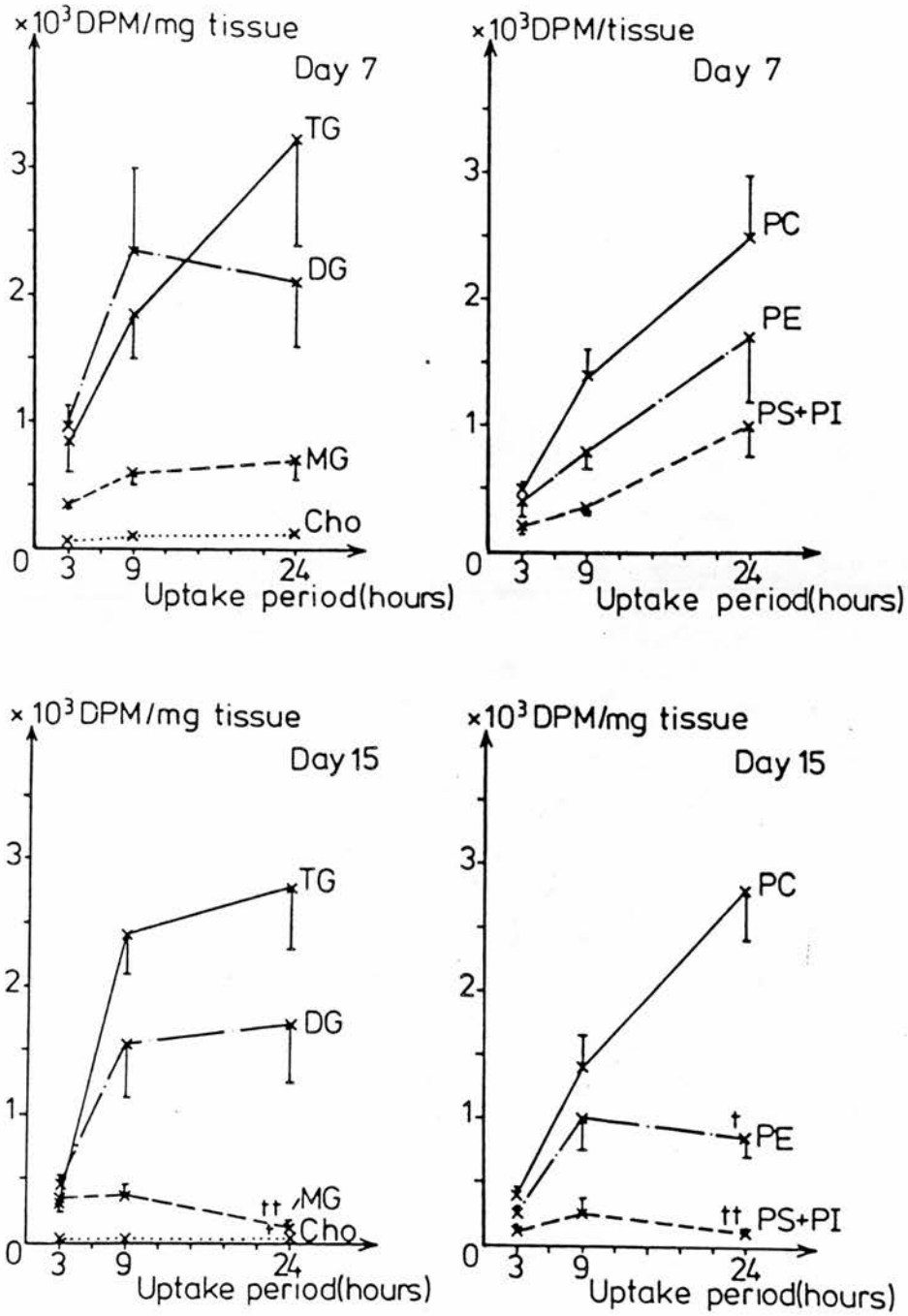


Figure 2.4 Mean (± s.e.m., n=8) uptake of ^{3}H-OA into triglyceride (TG), monoglyceride (MG), diglyceride (DG), cholesterol ester (cho), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine + phosphatidylinositol (PS + PI) in Day 7 and Day 15 guinea pig endometrium during a 24 hr culture period.

† ($P < 0.05$) †† ($P < 0.01$) Significantly lower than corresponding Day 7 value.

Conclusion

The data obtained from these experiments were more than adequate to answer the points raised in the introduction of the current section. It was demonstrated that all the neutral lipids and phospholipids examined were capable of incorporating arachidonic acid to a greater or lesser extent. Of the neutral lipids, triglycerides had the greatest capacity to incorporate arachidonic acid; of the phospholipids, phosphatidylcholine was observed to incorporate the largest amount of arachidonic acid. A comparison of the data from Day 7 and Day 15 indicated that the lipids therein incorporated arachidonic acid to a different extent, and that all the phospholipids with the exception of sphingomyelin of Day 15 tissue incorporated significantly more arachidonic acid than the phospholipids of Day 7 tissue.

Analysis of the second set of results indicated that, like arachidonic acid, oleic acid was incorporated into all the neutral lipids and phospholipids of Day 7 and Day 15 tissue. However the pattern of incorporation of oleic acid throughout the oestrous cycle differed markedly from that of arachidonic acid. The most notable differences were :-

1) there was a greater incorporation of oleic acid into monoglycerides on Day 7 than on Day 15 at 24 hr only. For arachidonic acid there was greater incorporation into monoglycerides on Day 15 than on Day 7 at 3 and 9 hr.

2) there was a lower incorporation of oleic acid into phosphatidylethanolamine and phosphatidylserine +

phosphatidylinositol at 24 hr on Day 15 than on Day 7. Arachidonic acid incorporation into phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol + phosphatidylserine was greater on Day 15 than on Day 7.

It was not possible to compare the actual amounts of ^{3}H-AA and ^{3}H-OA incorporated into the different lipid classes because the quantity of ^{3}H-OA used was much greater than the quantity of ^{3}H-AA used (10μCi of both tritiated compounds was used but the specific activity of ^{3}H-AA was 14 times higher than that of ^{3}H-OA). However, at 24hr, particularly on Day 15, more of the ^{3}H-OA was incorporated into neutral lipids while more of the ^{3}H-AA was incorporated into phospholipids. In vivo, there is a 10- to 15-fold higher incorporation of endogenous oleic acid than of endogenous arachidonic acid into triglycerides while the incorporation of both unsaturated fatty acids into phospholipids is similar (Leaver and Poyser, 1981). These two sets of observations indicate that there are differences in the incorporation and relative distribution of arachidonic acid and oleic acid among the different lipid classes in guinea pig endometrium. To conclude, the overall data indicated that there was a specific, increased incorporation of arachidonic acid into the phospholipids of Day 15 endometrial tissue, which is a day of high $PGF_{2\alpha}$ synthesis, compared to Day 7, which is a day of low $PGF_{2\alpha}$ synthesis.

Discussion

During the period of culture in the presence of tritiated unsaturated fatty acids, it was noted that the levels of free ^{3}H-OA in guinea pig endometrium were much higher than the levels of free ^{3}H-AA presumably due, at least in part, to the conversion of ^{3}H-AA into cyclooxygenase and possibly lipoxygenase products. ^{3}H-OA was not a precursor substrate for the prostaglandin synthetase enzymes and therefore was not metabolised. However approximately 10% of the ^{3}H-AA taken up into endometrial tissue underwent further metabolism.

The tritiated fatty acids, which had not undergone futher metabolism or removal from the uterine cells, were incorporated into the neutral lipids and phospholipids. Levasseur, Sun, Friedman and Burke (1983) have demonstrated that 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) was incorporated into triglycerides in much greater quantities than into phospholipids of mouse thyroid glands. In the present study, it was not known whether the radioactivity in neutral lipids was solely ^{3}H-AA, or whether a proportion of the radioactivity consisted also of ^{3}H-12-HETE which had been synthesized from ^{3}H-AA by lipoxygenase enzymes in the guinea pig endometrium. Further analysis into the identity of the radioactivity present in triglycerides is necessary to establish whether this is the case or not.

Over 90% of the total endogenous arachidonic acid present in the guinea pig uterus is contained in phospholipids

particularly phosphatidylcholine and phosphatidylethanolamine (Leaver and Poyser, 1981). Phospholipids may therefore be the source of precursor for prostaglandin synthesis by the guinea pig endometrium. The present experiments have shown that there is a specific and significantly increased incorporation of arachidonic acid into endometrial phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine, on Day 15 compared to Day 7 (days of high and low $PGF_{2\alpha}$ synthesis, respectively). Therefore the increased uptake of arachidonic acid into phospholipids may compensate for the release of arachidonic acid from phospholipids at the time of increased $PGF_{2\alpha}$ synthesis.

SECTION 3 PHOSPHOLIPIDS IN THE GUINEA PIG ENDOMETRIUM

Introduction

The previous section showed that the pattern of incorporation of arachidonic acid within the phospholipids of the guinea pig endometrium was of the order: phosphatidylcholine > phosphatidylethanolamine > phosphatidylserine + phosphatidylinositol > sphingomyelin. There was a significant increase in incorporation of arachidonic acid into the endometrial phospholipids (with the exception of sphingomyelin) of Day 15 tissue compared to Day 7 tissue. The rise in oestradiol output from the ovary towards the end of the oestrous cycle (Joshi et al.,1973) may be responsible especially as Aizawa and Mueller (1961) have demonstrated an increase in uterine phospholipid synthesis *in vivo* and *in vitro* after oestrogen administration to ovariectomised rats. The experiments presented in the current section were designed to investigate whether an increase in phospholipid synthesis was responsible for the increased incorporation of arachidonic acid into endometrial phospholipids at the time of increased $PGF_{2\alpha}$ production by the endometrium. Three avenues of investigation were explored:

a) The phospholipid content of the endometrium from guinea pigs on Day 7 and Day 15 of the oestrous cycle was measured.

b) The rate of synthesis of phosphatidylcholine in the endometrium from guinea pigs on Day 7 and Day 15 of the oestrous cycle was estimated.

c) The rate of synthesis of phosphatidylinositol in the

endometrium from guinea pigs on Day 7 and Day 15 of the oestrous cycle was monitored also.

a) Quantitative Estimation of Phospholipid Content In Endometrium of Guinea Pigs on Day 7 and Day 15 of the Oestrous Cycle

Introduction

The quantitative estimation of the phospholipid content of tissues used to involve histochemical methods, based on the binding of potassium dichromate to the phospholipid; however the drawback lay in the fact that the individual phospholipids could not be differentiated one from the other (see Thompson, 1966). Biochemical methods became available and were found to be more concise as they were based on the quantitation of changes in the lipid phosphorus. Phospholipids to be analysed were usually extracted into chloroform by the procedure of Bligh and Dyer (1959) and separated into their major classes by thin-layer chromatography. The quantity of each phospholipid was then measured by estimating their phosphorus content. The most popular technique for analysis of the phosphorus in each lipid class involved incubation of the sample with sulphuric acid together with a molybdenum reagent at 160°C for about 3hr (Bartlett, 1959). This step converted the "organic phosphorus" into "inorganic phosphorus" which subsequently bound to molybdenum to form phosphomolybdenum. The latter was reduced to a blue-coloured complex, the quantity of which was measured by monitoring ultraviolet absorbance at a wavelength of 830 nm. Although the method was

insensitive to small variations in the concentration of the reagents and the blue complex was stable at room temperature for about 24hr, it involved the dangerous procedure of boiling with acid as well as being a time consuming step. As a result the following method devised by Raheja, Kair, Singh and Bhatia (1973) was utilised in the present study to gain an accurate, reproducible quantitation of the mass amounts of phospholipids in the endometrium as well as monitoring any changes in the phospholipid content between the endometrium of Day 7 and Day 15 guinea pigs.

Method

Preparation of Chromogenic Solution

Ammonium molybdate (8g) was dissolved in 60ml water to give solution A. Mercury (5ml) and 10N hydrochloric acid (20ml) were shaken with 40ml solution A for 30 min. The mixture was filtered and the supernatant fluid collected was designated solution B. Solution B was added to the remainder of solution A, and 10N sulphuric acid (100ml) was then added to give solution C. Methanol (22.5ml), chloroform (2.5ml) and water (10ml) were added to 12.5ml solution C to give the chromogenic solution, which was stable for at least three months when stored at 5°C.

Preparation of Phospholipid Samples

Ten guinea pigs, five on Day 7 and five on Day 15 of the oestrous cycle, were killed by stunning and incising the neck. In each case the uterus was rapidly removed and the endometrium was separated from the myometrium by cutting away

1mm by 2mm pieces of endometrium. The myometrium was discarded. The endometrial tissue from each uterus was weighed and then homogenised in 15ml chloroform and methanol (2:1). Neutral lipids, arachidonate products and phospholipids were separated by silicic acid column chromatography as described in Section 1(e). The neutral lipids and arachidonate products were discarded.

The phospholipids were separated into their individual classes by thin-layer chromatography as described in Section 1(f). Comparison of the R_f values of the standards with the R_f values of the samples identified the phospholipid in each zone of the plate. These areas were scraped into Corning test tubes, to each of which was added 20ml chloroform and methanol (2:1). The tubes were stoppered, shaken vigorously by hand and then underwent centrifugation at 100g for 15 min to precipitate the silica gel. The solvent containing the phospholipid was removed by Pasteur pipette and the extraction procedure was repeated three times so that all the phospholipid was removed from the silica gel. The solvent collected was pooled for each phospholipid and was reduced in volume under vacuum until near dryness on a rotary evaporator at 35°C. The phospholipid samples were stored at -20°C under a layer of nitrogen gas to await further analysis.

Colorimetric Method

Each phospholipid sample in 1ml chloroform was added to a 15mm by 25mm Corning test tube and the solvent was evaporated. Chloroform (0.4ml) and chromogenic solution (0.1ml) were added to each sample test tube. A control test

tube containing no phospholipid, chloroform (0.4ml) and chromogenic solution (0.1ml) was also prepared. All tubes were placed in a boiling water bath from 1 to 1.5 min with marbles as loose-fitting stoppers. The tubes were then removed and allowed to cool to room temperature. After a further 5 min, chloroform (5ml) was added to each tube. The tubes were shaken gently by hand, and were then allowed to stand for 30 min to enable the lower chloroform layer to separate from the upper aqueous layer. By carefully manipulating each test tube, it was possible to remove the chloroform layer by use of a Pasteur pipette. The absorbance of the chloroform layer from each sample was read at 710nm against the control containing no phospholipid. A Unicam SP 800B ultraviolet spectrophotometer was used.

Calibration Graphs

Known quantities of standard phospholipids were also treated in the same way as the sample phospholipids. The resultant absorbances were plotted against the corresponding phospholipid concentrations to form a calibration graph. The observed absorbances of the sample phospholipids were then read off the calibration graph to give the quantitative amounts of phospholipid present in each sample (Fig. 3.1).

Mode of Action

The chromogenic solution reacted directly with the phospholipid phosphorus and a Prussian blue complex was formed which was stable for over 3hr. That the intact phospholipid reacted with the chromogenic solution was supported by the solubility of the coloured complex in

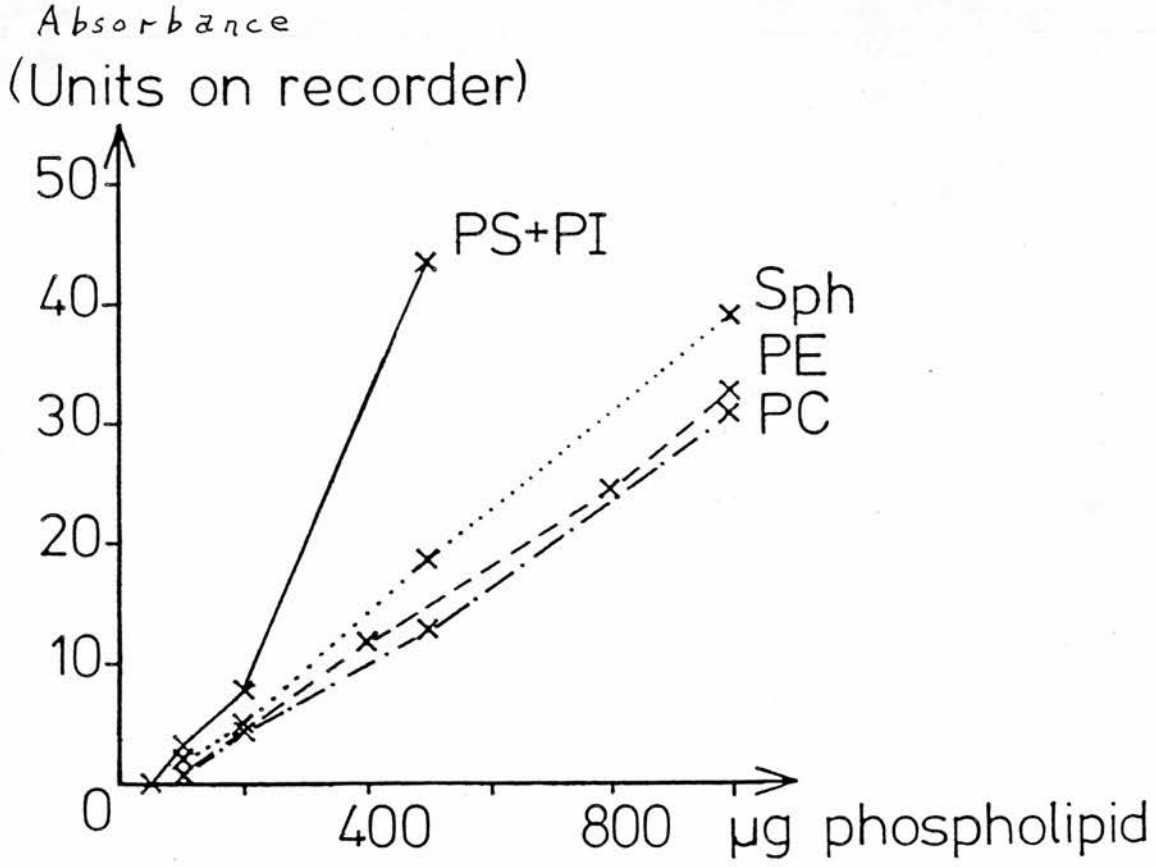


Figure 3.1 Graphical representation of the absorbances observed from known quantities of phosphatidylethanolamine (PE), phosphatidylserine and phosphatidylinositol (PS + PI), sphingomyelin (Sph) and phosphatidylcholine (PC)

chloroform, because digestion of the lipid sample would result in the conversion of organic phosphorus to inorganic phosphorus making the complex water soluble.

Statistical Analysis

All data were subjected to a variance ratio "F" test. If the variances were not unequal, the set of data was subjected to the Student's unpaired "t" test; if the variances were unequal, the modified "t" test was utilised.

Results

The data indicated that both Day 7 and Day 15 endometrium contained detectable quantities of phospholipids. In Day 7 endometrium phosphatidylcholine was the major phospholipid class present in the tissue i.e. this phospholipid made the most significant ($P<0.001$) contribution to the total phospholipid content compared to phosphatidylethanolamine, sphingomyelin and phosphatidylserine + phosphatidylinositol (Fig. 3.2). In Day 15 endometrium phosphatidylcholine also made the most significant ($P<0.05$) contribution to the total phospholipid content of the tissue compared to any other phospholipid (Fig. 3.2). Further calculation indicated that about 62% of the total phospholipid content of both tissues was phosphatidylcholine.

A comparison of the quantitative amounts of the individual phospholipids between Day 7 and Day 15 endometrial tissue was carried out using either a Student's "t" test or a modified "t" test if the variances of the sets of data were unequal. The statistical analyses revealed no significant differences in the content of any phospholipid between the two tissues.

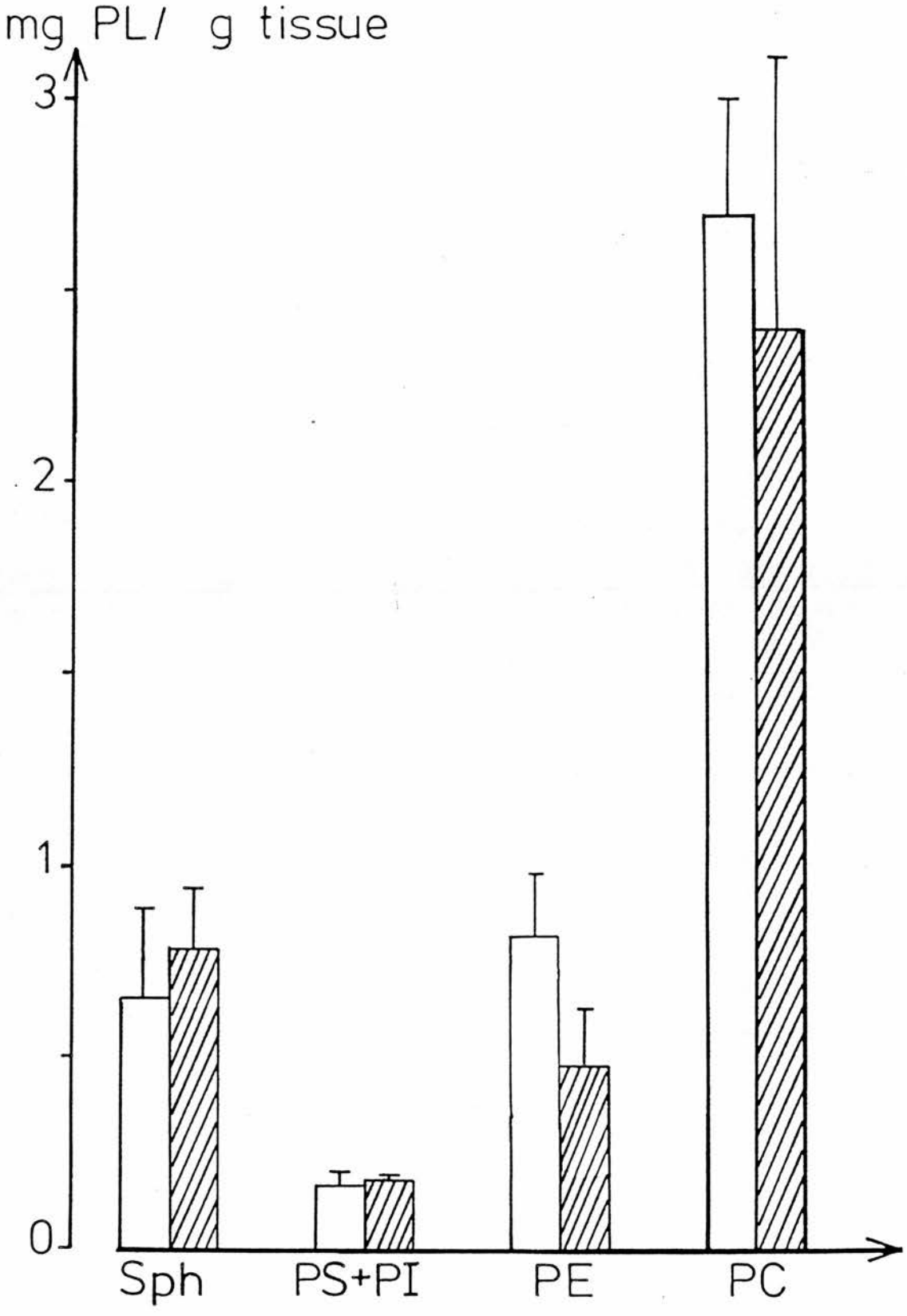


Figure 3.2 Histogram showing the mean quantitive amounts (± s.e.m., n=5) of sphingomyelin (Sph), phosphatidylserine and phosphatidylinositol (PS + PI), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in the endometrium of guinea pigs on Day 7 (blank boxes) and Day 15 (hatched boxes) of the oestrous cycle

Conclusion

Analysis of the phospholipid content in the endometrium of guinea pigs on Day 7 and Day 15 of the oestrous cycle indicated that:

a) phosphatidylcholine made the most significant contribution to the total phospholipid content of the tissue.

b) there was no significant change in the quantity of any phospholipid class.

The results presented in this section suggested that the increased arachidonic acid incorporation observed in the phospholipids of Day 15 endometrium (reported previously in Section 2) was probably not due to a change in phospholipid content of the uterine tissue.

b) Investigations Into Phosphatidylcholine Biosynthesis In Guinea Pig Endometrium

Introduction

The experiments in Section 3(a) demonstrated that the phospholipid content of endometrial tissue was not significantly different between Day 7 and Day 15 of the oestrous cycle. Although the mass content did not vary throughout the cycle it was possible that the turnover of the phospholipids varied i.e. the continual replacement of molecules by synthesis and breakdown has to be considered. The experiments described in the current section were designed to monitor the biosynthesis of phosphatidylcholine, the major lipid class in both Day 7 and Day 15 endometrial tissue.

Two separate pathways for phosphatidylcholine synthesis are known and have been described in detail in the General Introduction. Briefly, to reiterate, the first pathway is de novo synthesis of the phospholipid, whereby two fatty acids are esterified to the glycerol structure to form diglyceride, with the addition of a phosphorylated nitrogenous base completing the synthesis (Kennedy and Weiss, 1956). The second pathway requires three successive methylations of the ethanolamine moiety in phosphatidylethanolamine by S-adenosyl methionine (Bremer and Greenberg, 1961; Hirata, Concoran, Venkatsubramanian, Schiffmann and Axelrod, 1979). This transmethylation pathway of phosphatidylcholine biosynthesis has been identified in many cell types including rabbit leucocytes (Hirata et al., 1979), rat liver (Bremer and Greenberg, 1961; Kaytal and Lombardi, 1976), bovine adrenal medulla (Hirata, Viveros, Diliberto and Axelrod, 1978) and rabbit platelets (Kannagi, Koizumi, Hata-Tanoue and Masuda, 1980). The existence of the transmethylation pathway has not yet been investigated in the endometrium of any species.

It was important to determine therefore:

1) the relative rates of phosphatidylcholine synthesis by the de novo pathway between Day 7 and Day 15 endometrial tissue,

2) the relative activities of the transmethylation pathway of phosphatidylcholine synthesis between Day 7 and Day 15 endometrial tissue.

The findings from these experiments should indicate which pathway plays the major role in synthesis of

phosphatidylcholine in guinea pig endometrium and should also determine whether there are any differences in activities of the two pathways between Day 7 and Day 15 of the oestrous cycle which may account for the increased uptake of arachidonic acid into phospholipids on the latter day.

Methods

Experiment A

Estimation of De Novo Phosphatidylcholine Synthesis

Ten guinea pigs, five on Day 7 and five on Day 15 of the oestrous cycle, were killed by stunning and incising the neck. In each case the uterus was rapidly removed and placed in supplemented Medium 199. The endometrium was dissected from the myometrium by cutting away 1mm by 2mm pieces of endometrium. The myometrium was discarded. The pieces of endometrial tissue were equally distributed among six petri dishes each containing 10μCi tritiated choline chloride (^{3}H-choline chloride) in 4ml supplemented Medium 199, as described in Section 1(d). The petri dishes were then incubated at 37°C for 1, 3 and 6hr periods by a procedure previously described in Section 1(d). Two samples were cultured for each time period. Following incubation, the tissue from each dish was washed in fresh medium, blotted dry, weighed and stored in 5ml chloroform and methanol (2:1) at -20°C for further analysis.

Experiment B

Estimation of the Transmethylation Synthetic Pathway

Ten guinea pigs, five on Day 7 and five on Day 15 of the oestrous cycle, were killed by stunning and incising the neck. In each case, the uterus was rapidly removed and placed in supplemented Medium 199. The endometrium was dissected from the myometrium by cutting away 1mm by 2mm pieces of endometrium. The myometrium was discarded. The pieces of endometrial tissue were equally divided among six petri dishes, each containing 10μCi tritiated S-adenosyl methionine (^{3}H-SAM) in 4ml supplemented Medium 199, as described in Section 1(d). The dishes were incubated at 37°C for 1, 3 and 6hr periods by a procedure previously described in Section 1(d). Two samples were cultured for each time period. Following incubation, each tissue sample was washed in fresh medium, blotted dry, weighed and stored in 5ml chloroform and methanol (2:1) at -20°C for further analysis.

In both experiments A and B, each sample was homogenised in 15ml chloroform and methanol (2:1) and the phospholipids, neutral lipids and arachidonate products present were separated by silicic acid column chromatography as described in Section 1(e). The excess solvent in all the samples was removed by a rotary evaporator at 35°C. The pear-shaped flasks containing the neutral lipids and arachidonate product samples were rinsed with 10ml scintillation fluid and any radioactivity present in the samples were subsequently measured by liquid scintillation counting as described in Section 1(g).

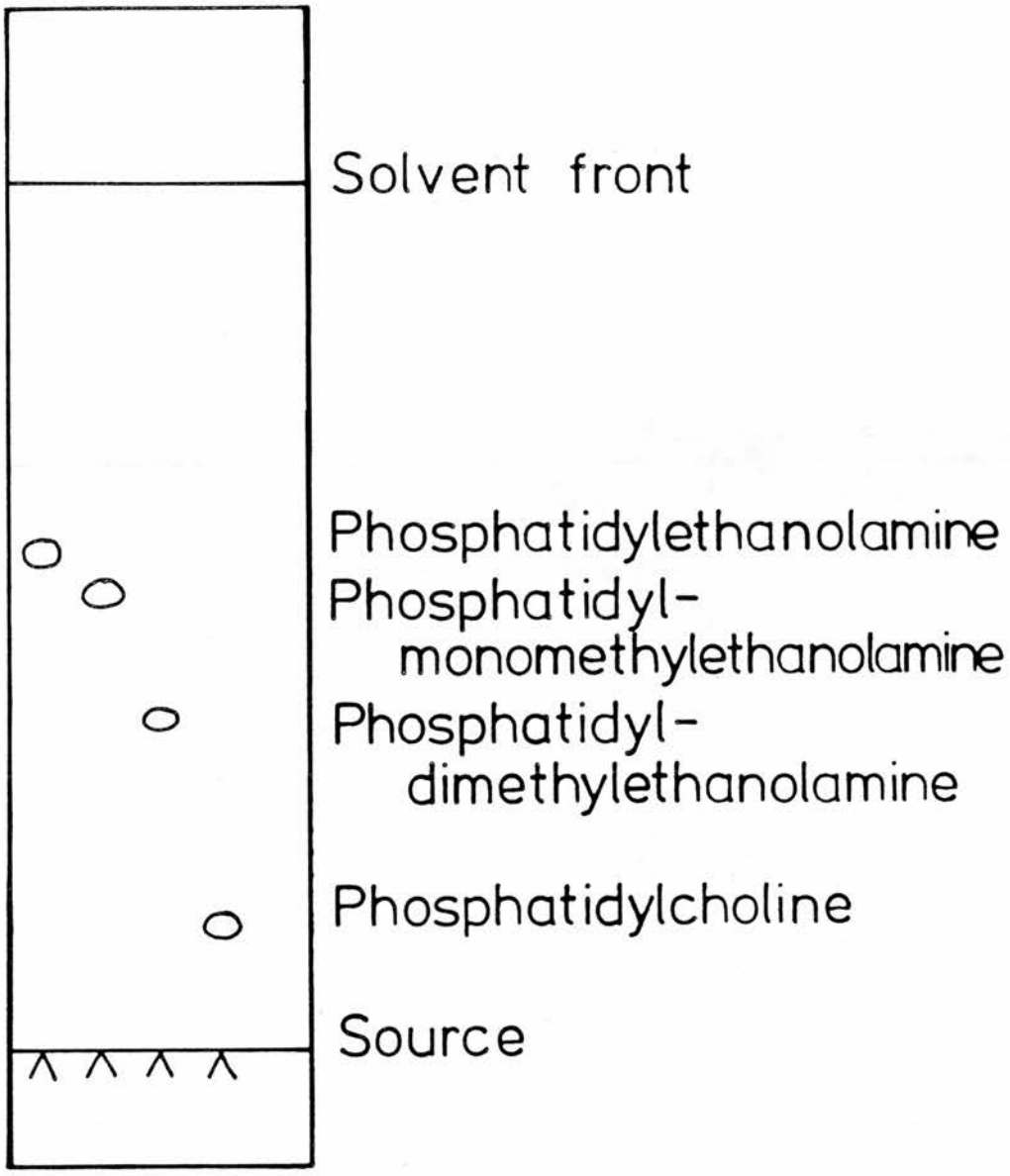


Figure 3.3 The standard phospholipids exhibited the above distribution pattern afer development in a solvent system of chloroform, methanol and water (100:40:6) and visualization by iodine vapour.

The calculated R_f value of

phosphatidylethanolamine	is	0.55 to 0.59
phosphatidylmonomethylethanolamine	is	0.50 to 0.54
phosphatidyldimethylethanolamine	is	0.30 to 0.39
phosphatidylcholine	is	0.12 to 0.16

The phospholipid samples were further separated by thin-layer chromatography (tlc). Each sample was "streaked" onto a precoated glass tlc plate (silica gel thickness 0.25mm, 5cm by 20cm) as described in Section 1(f). Standards (25μg of each) of phosphatidylethanolamine, phosphatidyl-N-monomethyl ethanolamine, phosphatidyl-N,N-dimethyl ethanolamine and phosphatidylcholine were spotted onto a separate tlc plate. All plates were developed in a solvent system consisting of chloroform, methanol and water (100:40:6) for 3hr (Fig. 3.3). They were then removed and left to dry in air at room temperature for 20 min. On each tlc plate containing sample phospholipids, lanes were marked at 0.5cm intervals from the baseline to 7cm from the solvent front; lanes were then marked at 1cm intervals to the solvent front. Subsequently, each lane was measured for radioactivity by liquid scintillation counting as described in Section 1(g). The phospholipid standards were visualised by iodine vapour and the R_f values were calculated (Fig. 3.3).

Statistical Analysis

All data underwent analysis by the variance ratio 'F' test. If the variances were not unequal then data were analysed subsequently by the Student's unpaired 't' test; if the variances were unequal then data were analysed subsequently be a modified 't' test.

Results

Very little radioactivity was measured in the first two fractions eluted from the silicic acid columns indicating that neither the ^{3}H-choline nor the ^{3}H-SAM were incorporated

into the neutral lipids or converted into cyclooxygenase products (as would be expected).

The radioactivity from the ^{3}H-choline incorporated into the phosphatidylcholine class of Day 7 and Day 15 endometrial tissue was monitored as DPM and converted into picomoles phosphatidylcholine synthesized per gram endometrial tissue using the following equation:

$$\frac{\text{DPM per mg tissue} \times 10^{6}}{(2.2\times10^{12}\ \text{DPM}) \times \text{specific activity of radiolabel}}$$

where 2.2×10^{12} DPM $\doteq$ 1Ci, and the units of specific activity were Ci/mmol.

The radioactivity monitored in the investigations into the transmethylation pathway indicated that ^{3}H-SAM was incorporated into three products: the addition of one molecule of ^{3}H-SAM to phosphatidylethanolamine formed phosphatidylmonomethylethanolamine, the addition of a further molecule of ^{3}H-SAM to phosphatidylmonomethylethanolamine formed phosphatidyldimethylethanolamine, and the addition of a final molecule of ^{3}H-SAM to phosphatidyldimethylethanolamine resulted in phosphatidylcholine. All radioactivity was monitored as DPM and converted into picomoles phosphatidylethanolamine methylated per gram endometrial tissue using the equation mentioned previously. For phosphatidylcholine (formed by the addition of three ^{3}H-SAM to phosphatidylethanolamine) and phosphatidyldimethyl-

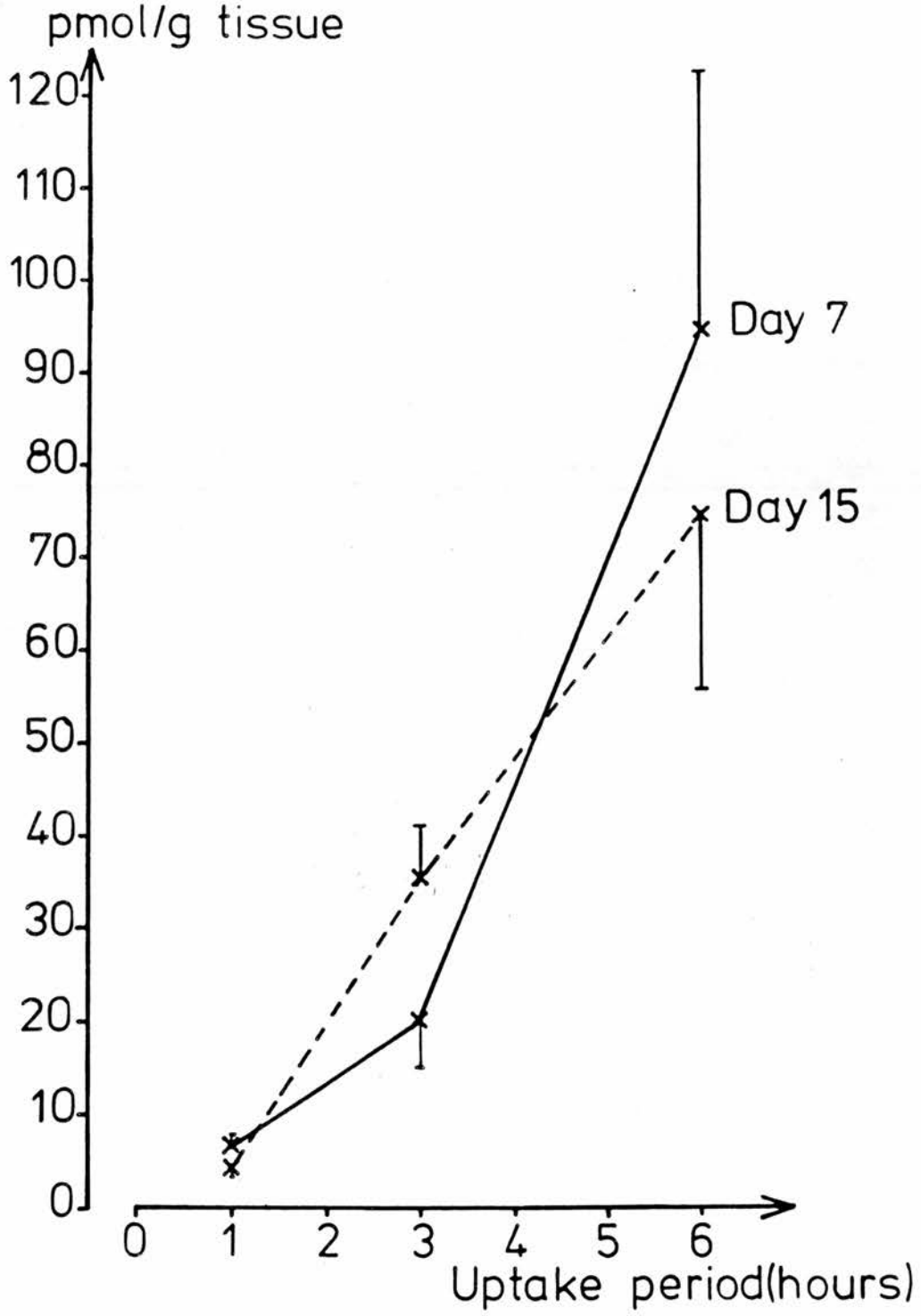


Figure 3.4 Graph demonstrating the mean amount (±s.e.m., n=10) of phosphatidylcholine formed from ^{3}H-choline taken up into Day 7 and Day 15 endometrial tissue over a period of 6hr.

ethanolamine (formed by the addition of two ^{3}H-SAM to phosphatidylethanolamine) it was necessary to divide the number of DPM by three and two respectively.

De novo synthesis as demonstrated by the choline pathway was active in both Day 7 and Day 15 endometrial tissue, (Fig. 3.4).

Statistical analysis using the Student's unpaired 't' test demonstrated that there was no significant difference in the amounts of phosphatidylcholine formed at any time period between Day 7 and Day 15 endometrial tissue i.e. both tissues possessed an equivalent capacity to produce phosphatidylcholine via the de novo synthesis pathway.

The data obtained from experiment B indicated that phosphatidylethanolamine was methylated by transmethylation on both Day 7 and Day 15 endometrial tissue (Fig. 3.5). The ratio of the resultant products, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine and phosphatidylcholine from Day 7 endometrial tissue did not vary over the 6hr time period. At 1hr the ratio of the mean amounts of these products formed was 71:10:19, whilst at 6hr the ratio was 69:14:17.

A similar ratio of methylated products was exhibited by Day 15 endometrial tissue. At 1hr the ratio was 69:12:19 whilst at 6hr the ratio was 75:10:15. In both tissues the methylation of phosphatidylethanolamine to phosphatidylmonomethylethanolamine appeared to be quite active but the conversion of phosphatidylmonomethylethanolamine to phosphatidyldimethylethanolamine and phosphatidylcholine was much slower.

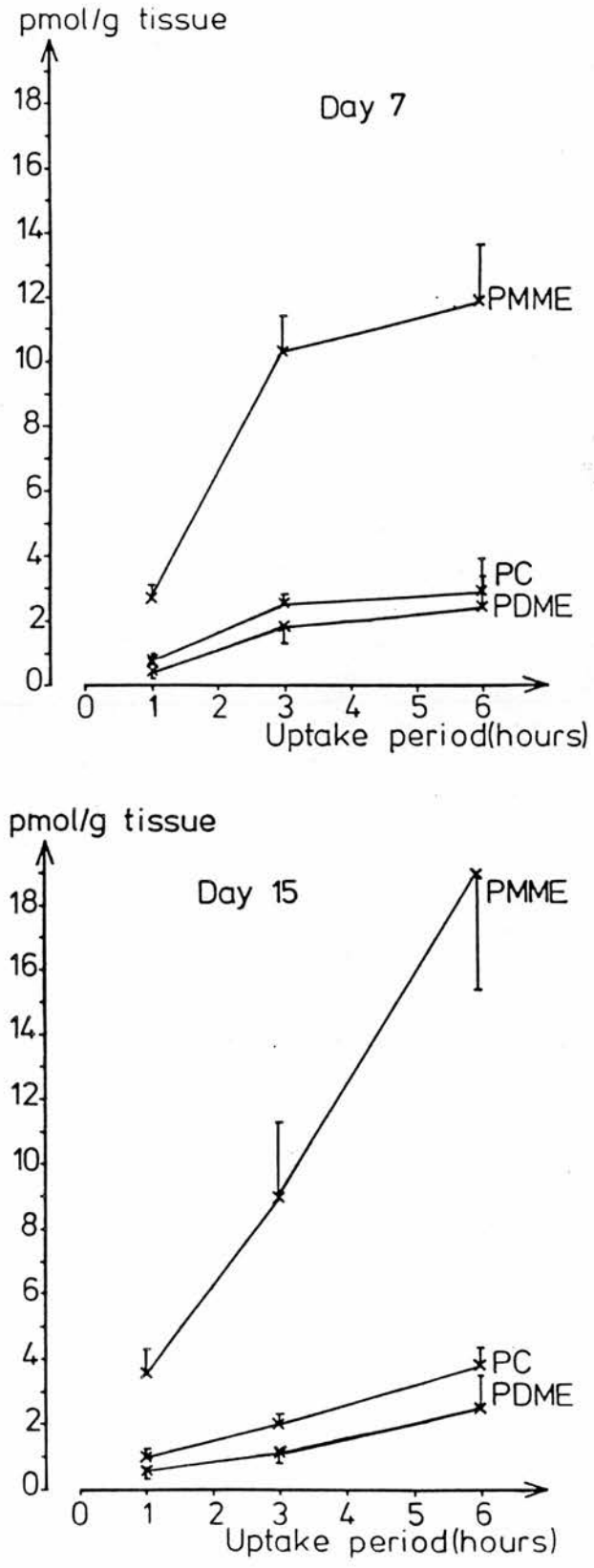


Figure 3.5 The graphs demonstrate the mean amounts (±s.e.m., n=10) of phosphatidylmonomethylethanolamine (PMME), phosphatidyldimethylethanolamine (PDME) and phosphatidylcholine (PC) formed from ^{3}H-SAM taken up over a period of 6 hr.

In Day 7 endometrial tissue, using a Student's unpaired 't' test it was demonstrated that the quantitative amounts of phosphatidylcholine formed by transmethylationof phosphatidylethanolamine increased significantly ($P < 0.0005$) during the 1hr to 3hr uptake period with a mean rate of 0.87 pmol/g tissue/hr. Between the 3hr and 6 hr uptake periods, the mean amount of phosphatidylcholine formed was 0.133 pmol/g tissue/hr.

In Day 15 endometrial tissue, again using a Student's unpaired 't' test it was demonstrated that the quantitative amounts of phosphatidylcholine formed by transmethylation of phosphatidylethanolamine increased significantly ($P < 0.025$) during the 1hr to 3hr uptake period with a mean rate of 0.528 pmol/g tissue/hr. Between the 3hr and 6hr uptake period, there was a significant ($P < 0.01$) production of phosphatidylcholine at a mean rate of 0.584 pmol/g tissue/hr.

Using a modified 't' test it was demonstrated that there was no significant difference in the amounts of phosphatidylcholine formed between Day 7 and Day 15 endometrial tissue at any time period i.e. both tissues possessed an equivalent capacity to synthesize phosphatidylcholine via the transmethylation of phosphatidylethanolamine.

Subsequently, a comparison of the amounts of phosphatidylcholine formed by the choline pathway with the amounts of phosphatidylcholine formed by transmethylation of phosphatidylethanolamine was carried out. Analysis by the modified 't' test demonstrated that the former pathway synthesized significantly ($P < 0.01$) greater amounts of

phosphatidylcholine than the latter pathway at all times on both days.

Conclusion

The results reported here have demonstrated the greater importance of the choline pathway, reflecting de novo synthesis,for the production of phosphatidylcholine in the endometrium of guinea pigs on Day 7 and Day 15 of the oestrous cycle. The transmethylation pathway appears to be a minor route for phosphatidylcholine synthesis in the guinea pig uterus. There was also no significant differences in the activities of either the transmethylation pathway or the choline pathway between Day 7 and Day 15 endometrial tissues. Therefore, the increased incorporation of ^{3}H-AA into phosphatidylcholine on Day 15 as compared to Day 7, observed in Section 2, appears not to have involved the stimulation of phosphatidylcholine synthesis by either of the pathways monitored in these experiments.

c) Studies Into Phosphatidylinositol Synthesis In Guinea Pig Endometrium

Introduction

Myo-inositol (also called meso-inositol) was isolated from biological sources over a hundred years ago. It was required in the medium for survival of most tissue cultures as well as cultured cell lines (Eagle, Oyama, Levy and Freeman, 1957). With every one of eighteen cultured human strains, including liver cells and embryonal fibroblasts, growth ceased in inositol-deficient media and there was

cytopathogenic evidence of cell injury in cell death and dissolution. To maintain growth, 10μM exogenous myo-inositol had to be added and no other inositol isomer proved capable of substituting for it.

It was also demonstrated that a concentration gradient for free inositol existed between cellular and extracellular fluids (Dawson and Freinkel, 1961). The reason for the high concentration of inositol in tissue was thought to be due to a combination of active transport and synthesis within the tissues. The only known function of inositol is as a precursor of phosphatidylinositol (see Hawthorne, 1960), a minor phospholipid of membranes which accounts for less than 10% of the total phospholipids in most cells. The importance of phosphatidylinositol was highlighted when it was demonstrated that inositol deficiency caused an increasing concentration of metabolic products in the cytoplasm of cells that were constrained by a cell wall unable to grow (Henry, Atkinson, Kolat and Culbertson, 1977). Cell surface expansion was terminated after one doubling of whole cells; membrane properties and functions were probably affected.

The phosphatidylinositol present in membranes is formed by de novo synthesis, but also undergoes a constant degradation and resynthesis by the phosphatidylinositol cycle, mentioned previously in the General Introduction. An increase in activity of this cycle has been implicated in providing free arachidonic acid for prostaglandin synthesis and release in several cell types such as human platelets (Rittenhouse-Simmons, Russell and Deykin, 1977), rat peritoneal

macrophages (Matsubara and Hirohata, 1983) and human neutrophils (Walsh, Dechatelet, Chilton, Wykle and Waite, 1983). Since phosphatidylinositol synthesis has not been studied previously in the endometrium of any species, the following set of experiments were designed to determine the relative uptake of tritiated inositol (^{3}H-inositol) with time into Day 7 and Day 15 guinea pig endometrium.

Method

Ten guinea pigs, five on Day 7 and five on Day 15 of the oestrous cycle, were killed by stunning and incising the neck. In each case the uterus was rapidly removed and placed in supplemented Medium 199. The endometrium was separated from the myometrium by cutting away 1mm by 2mm pieces of endometrium. The myometrium was discarded. The endometrial tissue from each uterus were then equally divided amongst ten petri dishes, each containing 10μCi ^{3}H-inositol in 4ml supplemented Medium 199 as described in Section 1(d). Duplicate samples of tissue were cultured for 0.5hr, 1hr, 3hr, 4hr and 6hr periods respectively, as described in Section 1(d). After each culture period, the tissue was washed in fresh medium, blotted dry, weighed and homogenised in 15ml chloroform and methanol (2:1). Each homogenised sample underwent silicic acid column chromatography as described in Section 1(e) to separate the neutral lipids and arachidonate products from the phospholipids. The excess solvent in the samples from the column was removed by a rotary evaporator at 35°C and, after rinsing the appropriate flasks with 10ml scintillation fluid, the radioactivity in

the neutral lipids and arachidonate product samples was measured by liquid scintillation counting as described in Section 1(g).

The phospholipid samples from the column were further separated by a thin-layer chromatography (tlc) procedure devised by Jolles, Zwiers, Dekker, Wirtz and Gispen (1981). Precoated glass tlc plates (silica gel thickness 0.25mm, 5cm by 20cm) were impregnated with potassium oxalate by development in 100ml methanol and water (2:3) containing 1% potassium oxalate. The plates were then air dried at room temperature for 15 min and activated at 110°C for a further 15 min. Lipid extracts were applied to these tlc plates in a volume of 15 to 20µl chloroform and methanol (2:1) using a 100µl syringe. Standards (25µg each) phosphatidylinositol (I), diphosphoinositide (II) and triphosphoinositide (III) were applied to a separate tlc plate and developed at the same time as the samples (Fig. 3.6 and Fig. 3.7). All tlc plates were developed in a chromatographic chamber containing a solvent system of chloroform, acetone, methanol, acetic acid and water (40:15:13:12:8) for 2hr. The plates were then left to dry in air at room temperature for 30 min. On each 'sample' tlc plate, lanes were marked at 0.5cm intervals from the baseline to 7cm from the solvent front; lanes were then marked at 1cm intervals to the solvent front on each tlc plate. Subsequently, the radioactivity in each lane was measured by liquid scintillation counting as described in Section 1(g). Standards were visualised by iodine vapour and the R_f values were calculated (Fig. 3.6).

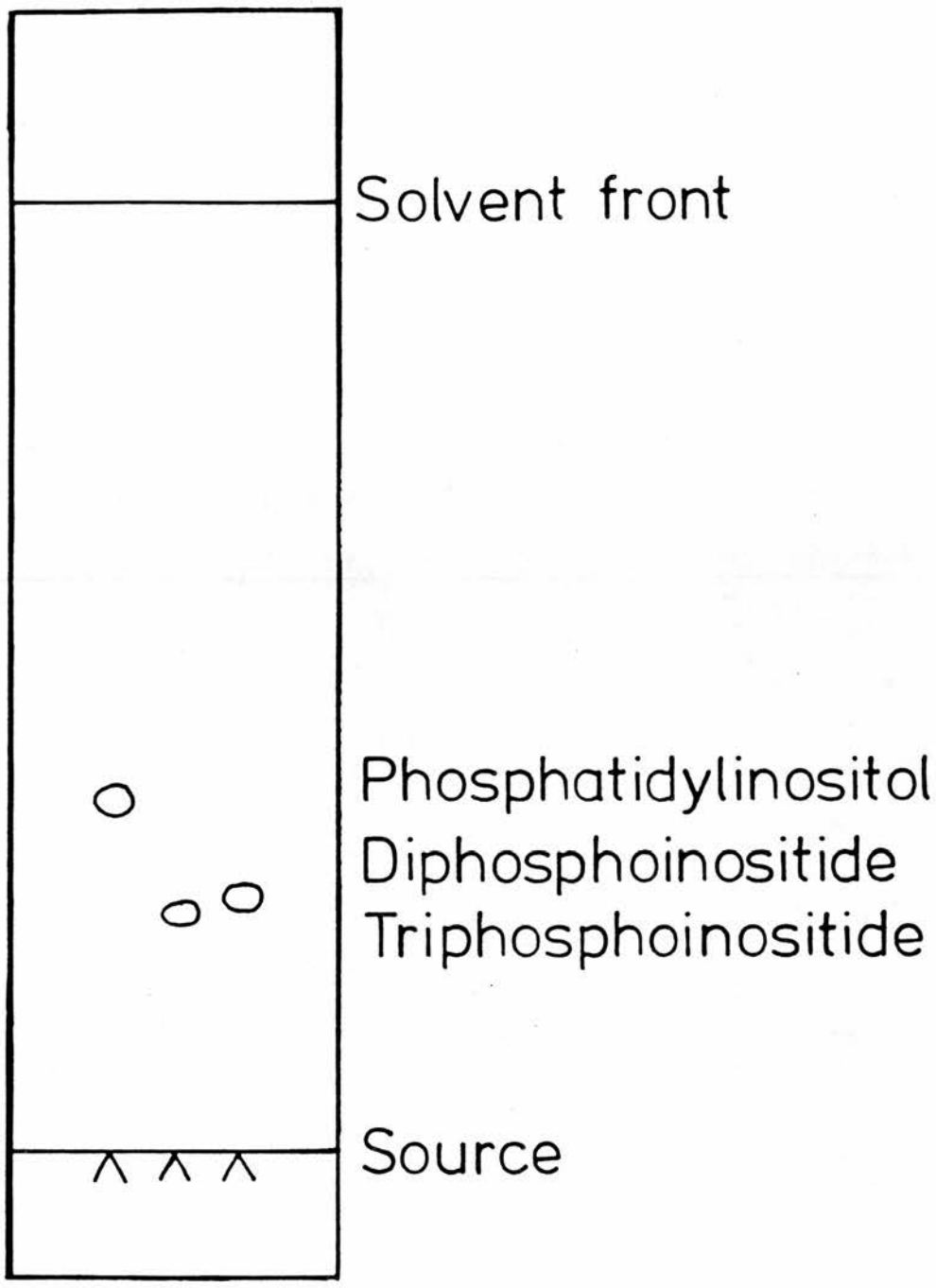


Figure 3.6 The standard phospholipids exhibited the above distribution pattern after development in a solvent system of chloroform, acetone, methanol, acetic acid and water (40:15:13:12:8). Phospholipids were visualised by iodine vapour.

The calculated R_f value of

phosphatidylinositol	is	0.35 to 0.39
diphosphoinositide	is	0.25 to 0.28
triphosphoinositide	is	0.23 to 0.26

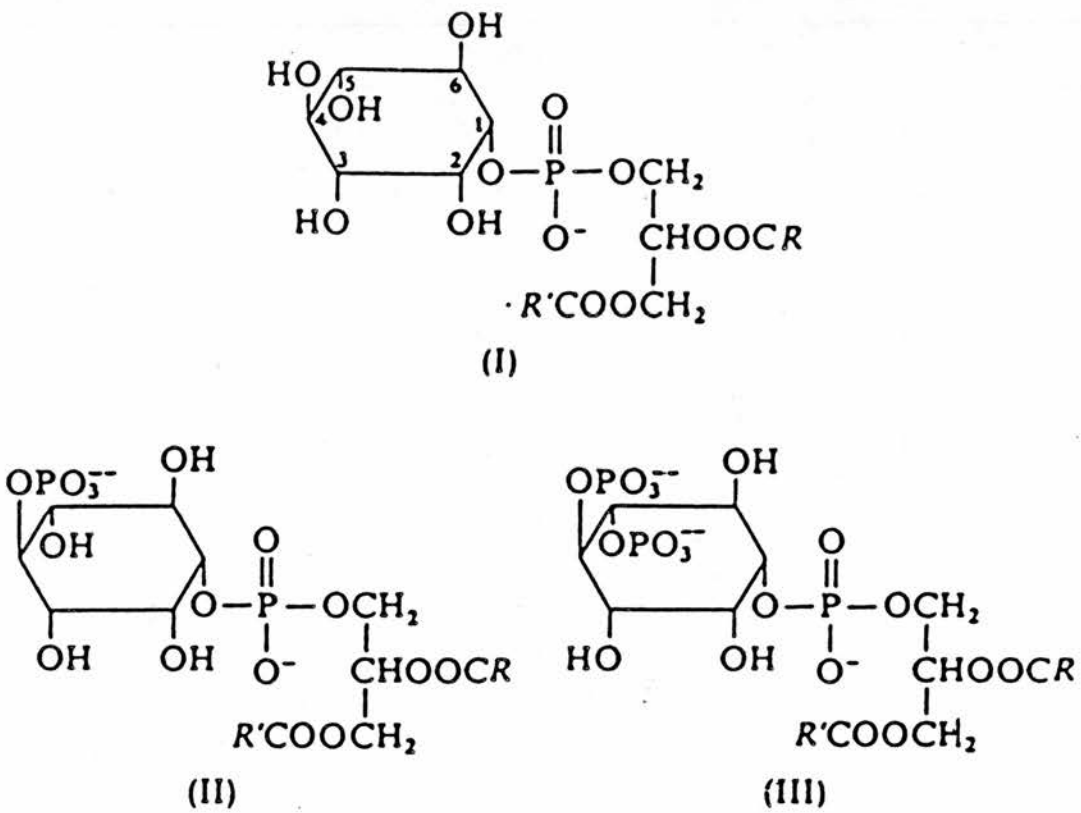


Figure 3.7 Strucures of phosphatidylinositol (I), diphosphoinositide (II) and triphosphoinositide (III) where R and R' are fatty acid chains.

Statistical Analysis

All data were analysed by the variance ratio 'F' test. If the variances were not unequal then data were analysed subsequently by the Student's unpaired 't'test. If the variances were unequal then data were analysed by the modified 't' test.

Results

Very little radioactivity was measured in the first two fractions eluted from the silicic acid columns indicating that the ^{3}H-inositol was not incorporated into either the neutral lipids or converted into cyclooxygenase products (as would be expected).

The radioactivity from the ^{3}H-inositol incorporated into the phosphatidylinositol classes of Day 7 and Day 15 endometrial tissue was monitored as DPM and converted into picomoles phospholipid per gram endometrial tissue using the following equation:

$$\frac{\text{DPM per mg tissue} \times 10^{6}}{(2.2\times10^{12}\text{DPM}) \times \text{specific activity of radiolabel}}$$

where 2.2×10^{12} DPM = 1Ci, and the units of specific activity were Ci/mmol.

It was demonstrated in rat liver that a small amount of phosphatidylinositol underwent further metabolism to diphosphoinositide (Michell and Hawthorne, 1965). Isotope studies suggested that triphosphoinositide was formed also by a two-step phosphorylation of phosphatidylinositol

(Brockerhoff and Ballou, 1962). It was necessary to monitor the amounts of any polyphosphoinositides formed. Therefore any radioactivity from ^{3}H-inositol incorporated into the polyphosphoinositides was converted into picomoles phospholipid per gram endometrial tissue using the above equation. As diphosphoinositide and triphosphoinositide exhibited similar R_f values, the data was combined to form the "remainder", a measure of the amount of phosphatidylinositol which underwent further metabolic conversion.

The resulting data indicated that, in both Day 7 and Day 15 endometrial tissue, the total amount of phosphatidylinositol synthesized increased with time (Fig. 3.8). Between 0.5hr and 6hr, the amounts of phosphatidylinositol in Day 7 tissue increased significantly ($P<0.01$) with time at an average rate of 2.036 pmol/g tissue/hr. The "remainder" i.e. polyphosphoinositides did not increase with time, the values never exceeding 3 pmol/g tissue at any stage.

Between 0.5hr and 6hr, the amounts of phosphatidylinositol in Day 15 tissue increased significantly ($P<0.01$) with time at an average rate of 1.23 pmol/g tissue/hr. The "remainder" also increased significantly ($P<0.05$) with time at an average rate of 0.078 pmol/g tissue/hr. There was about twelve times more phosphatidylinositol present after 6hr than after 0.5hr in Day 7 tissue; in Day 15 tissue there was about fourteen times more phosphatidylinositol present after 6hr than after 0.5hr. The Student's unpaired 't' test revealed that there was no significant difference in the amounts of

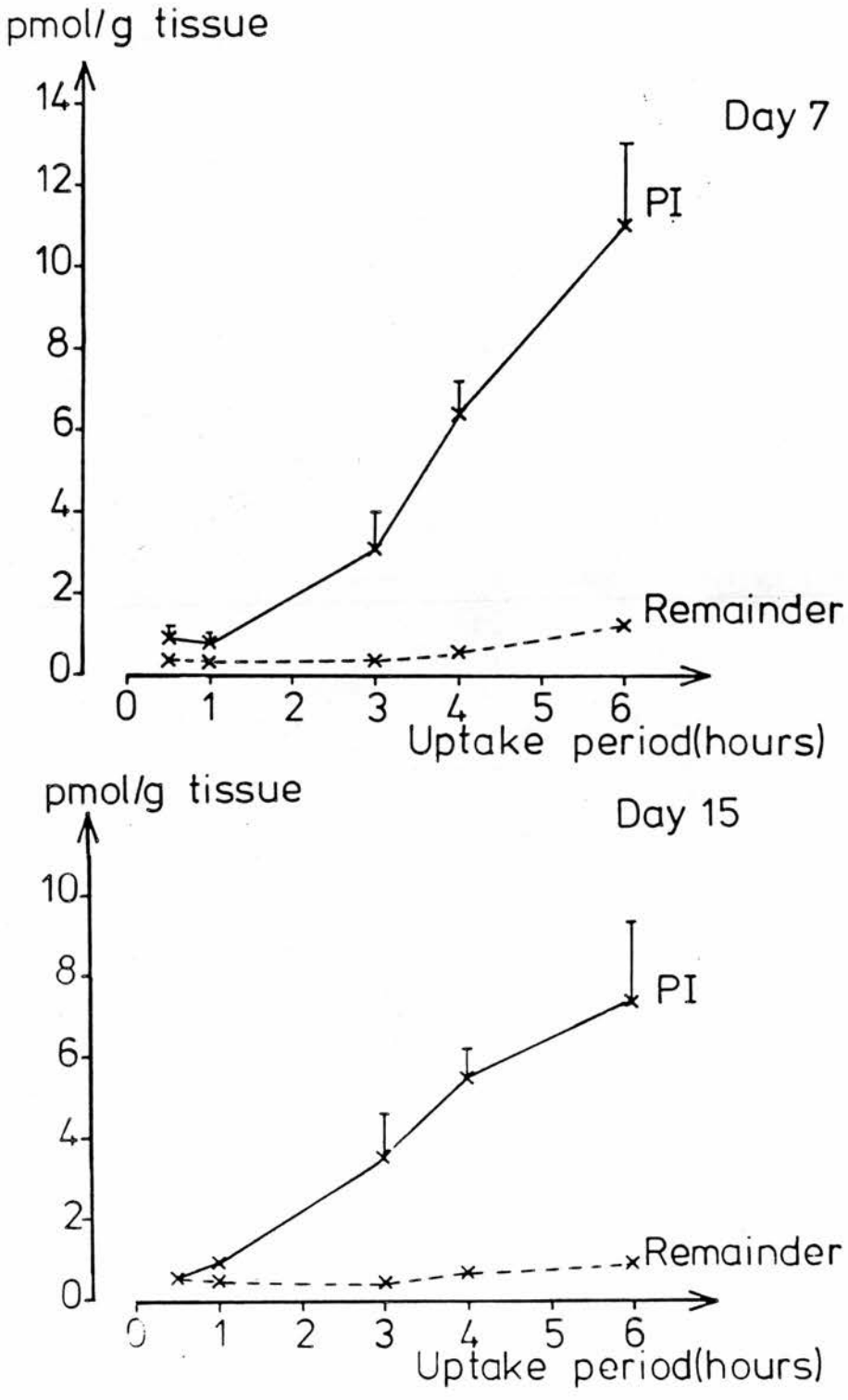


Figure 3.8 Graphs indicate the mean quantative amounts (± s.e.m., n=8) of phosphatidylinositol (PI) formed from ^{3}H-inositol taken up over a 6 hr period into Day 7 and Day 15 endometrial tissue. The remainder is an indication of the mean quantative amounts of PI which underwent further metabolism to polyphosphoinositides.

phosphatidylinositol synthesized between Day 7 and Day 15 endometrium at all times.

The combined use of the modified 't' test and the Student's unpaired 't' test revealed that there was no significant difference in the amounts of polyphosphoinositides synthesized between Day 7 and Day 15 endometrial tissue at all times.

Conclusion

The results reported here have demonstrated that the inositol was incorporated into phosphatidylinositol by Day 7 and Day 15 endometrial tissue. Route of inositol incorporation may be by de novo phosphatidylinositol synthesis and/or via the phosphatidylinositol cycle, i.e. by addition of inositol to phosphatidic acid formed by breakdown of endogenous phosphatidylinositol. Since there was no difference in incorporation of inositol into phosphatidylinositol between Day 7 and Day 15 endometrial tissue, it can be concluded that neither de novo phosphatidylinositol synthesis nor the phosphatidylinositol cycle underwent stimulation. Therefore the increased incorporation of ^{3}H-AA into the phospholipids on Day 15 as compared to Day 7, observed in Section 2, had not involved stimulation of the pathways associated with phosphatidylinositol synthesis.

Discussion

In Section 2 it was demonstrated that there was a significant incorporation of arachidonic acid into all the endometrial phospholipids (with the exception of sphingomyelin) of Day 15

tissue compared to Day 7 tissue. It is possible that the storage capacity of the phospholipids was increased by an increase in the amount of phospholipids present in the tissue membranes. The experiments in Section 3(a) were designed to quantitatively estimate any differences in the mass amount of phospholipids between Day 7 and Day 15 endometrial tissue. The data indicated that none of the phospholipid classes varied in amount throughout the oestrous cycle. Therefore the increased incorporation of arachidonic acid into phospholipids of Day 15 tissue compared to Day 7 tissue cannot be explained by a change in phospholipid content. However it is possible that there may have been increased breakdown and increased synthesis resulting in an apparent lack of change in content.

The lack of mass change in phosphatidylinositol content of the endometrium between Day 7 and Day 15 of the cycle may indicate that arachidonic acid release from phosphatidylinositol by stimulation of phospholipase C and working in conjunction with diglyceride lipase was probably not operating or was operating at a very low rate, i.e. there was no breakdown of the phospholipid to monoglyceride and free fatty acids (Bell et al., 1979). Phosphatidylinositol-specific phospholipase C has also been shown to play a minor role in arachidonic acid liberation from phosphatidylinositol in human platelets (Imai et al., 1982). However it was possible that the diglyceride was reincorporated into phosphatidylinositol via the phosphatidylinositol cycle (Lapetina, 1982) and arachidonic acid was indirectly released by phosphatidic acid enroute (see General Introduction). An

apparent lack in phosphatidylinositol content, does not necessarily mean that there were no changes in the rate of phosphatidylinositol turnover via the phosphatidylinositol cycle in Day 15 tissue compared to Day 7 tissue. Consequently it was important to measure the rates of phospholipid synthesis.

Leaver and Poyser (1981) reported that, of the total arachidonate bound to phospholipids in the uterus, phosphatidylethanolamine contained more arachidonate than any other phospholipid on both Day 7 and Day 15. However data presented in Section 3(a) indicated that the quantity of phosphatidylcholine on both Day 7 and Day 15 was significantly greater ($P<0.05$) than phosphatidylethanolamine. It has been suggested that there may be exchange of acyl fatty acids from arachidonate-rich phosphatidylethanolamine to phosphatidylcholine (Trotter and Ferber, 1981) resulting in enrichment of the latter phospholipid with arachidonic acid.

Enrichment of phosphatidylcholine with arachidonic acid can also be brought about by conversion of phosphatidylethanolamine to phosphatidylcholine via the transmethylation pathway (Bremer and Greenberg, 1961). Therefore the experiments in Section 3(b) were carried out to determine the importance of this pathway in Day 7 and Day 15 endometrial tissue by measuring the amounts of phosphatidylcholine formed from phosphatidylethanolamine. The amounts of phosphatidylcholine formed from the <u>de novo</u> synthesis involving choline incorporation was also measured. The results indicated that

the conversion of phosphatidylethanolamine to phosphatidylcholine played a minor role in the total synthesis of phosphatidylcholine in the guinea pig endometrium on both Day 7 and Day 15 of the oestrous cycle.

A methyl transferase enzyme is responsible for the transmethylation of phosphatidylethanolamine to phosphatidylmonomethylethanolamine. The conversion of phosphatidylmonomethylethanolamine to phosphatidyldimethylethanolamine and then to phosphatidylcholine is catalysed by a second methyltransferase enzyme. Investigations using rat liver microsomes (Bremer and Greenberg, 1961) have determined that incorporation of the first methyl group was the rate-limiting step in transmethylation. The current investigations demonstrated that phosphatidylmonomethylethanolamine was present in greater quantities than phosphatidyldimethylethanolamine, indicating that the second methyltransferase enzyme was the rate-limiting step in transmethylation in the guinea pig endometrium. The discrepancy between the current investigations and that which was reported in the literature may be explained by the type of tissue used, species difference or differences in enzyme distribution.

The experiments in Section 3(b) clearly demonstrated the importance of the choline pathway (reflecting <u>de novo</u> synthesis) in the formation of phosphatidylcholine, the pathway being five times as active as the transmethylation pathway. However there was no significant difference in the rate of incorporation of choline into phosphatidylcholine between Day 7 and Day 15 endometrial tissue. It appears therefore that increased arachidonic acid incorporation

cannot be explained by an increase in de novo synthesis of phosphatidylcholine in Day 15 tissue.

Increased incorporation of arachidonic acid into phosphatidylinositol of Day 15 tissue was demonstrated in Section 2, therefore further experiments were carried out to determine if an increased rate of synthesis of phosphatidylinositol was responsible. The investigations into phosphatidylinositol biosynthesis could have been complicated by the fact that a small amount of phosphatidylinositol underwent further metabolism to diphosphoinositide and triphosphoinositide. Reproducible extractions of the polyphosphoinositides are extremely difficult, good extraction efficiency is managed with the use of acid solvents (Michell R.,personal communication). The procedures in these experiments however did not utilise acid conditions and it was possible that not all the polyphosphoinositides formed were extracted. Nevertheless since diphosphoinositide and triphosphoinositide are not usually considered to occur in appreciable amounts outside the nervous system (see Hawthorne and Kai, 1970) the formation of these two compounds was not considered a complication in this study.

The data obtained from the experiments in Section 3(c) indicated that there was no significant difference in the amount of inositol inorporated into phosphatidylinositol between Day 7 and Day 15 endometrial tissue. Thus it appears that there was no stimulation of de novo phosphatidylinositol synthesis or of the phosphatidylinositol cycle between Day 7 and Day 15 of the cycle, and that the increased uptake of

arachidonic acid into phosphatidylinositol between these two days is not due to increased phosphatidylinositol synthesis.

A comparison of the quantitative amounts of phosphatidylinositol formed with the quantitative amounts of phosphatidylcholine formed resulted in a phospholipid ratio of one to eight. It is suggested that the difference in phospholipid synthesizing capacity of the tissue was a reflection of the importance of phosphatidylcholine synthesis in providing arachidonic acid for prostaglandin synthesis, especially as phosphatidylcholine contains much more arachidonic acid than phosphatidylinositol (Leaver and Poyser, 1981).

To summarise, the increased arachidonic acid incorporation into the phospholipids of Day 15 tissue compared to Day 7 tissue (reported in Section 2) cannot be explained by a change in phospholipid content or by an increase in the phospholipid synthesizing capacity of the endometrium. In addition the lack of increased inositol incorporation into phosphatidylinositol on Day 15 compared to Day 7 also suggests that the increased output of $PGF_{2\alpha}$ from the uterus on Day 15 does not involve stimulation of the phosphatidylinositol cycle, in contrast to the stimulation of TXA_2 production by thrombin in platelets (Lapetina, 1982).

SECTION 4 THE CONTROL OF ARACHIDONIC ACID RELEASE FROM THE GUINEA PIG ENDOMETRIUM

Introduction

Studies carried out by Leaver and Poyser (1981) have demonstrated that phosphatidylcholine and phosphatidylethanolamine contain 80% of the arachidonic acid bound to phospholipids in the uterus. However there was a significant ($P<0.05$) decrease in the amount of arachidonic acid esterified to phosphatidylcholine on Day 15 of the oestrous cycle. These studies suggested that there had been a greater release of arachidonic acid from phosphatidylcholine on Day 15 than on Day 7 of the oestrous cycle.

Because the ovarian venous plasma levels of oestradiol increased in the guinea pig after Day 10 of the cycle prior to release of $PGF_{2\alpha}$ (Joshi et al.,1973) it was thought that the release of arachidonic acid may in some way be controlled by this hormone. Oestradiol (10μg) injected into the tail vein of ovariectomised rats (Mueller et al., 1961) was able to increase protein synthesis of the uterus. Activation, mobilization or possibly *de novo* synthesis of the enzymes involved in the release of arachidonic acid may have occurred.

The experiments reported in the following section were designed to measure:

a) the release of ^{3}H-AA from Day 15 endometrial tissue compared to Day 7 endometrial tissue as these are days of high and low endometrial $PGF_{2\alpha}$ synthesis respectively.

b) the release of ^{3}H-AA in the presence of a general stimulus of endometrial $PGF_{2\alpha}$ synthesis, such as calcium ionophore A23187, from Day 7 and Day 15 endometrial tissue.

c) the release of ^{3}H-AA in the presence of either oestradiol or progesterone from Day 7 and Day 15 endometrial tissue. These steroids are known to stimulate and inhibit, respectively, $PGF_{2\alpha}$ synthesis from the guinea pig endometrium in vitro (Leaver and Seawright, 1982).

a) Release of Arachidonic Acid from the Guinea Pig Endometrium on Day 7 and Day 15 of the Oestrous Cycle

Introduction

Endogenous oestradiol released from the developing follicles in the ovaries increases after Day 10 in the guinea pig (Joshi et al.,1973). It has been proposed that oestradiol acting on a progesterone-primed uterus is the stimulus for increased $PGF_{2\alpha}$ production in the guinea pig (Blatchley and Poyser, 1974). The extent to which the endogenous steroid hormones have an effect on arachidonic acid turnover within the uterine cells is not fully known. Therefore the experiments presented in this subsection were designed to measure the release of ^{3}H-AA from the neutral lipids and phospholipids of Day 7 and Day 15 endometrial tissue.

Methods

Ten guinea pigs, five on Day 7 and five on Day 15 of the oestrous cycle, were killed by stunning and incising the

neck. In each case the uterus was rapidly removed and the endometrium was dissected from the myometrium by cutting away 1mm by 2mm pieces of endometrium. The myometrium was discarded.

The endometrial tissue from each guinea pig was placed in a single petri dish, containing 4ml supplemented Medium 199 and 62.5μCi ^{3}H-AA (as the sodium salt), and was cultured for 24hr using a modified tissue culture technique, as described in Section 1(d). This enabled all the different lipid classes to become "labelled" with ^{3}H-AA. The endometrial tissue was then equally distributed among six further petri dishes, each containing 4ml supplemented Medium 199 plus 0.1% bovine serum albumin, and two glass bottles each containing 5ml chloroform and methanol (2:1). The last two control samples were weighed before being placed in the bottles, and were then stored in the chloroform and methanol (2:1) at -20°C. The remaining six samples were incubated as described in Section 1(d) for a further 0.5, 3 or 24hr. Two cultured samples were removed at the end of each time period, weighed and stored in 5ml chloroform and methanol (2:1) at -20°C to await further analysis.

Each sample was then homogenised in 15ml chloroform and methanol (2:1) and subjected to silicic acid column chromatography as described in Section 1(e). Thin-layer chromatography as described in Section 1(f) was used to separate the individual components of the neutral lipid and phospholipid fractions obtained after silicic acid column chromatography. The excess solvent in the fraction

containing arachidonate products from the silicic acid column was removed by a rotary evaporator at 35°C, and, after rinsing the flask with 10ml scintillation fluid, radioactivity was measured by liquid scintillation counting, as described in Section 1(g). The radioactivity in the separated components of the neutral lipids and phospholipids were also monitored by liquid scintillation counting.

Statistical Analysis

All data was analysed by the Student's unpaired 't' test and Duncan's multiple range test.

Results

About 0.7 x 10^3 to 5.3 x 10^3 DPM/mg tissue was measured in the second fraction eluted from the silicic acid columns. The results were not incorporated in the graphs so that the other results were more clearly observed. The amounts of ^{3}H-AA incorporated after 24hr into Day 7 and Day 15 tissue was used as the control value for the release experiments (Fig. 4.1).

The results from the release experiments indicated that there was no apparent release of ^{3}H-AA from any lipid class in Day 7 endometrial tissue over the time period studied except for a significant decrease ($P<0.05$) in the amount of ^{3}H-AA bound to diglyceride and monoglyceride at 24hr compared to 0hr (Fig. 4.1 and Fig. 4.2). On Day 15, there was a general 50 to 80% decrease in ^{3}H-AA content of all lipid classes over the release period, particularly at 3hr (Fig. 4.1 and Fig. 4.2). At 24hr, there was an apparent reincorporation of ^{3}H-AA into the phospholipid classes, particularly phosphatidyl-

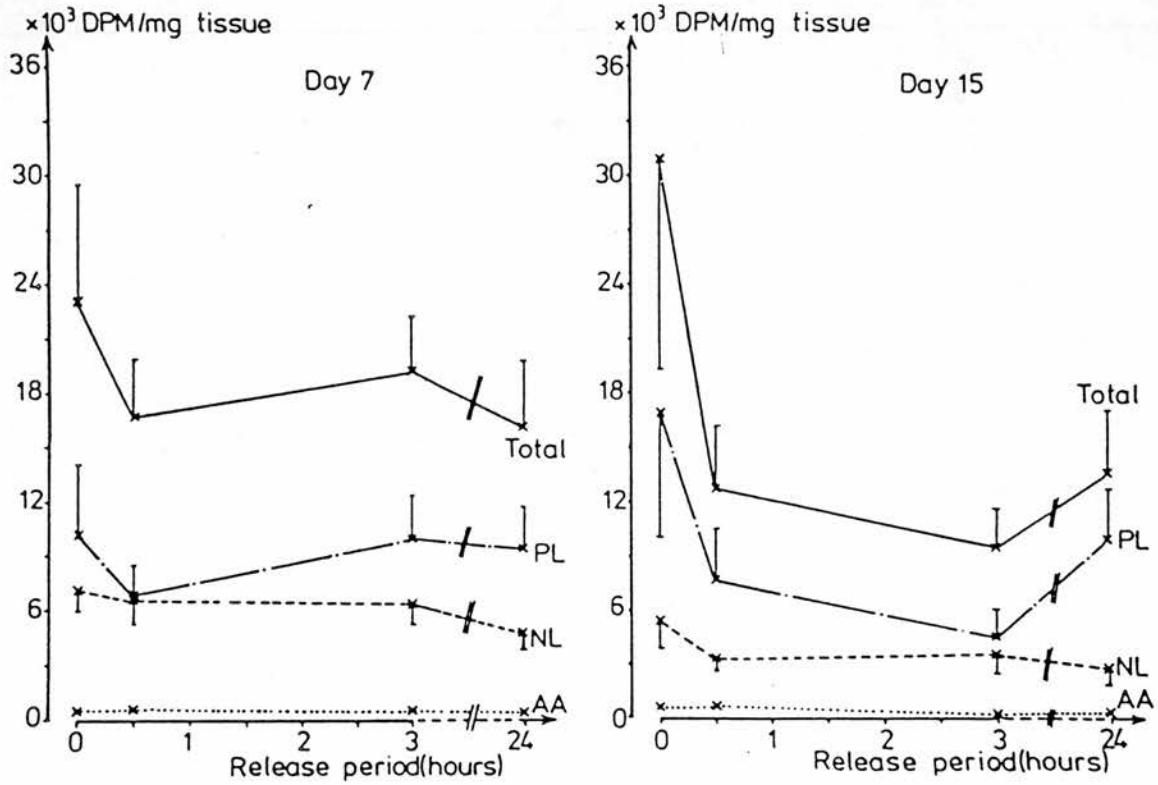


Figure 4.1 Mean (± s.e.m., n=10) total content of ^{3}H-arachidonic acid in Day 7 and Day 15 guinea pig endometrium, and its distribution among phospholipids (PL), neutral lipids (NL) and free ^{3}H-arachidonic acid (AA) during a 24 hr release period following a 24 hr uptake period (Standard errors for AA are too small to include).

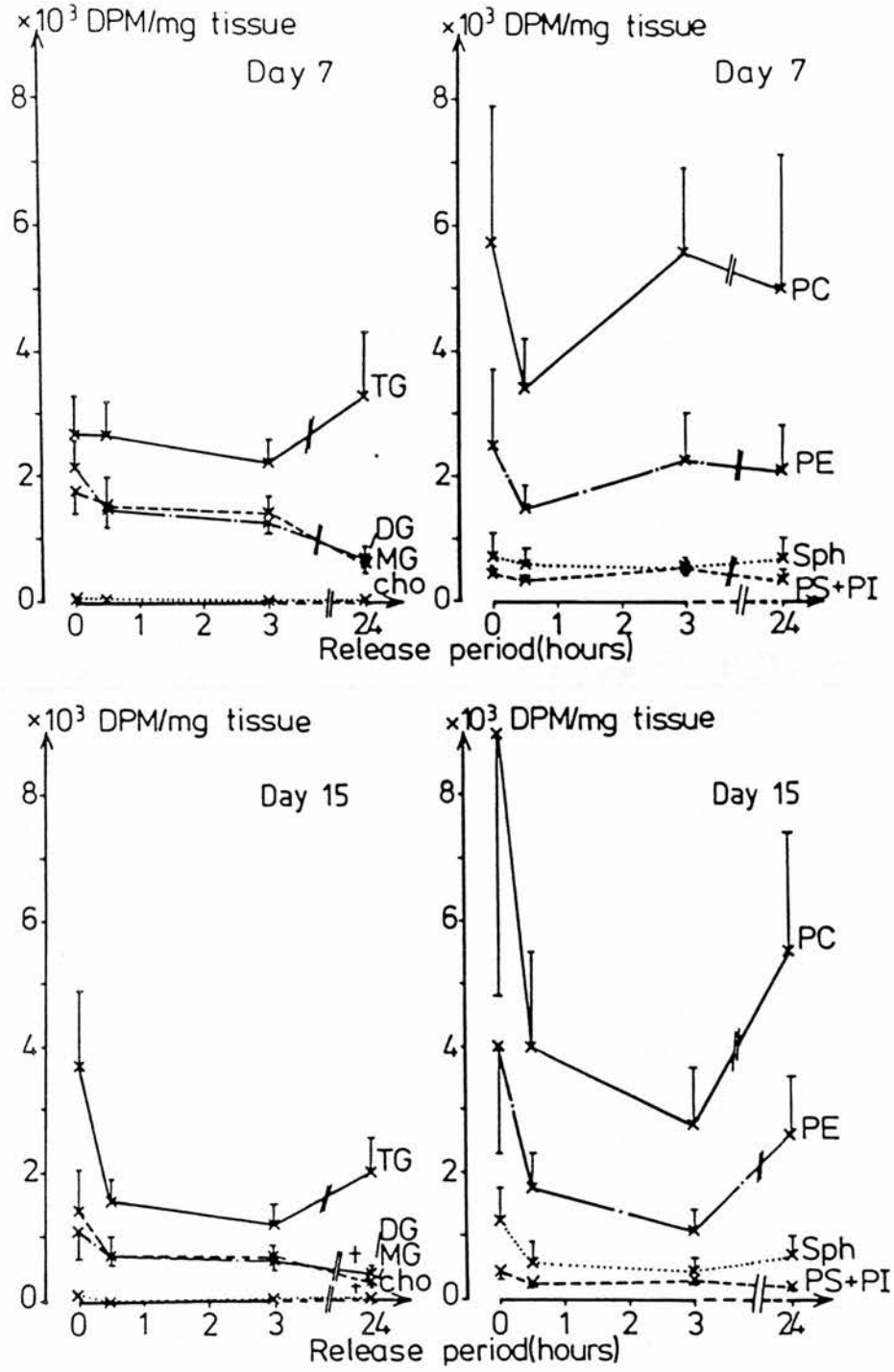


Figure 4.2 Mean (± s.e.m., n=10) total content of ^{3}H-arachidonic acid in triglyceride (TG), monoglyceride (MG), diglyceride (DG), cholesterol ester (cho), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine + phosphatidylinositol (PS + PI) and sphingomyelin (Sph) in Day 7 and Day 15 guinea pig endometrium during a 24 hr release period following a 24 hr uptake period (Standard errors for cho are too small to include).

† Significantly lower ($P < 0.05$) than corresponding Day 7 value.

choline and phosphatidylethanolamine (Fig. 4.2). However none of the values at 0.5, 3 and 24hr were significantly different from the corresponding value at 0hr due to the wide scatter of results.

Conclusion

The results show that the endogenous steroids did not have any apparent significant effect on ^{3}H-AA release from the neutral lipids and phospholipids of Day 7 and Day 15 endometrial tissue.

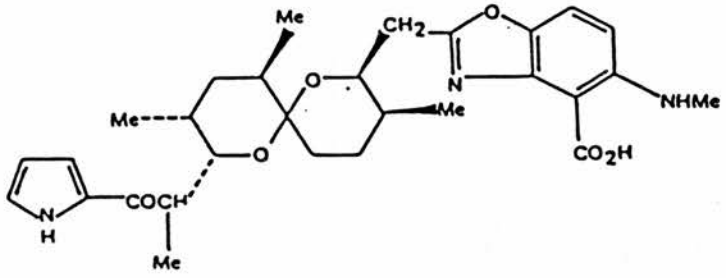
b) Release of Arachidonic Acid From the Guinea Pig Endometrium In the Presence of a Calcium Ionophore

Introduction

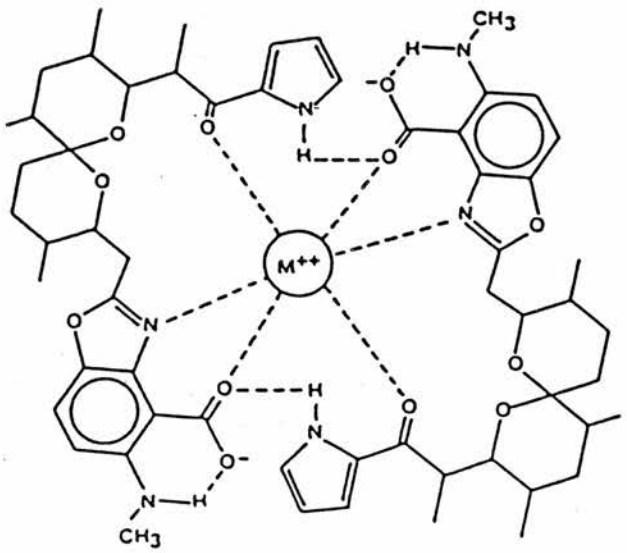
The radiolabelling studies carried out in Section 4(a) utilised ^{3}H-AA which had been made available to the endometrial tissue in small quantities. In those studies there was no significant loss of ^{3}H-AA from any of the lipid stores. It was considered necessary therefore to stimulate the tissue so that perhaps a larger release of ^{3}H-AA occurred.

Agents influencing the output of $PGF_{2\alpha}$ have already been investigated by modified tissue culture (Leaver and Seawright, 1982). Of particular interest was the use of calcium ionophore A23187 as a cell stimulant (see Van den Bosch, 1980). At a concentration of 5µg/ml the calcium ionophore A23187 stimulated $PGF_{2\alpha}$ synthesis from the endometrium of guinea pigs on both Day 7 and Day 15 of the

Structural Formula of A23187



Free Acid



Schematic representation of the A23187-metal complex

Empirical Formula
$C_{29}H_{37}N_3O_6$
Molecular Weight
523.6

Figure 4.3 The figure illustrates the free acid of A23187 together with the schematic representation of the A23187-metal complex

oestrous cycle. This compound was first discovered as a carboxylic acid antibiotic which simultaneously uncouples oxidative phosphorylation and inhibits ATPase of rat liver mitochondria incubated in a magnesium-free medium (Reed and Lardy, 1972). The antibiotic acts as a freely mobile carrier of calcium and magnesium. Pickett, Jesse and Cohen (1977) using human platelets were able to demonstrate that ethyleneglycol-bis-(β-aminoethylether)-N,N,N^1,N^1,-tetraacetic acid (EGTA; a chelating agent) in the extracellular medium did not greatly affect the stimulating action of A23187 on arachidonate release i.e. A23187 had the ability to mobilise intracellular calcium stores as well as stimulating the influx of extracellular calcium. However, further investigations carried out by Poyser (1983) demonstrated that the stimulating effect of A23187 on prostaglandin release from the guinea pig endometrium was abolished by removing extracellular calcium. The discrepancy observed in the former report was probably due to the inability of EGTA to remove the calcium ions completely from the extracellular medium.

It was now generally accepted that the action of A23187 is to greatly increase the ability of divalent ions to cross biological membranes. Stable complexes (Fig. 4.3) consisting of two molecules of A23187 per divalent cation are formed which allow the ions to be soluble in organic solvents (Pfeiffer and Deber, 1979). The following order of ion specificity is observed with A23187:

$$Mn^{2+} \gg Ca^{2+} = Mg^{2+} \gg Sr^{2+}.$$

The series of experiments reported in this subsection involved the culture of endometrial tissue in the presence of A23187 to determine the effects of the calcium ionophore on the release of arachidonate from the different lipid classes within Day 7 and Day 15 endometrial tissue

Method

Ten guinea pigs, five on Day 7 and five on Day 15 of the oestrous cycle, were killed by stunning and incising the neck. In each case, the uterus was rapidly removed and the endometrium was dissected from the myometrium by cutting away 1mm by 2mm pieces of endometrium. The myometrium was then discarded. The endometrial tissue from each guinea pig was incubated for 24hr in the presence of 62.5μCi ^{3}H-AA (as the sodium salt) in 4ml supplemented Medium 199 as described previously in Section 1(d). After the neutral lipids and phospholipids were labelled with ^{3}H-AA, the endometrial tissue was equally distributed among twelve petri dishes. Each of six petri dishes contained 4ml supplemented Medium 199, which contained 0.1% bovine serum albumin and 20μg A23187 in 20μl ethanol. Each of the remaining six control petri dishes contained 20μl ethanol in 4ml supplemented Medium 199 containing 0.1% bovine serum albumin. This arrangement ensured that only the effects of the calcium ionophore were observed. Two samples of endometrial tissue were also stored in two glass bottles, each containing 5ml chloroform and methanol (2:1) at -20°C for further analysis. Two control petri dishes and two petri dishes containing calcium ionophore were removed 0.5hr, 3hr or 6hr after the start of the second incubation period, blotted dry, weighed

and homogenised in 15ml chloroform and methanol (2:1). The neutral lipids, arachidonate products and phospholipids were separated by silicic acid column chromatography as described in Section 1(e). The solvent used to elute the arachidonate products was removed by a rotary evaporator at 35°C and the radioactivity was measured by rinsing the container with 10ml scintillation fluid which underwent liquid scintillation counting as described in Section 1(g). The neutral lipids and phospholipids were separated into their individual components by thin-layer chromatography (tlc) as described in Section 1(f). Radioactivity on each tlc plate was analysed by liquid scintillation counting as described in Section 1(g).

Statistical Analysis

All data were analysed by the variance ratio "F" test to determine if the variances of any groups were unequal. Where variances were not unequal, data was analysed by the Student's unpaired 't' test; where variances were unequal, data was analysed by a modified 't' test for unequal variances.

Results

The incorporation of ^{3}H-AA after 24hr into the neutral lipids and phospholipids of Day 7 and Day 15 endometrial tissue was used as the control for the release of ^{3}H-AA in the absence and presence of A23187. The data from Day 7 endometrial tissue indicated that in the absence of A23187 there was no significant release of ^{3}H-AA from any lipid class over the

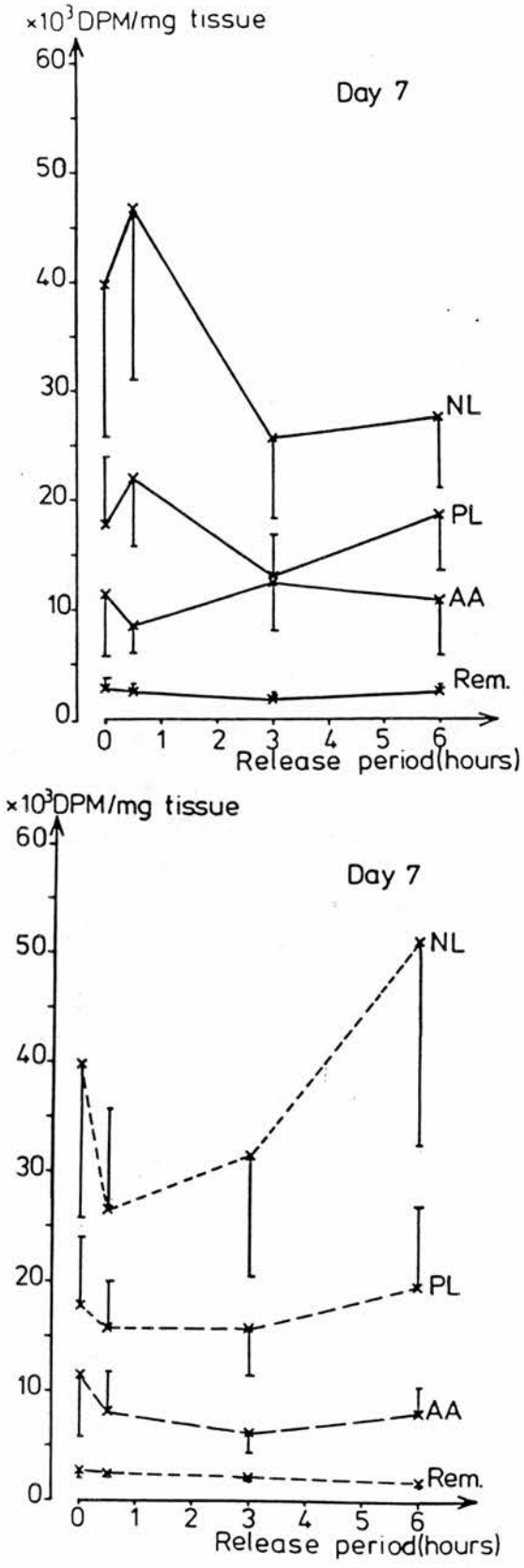


Figure 4.4 The graphs indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid esterified to neutral lipids (NL), phospholipids (PL), free ^{3}H-arachidonic acid (AA) and arachidonate products (Rem) of Day 7 endometrial tissue over a release period of 6 hr in the absence (——) and presence (- - -) of 5 μg/ml A23187.

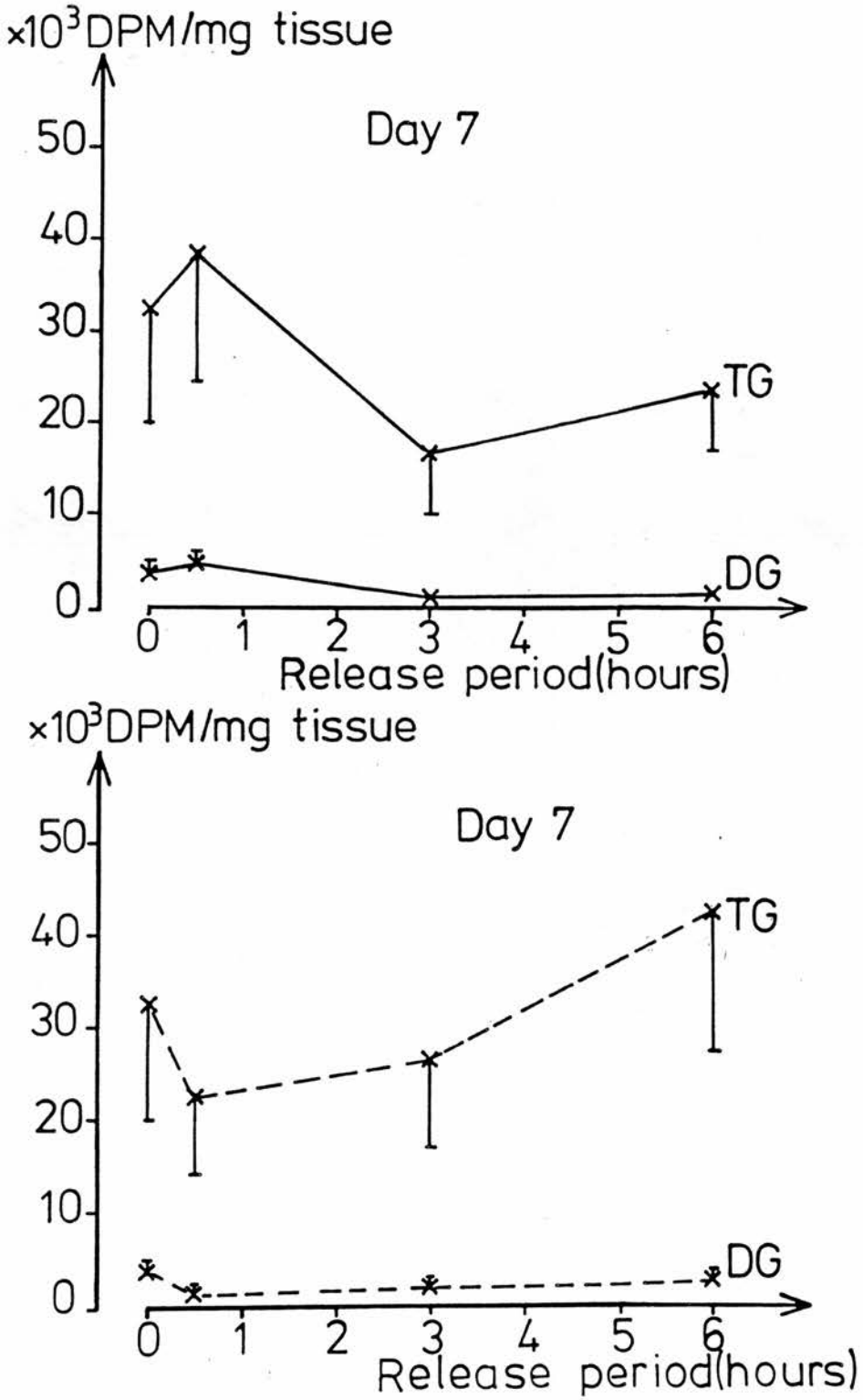


Figure 4.5 Graphs indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid esterified to triglyceride (TG) and diglyceride (DG) of Day 7 endometrial tissue over a release period of 6 hr in the absence (——) and presence (- - -) of 5μg/ml A23187.

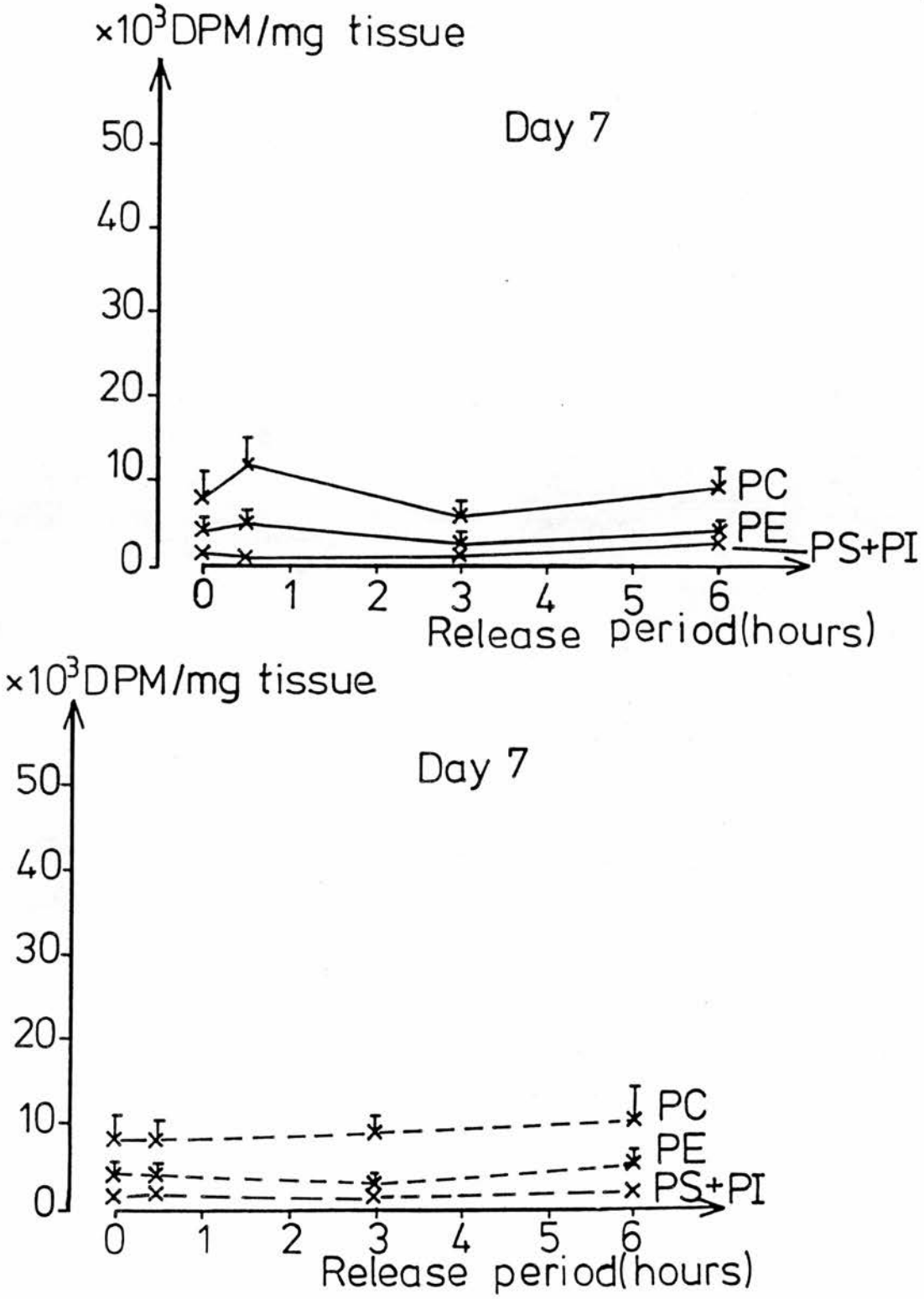


Figure 4.6 Graphs indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid esterified into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine + phosphatidylinositol (PS + PI) of Day 7 endometrial tissue over a release period of 6 hr in the absence (——) and presence (- - - -) of 5µg/ml A23187.

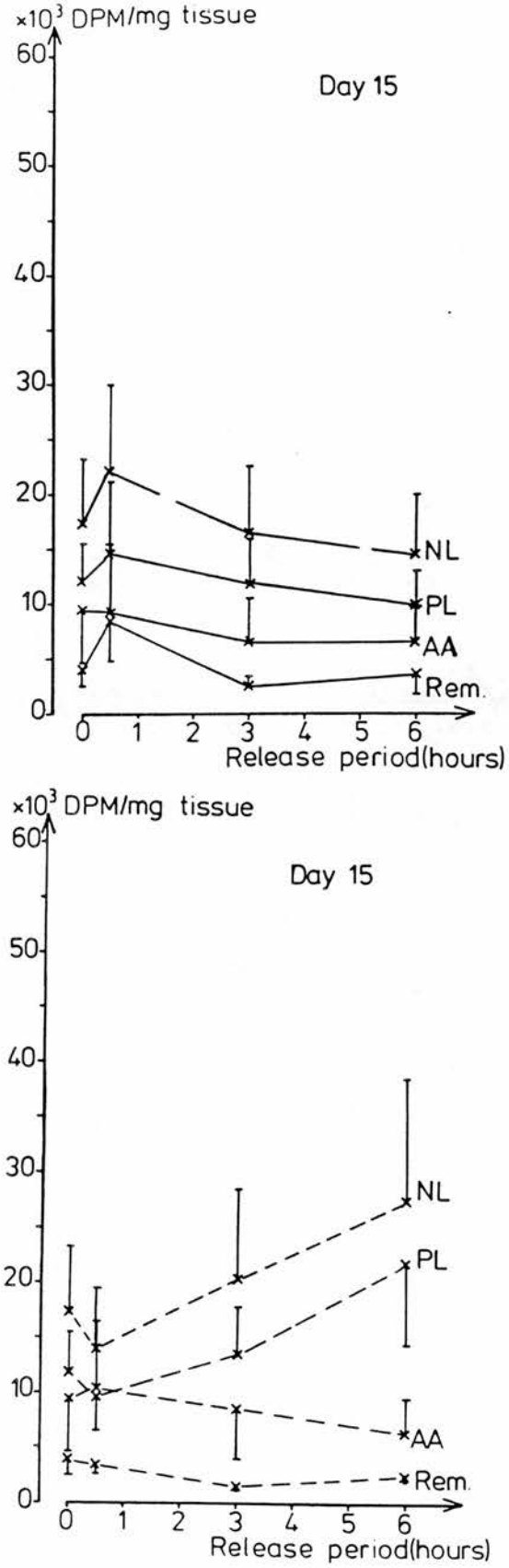


Figure 4.7 Graphs indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid esterified to neutral lipids (NL), phospholipids (PL), free ^{3}H-arachidonic acid (AA) and arachidonate products (Rem) of Day 15 endometrial tissue over a period of 6 hr in the absence (——) and presence (- - -) of 5 μg/ml A23187

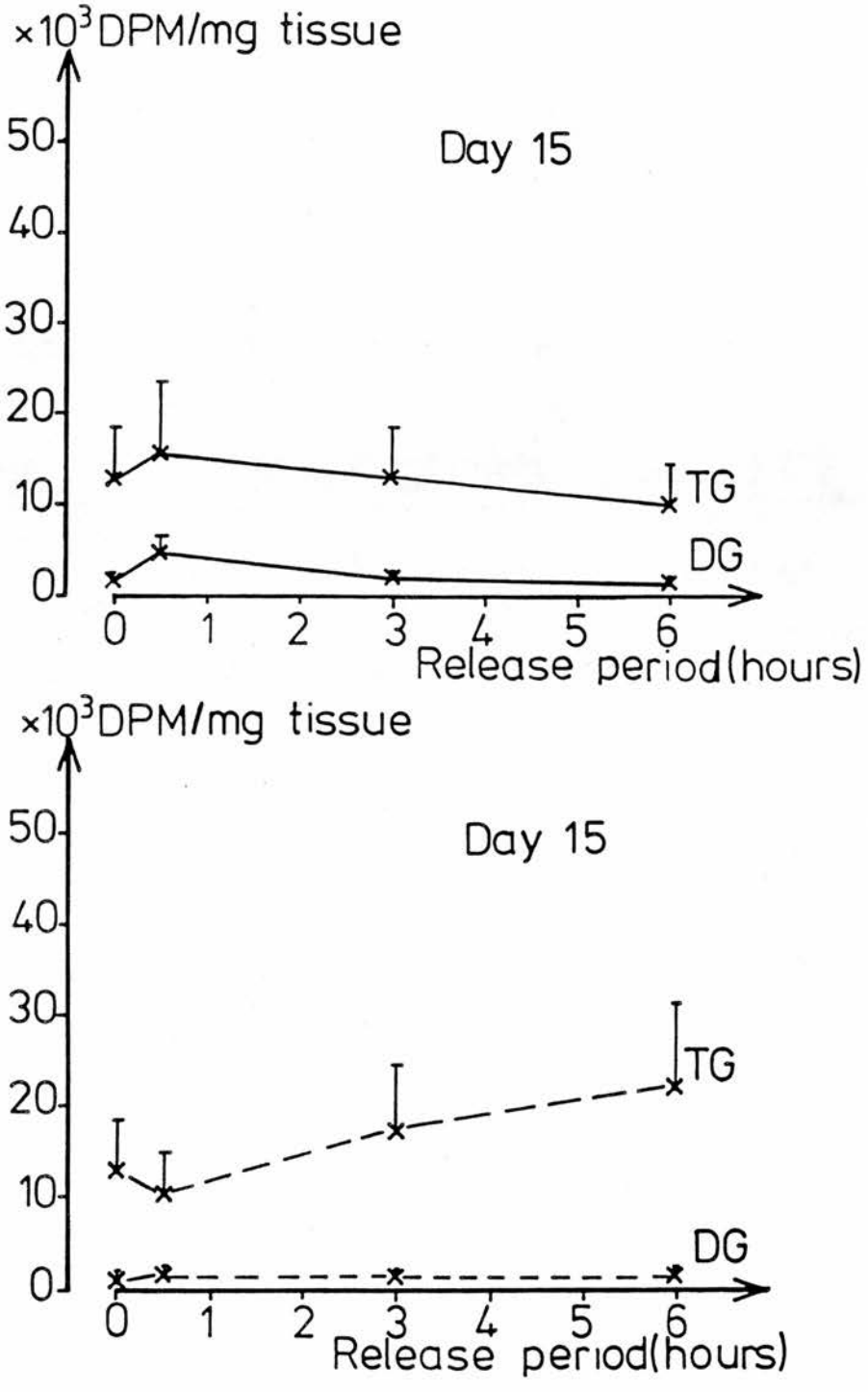


Figure 4.8 Graphs indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid incorporated into triglyceride (TG) and diglyceride (DG) of Day 15 endometrial tissue over a period of 6 hr in the absence (——) and presence (- - - -) of 5µg/ml A23187.

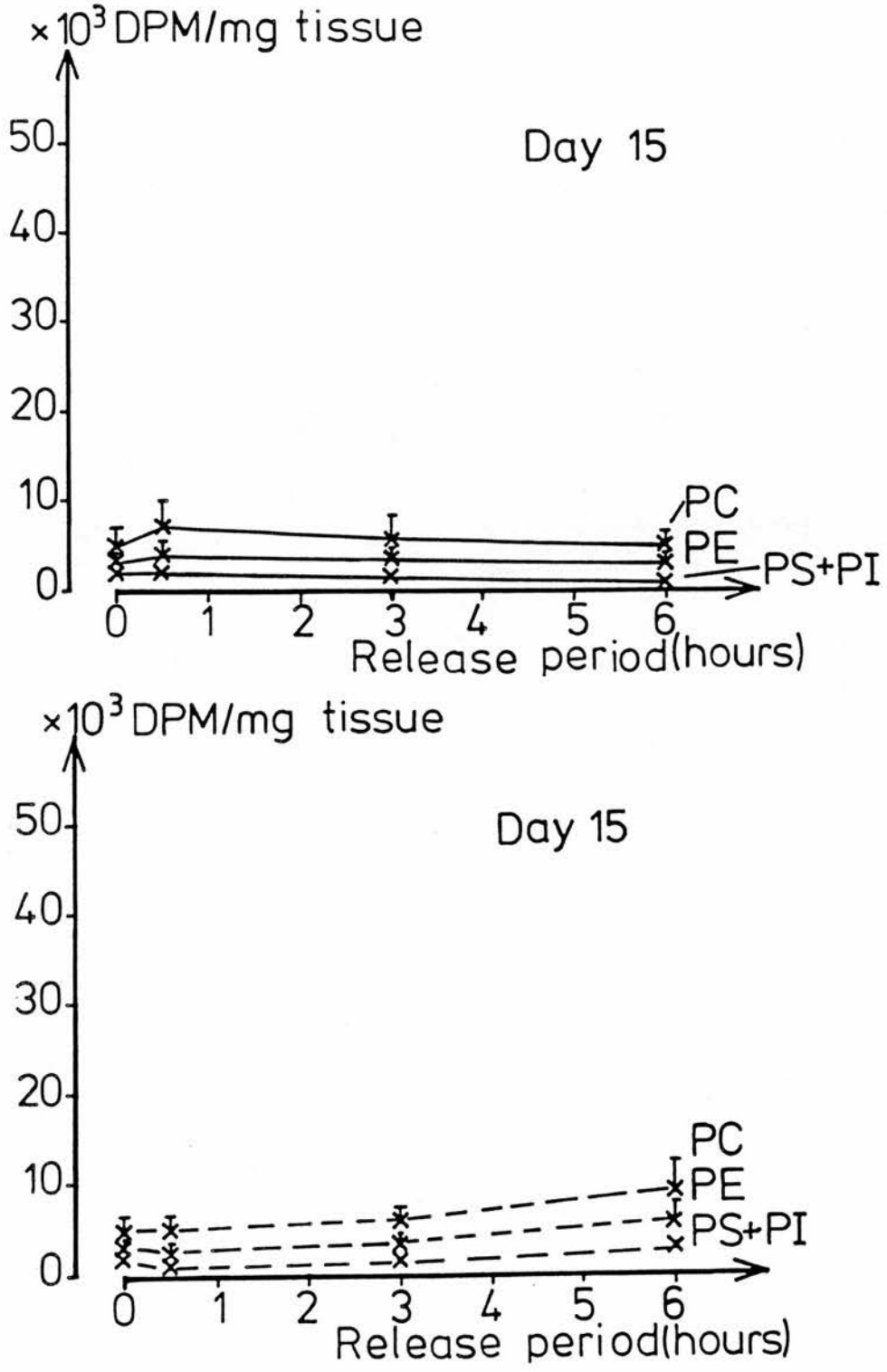


Figure 4.9 Graphs indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid incorporated into phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine + phosphatidylinositol (PS + PI) of Day 15 endometrial tissue over a release period of 6 hr in the absence (——) and presence (- - - -) of 5µg/ml A23187.

time period studied (Fig. 4.4). In the presence of A23187 there was also no significant release of ^{3}H-AA from the neutral lipid or phospholipid classes. In fact, ^{3}H-AA content in neutral lipids seemed to increase.

The absence or presence of A23187 did not significantly affect the levels of free ^{3}H-AA and the relatively lower levels of arachidonate products at any time.

Separation of the neutral lipids and phospholipids of Day 7 tissue into their individual components indicated that all the lipids incorporated ^{3}H-AA with the exception of sphingomyelin, cholesterol ester and monoglyceride. Since the three lipids classes mentioned had incorporated negligible amounts of ^{3}H-AA, their results were not included in Fig. 4.5 and Fig. 4.6.

In the presence of A23187 it was clear that there was no significant release of ^{3}H-AA from triglyceride, diglyceride, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol at any time period studied. However the triglyceride class seemed to incorporate more ^{3}H-AA than any other lipid class at 6hr.

The data from Day 15 endometrial tissue indicated that in the absence of A23187 there was no significant release of ^{3}H-AA from either the neutral lipids or the phospholipids (Fig. 4.7). The presence of A23187 seemed to have no significant effect on the release of ^{3}H-AA from any lipid class. The absence or presence of A23187 did not significantly affect the levels of free ^{3}H-AA and the relatively lower levels of arachidonate products at any time. Further separation of

the neutral lipids and phospholipids of Day 15 tissue revealed that none of the individual lipid classes exhibited a significant decrease in ^{3}H-AA content at any time period studied (Fig. 4.8 and Fig. 4.9). In fact, there seemed to be an increase in ^{3}H-AA content of triglyceride at 6hr.

The monoglyceride, cholesterol ester and sphingomyelin classes incorporated negligible amounts of ^{3}H-AA therefore their data were omitted from Fig. 4.8 and Fig. 4.9.

Conclusion

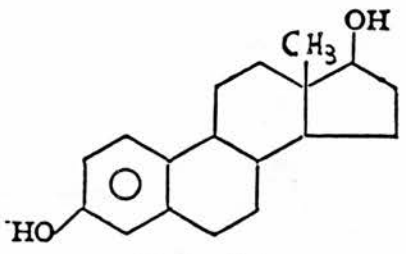
The overall results indicated that the addition of 5µg/ml calcium ionophore A23187 to the culture medium did not appear to have a significant effect on ^{3}H-AA release from any of the lipid classes of Day 7 and Day 15 endometrial tissue.

c) Release of Arachidonic Acid From the Guinea Pig Endometrium In The Presence of Oestradiol And Progesterone

Introduction

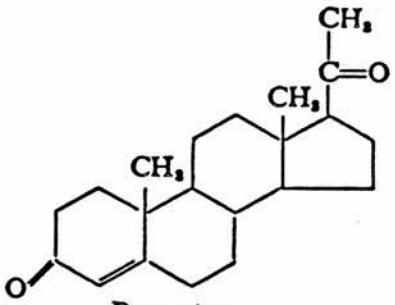
It has been established that one of the most consistent histochemical findings in the rat uterus is that the quantities of stainable neutral lipids fluctuate during the oestrous cycle (see Beall, 1972). Further biochemical analysis of the rat uterus confirmed that the decrease in neutral lipids paralleled the increase in phospholipids during oestrus whilst the reverse was true at dioestrus (Biswas and Mukherjea, 1973). In the guinea pig, histochemical findings demonstrated that minimal amounts of

A



Oestradiol

B



Progesterone

Figure 4.10
Structures of oestradiol-17β(A)and progesterone (B)

neutral lipids were present in the uterine epithelial cells at oestrus and maximal amounts were present during dioestrus (Nicol and Snell, 1955). However further biochemical analysis of the guinea pig uterus indicated that the decrease caused by the triglyceride component of the neutral lipids at oestrus was not paralleled by an increase in phospholipid content (Leaver and Poyser, 1981).

It was demonstrated that the deposition of stainable neutral lipids in the guinea pig uterus required oestrogen and progesterone administration in a specific time-related manner (Nicol and Snell, 1955; Snell and Nicol, 1955). Thus uterine lipid was produced in spayed guinea pigs with administration of progesterone but only if sensitizing amounts of oestrogen were given first. Although the phospholipid content of the guinea pig uterus did not vary throughout the oestrous cycle there was a significant ($P<0.05$) decrease in the amount of arachidonic acid esterified to phosphatidylcholine on Day 15 of the oestrous cycle (Leaver and Poyser, 1981). This study suggested that release of arachidonic acid from phosphatidylcholine was higher on Day 15 than on Day 7 of the oestrous cycle. As oestradiol and progesterone are known to be involved with lipid metabolism, the steroid hormones may be implicated with the release of arachidonic acid from phospholipids on Day 15 of the cycle, which is a day of high $PGF_{2\alpha}$ synthesis. Leaver and Seawright (1982) using a modified tissue culture demonstrated that oestradiol stimulated $PGF_{2\alpha}$ output from the guinea pig endometrium whilst progesterone inhibited $PGF_{2\alpha}$ output.

The experiments reported in this subsection were carried out to determine whether oestradiol (A) and progesterone (B) (see Fig. 4.10) affected the release of ^{3}H-AA from the endometrium of guinea pigs on Day 7 (day of low $PGF_{2\alpha}$ synthesis) and Day 15 (day of high $PGF_{2\alpha}$ synthesis) of the oestrous cycle.

Methods

Ten guinea pigs, five on Day 7 and five on Day 15 of the oestrous cycle, were killed by stunning and incising the neck. In each case the uterus was removed and the endometrium was dissected from the myometrium by cutting away 1mm by 2mm pieces of endometrial tissue. The myometrium was discarded. The endometrial tissue from each guinea pig was cultured in the presence of 25μCi ^{3}H-AA (as the sodium salt) for 24hr as described previously in Section 1(d). After the neutral lipids and phospholipids were labelled with ^{3}H-AA, the endometrial tissue was divided into eight samples: two samples were washed, placed in two glass bottles each containing 5ml chloroform and methanol (2:1) and stored at -20°C for further analysis; two samples were placed in separate petri dishes each containing 4ml supplemented Medium 199 (plus 0.1% bovine serum albumin) and 40ng oestradiol which had been added in 20μl ethanol; two samples were placed in two petri dishes each containing 4ml supplemeted Medium 199 (plus 0.1% bovine serum albumin) and 4μg progesterone which had been added also in 20μl ethanol. The remaining two samples were used as controls and each was incubated in 4ml supplemented Medium 199 (plus 0.1% bovine serum albumin) and 20μl ethanol. The six samples from each guinea pig were

incubated for 24hr as described in Section 1(d). At the end of this time period the gas in the Kilner jars was renewed and the samples were incubated for a further 24hr. At the end of the 48hr culture period each tissue sample was removed, washed with fresh medium, blotted dry, weighed and homogenised in 15ml chloroform and methanol (2:1). Neutral lipids, arachidonate products and phospholipids were separated by silicic acid column chromatography, as described in Section 1(e). The excess solvent in the arachidonate product fraction was removed by a rotary evaporator at 35°C and 10ml scintillation fluid was used to remove the radioactivity from each container. Radioactivity was measured by liquid scintillation counting as described in Section 1(g). The neutral lipids and phospholipids were separated into their individual components by thin-layer chromatography as described in Section 1(f). Radioactivity in each lipid component was monitored by liquid scintillation counting as described in Section 1(g).

Statistical Analysis

All data were analysed by the variance ratio 'F' test to determine if the variances of any groups were unequal. Where variances were not unequal data was analysed by the Student'sunpaired 't' test; where variances were unequal, data was analysed by a modified 't' test for unequal variances.

Results

After the initial 24hr uptake period it was observed that both Day 7 and Day 15 tissue had incorporated ^{3}H-AA as

expected. The values for the amounts of ^{3}H-AA present in neutral lipids and phospholipids after this 24hr labelling period were then used as the control values in the release experiments. The results obtained from these release experiments were divided into two sections:

i) basal release of ^{3}H-AA from lipids of Day 7 and Day 15 endometrial tissue after a 48hr incubation period.

ii) release of ^{3}H-AA from lipids of Day 7 and Day 15 endometrial tissue after a 48hr incubation period in the presence of 10ng/ml oestradiol or 1000ng/ml progesterone.

i) Analysis of data by the modified 't' test indicated that there was a significant ($P<0.05$) decrease in ^{3}H-AA content from the total amount of lipid present in Day 7 endometrial tissue after a 48hr incubation period (Fig. 4.11). Further analysis revealed that ^{3}H-AA seemed to be released in significant ($P<0.05$) quantities from the neutral lipid fraction only. There was also a significant ($P<0.01$) decrease in ^{3}H-AA content of the arachidonate products after 48hr (Fig. 4.11).

Separation of the neutral lipids of Day 7 tissue into their individual components revealed that there was an apparent decrease in ^{3}H-AA content of triglycerides, diglycerides and monoglycerides over the 48hr time period (Fig. 4.12). Analysis of these results using the modified 't' test revealed that there was no significant release of ^{3}H-AA from any of the neutral lipid classes. Separation of the phospholipids of Day 7 tissue into their individual

components confirmed that there was no significant release of ^{3}H-AA from any of the phospholipid classes after 24hr (Fig. 4.12).

Analysis of data by the unpaired 't' test indicated that there was no significant decrease in ^{3}H-AA content from the total amount of lipid present in Day 15 endometrial tissue after a 48hr incubation period (Fig. 4.13). Further analysis confirmed that ^{3}H-AA was not released in significant quantities from the neutral lipids, phospholipids or arachidonate products fractions.

Separation of the neutral lipids of Day 15 tissue into their individual components confirmed that there was no significant release of ^{3}H-AA from any of the neutral lipid classes (Fig. 4.14). However separation of the phospholipids of Day 15 tissue into their individual components revealed that there was a significant($P<0.05$) release of ^{3}H-AA from the phosphatidylcholine class (Fig. 4.14). Using a modified 't' test, it was demonstrated that there was also a significant ($P<0.05$) decrease in ^{3}H-AA content in the phosphatidyl-ethanolamine class of Day 15 tissue after 48hr. There was no significant change in ^{3}H-AA content of phosphatidylserine and phosphatidylinositol after the 48hr incubation period (Fig. 4.14).

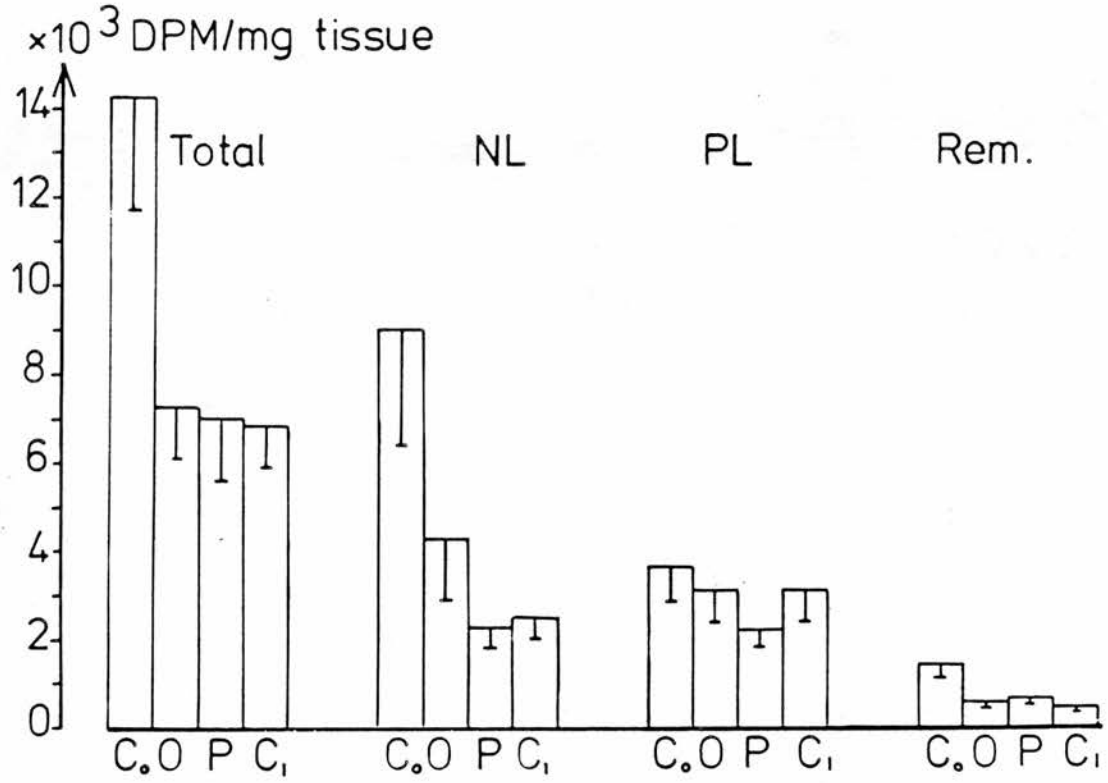


Figure 4.11
The above histograms indicate the mean total (± s.e.m., n=10) of ^{3}H-arachidonic acid in the total lipids (total), neutral lipids (NL), phospholipids (PL) and arachinonate products (Rem.) of Day 7 endometrial tissue. Incubations were in the absence (Co) of steroids, in the presence of 10ng/ml oestradiol (O) for 48 hr, in the presence of 1000ng/ml progesterone (P) for 48 hr and in the absence (C_1) of steroids for 48 hr.

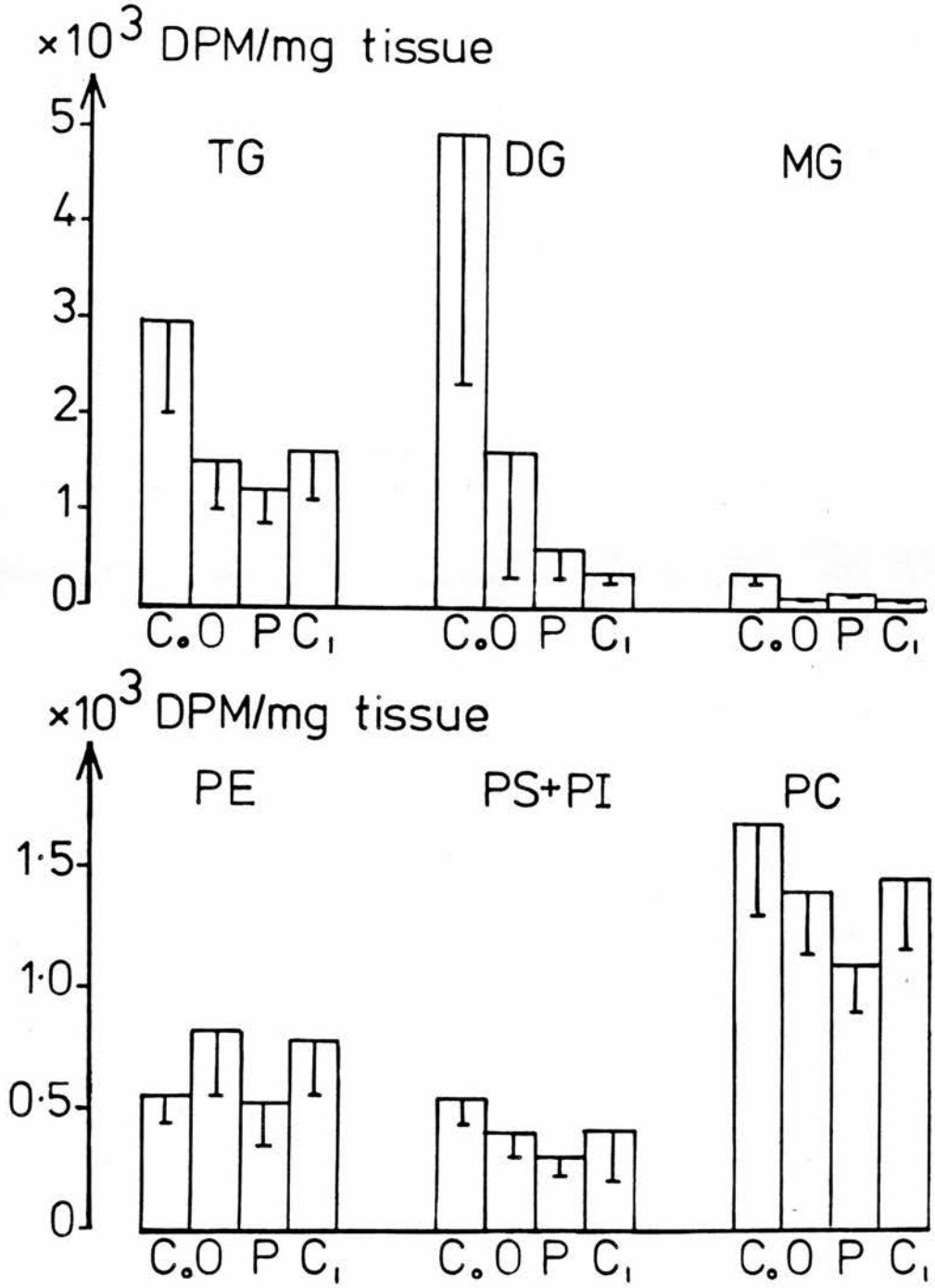


Figure 4.12
The above histograms indicate the mean total content (±) s.e.m., n=10) of ^{3}H-arachidonic acid in the triglyceride (TG), diglyceride (DG), monoglyceride (MG), phosphatidylethanolamine (PE), phosphatidylserine and phosphatidylinositol (PS + PI) and phosphatidylcholine (PC) of Day 7 endometrial tissue. Incubations were in the absence (Co) of steroids, in the presence of 10ng/ml (O) for 48 hr, in the presence of 1000ng/ml progesterone (P) for 48 hr and in the absence (C_1) of steroids for 48 hr.

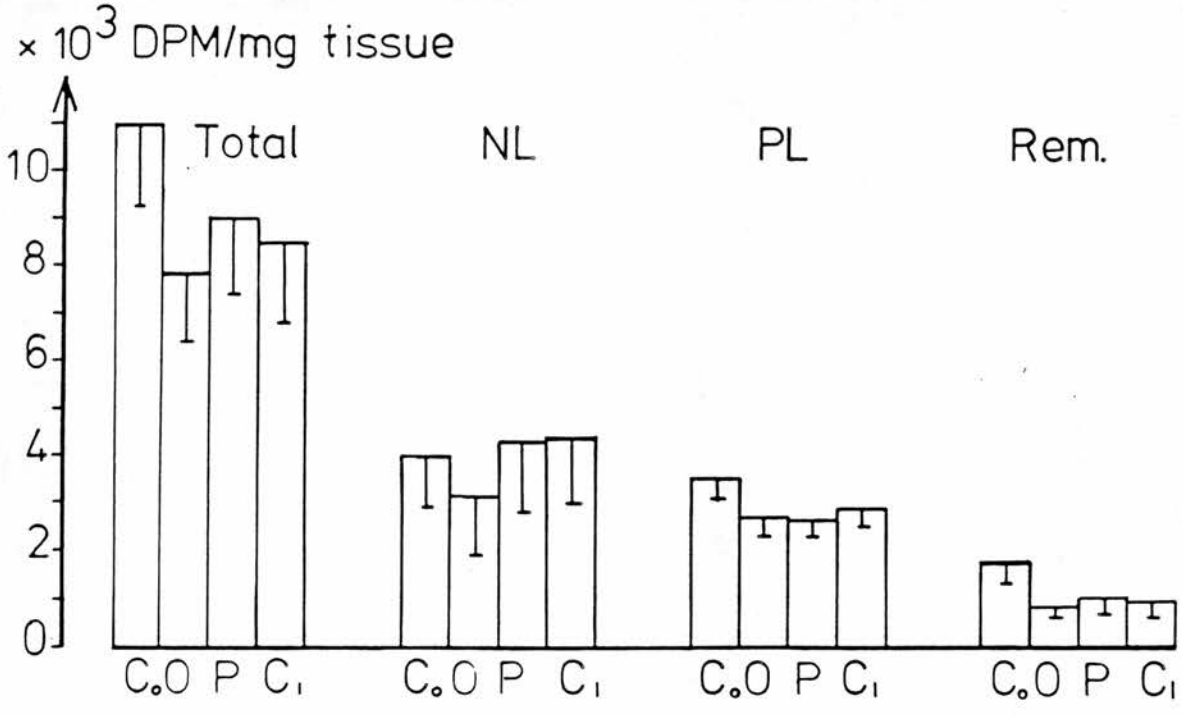


Figure 4.13
The above histograms indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid in the total lipids (total), neutral lipids (NL), phospholipids (PL) and arachidonate products (Rem.) of Day 15 endometrial tissue. Incubations were in the absence (Co) of steroids, in the presence of 10ng/ml oestradiol (O) for 48 hr, in the presence of 1000ng/ml progesterone (P) for 48 hr and in the absence (C_1) of steroids for 48 hr.

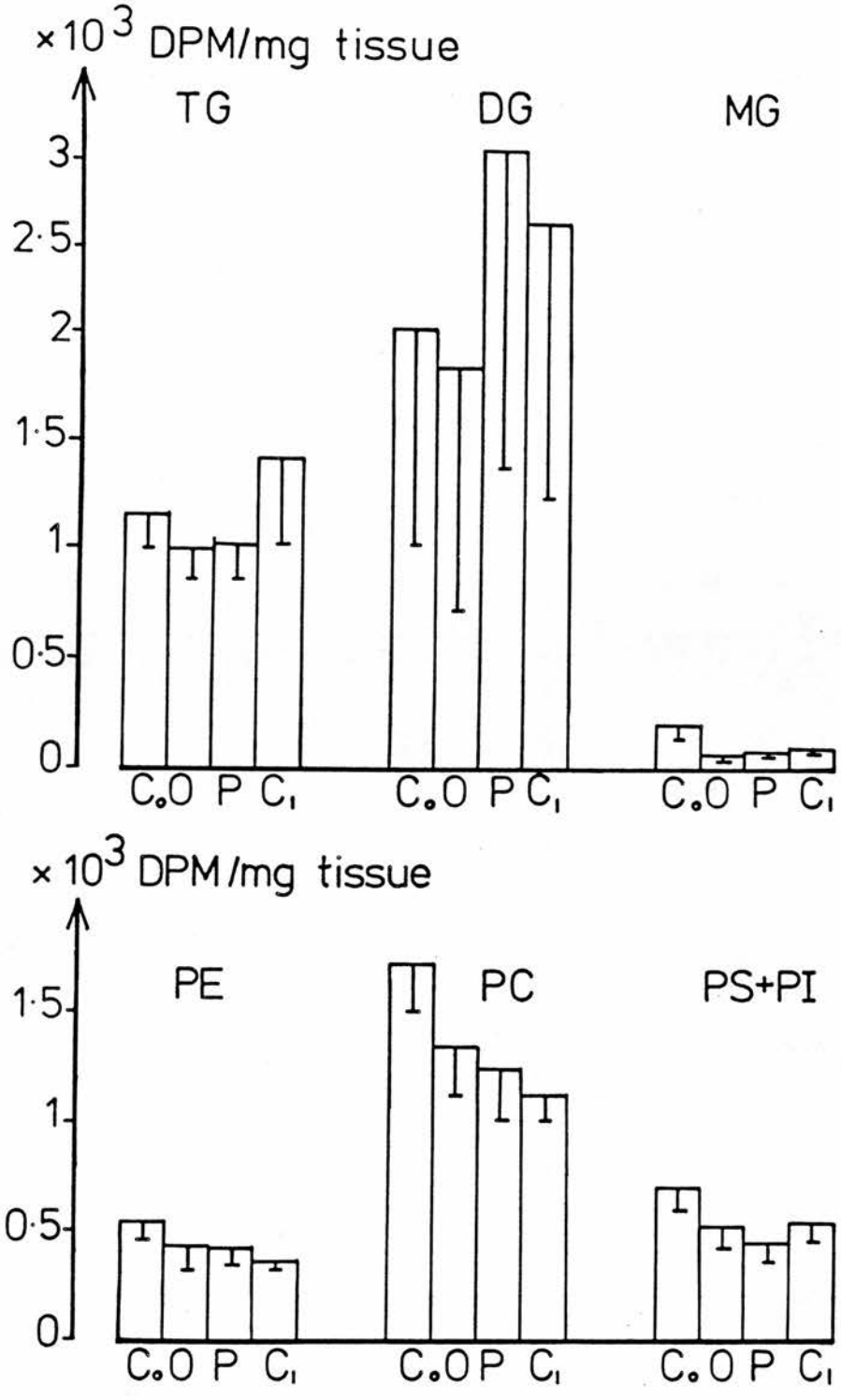


Figure 4.14.
The above histograms indicate the mean total content (± s.e.m., n=10) of ^{3}H-arachidonic acid in triglyceride (TG), diglyceride (DG), monoglyceride (MG), phosphatidylserine and phosphatidylinositol (PS + PI). Incubations were in the absence of steroids (Co), in the presence of 10ng/ml oestradiol (O) for 48 hr, in the presence of 1000ng/ml progesterone (P) for 48 hr and in the absence of steroids (C_1) for 48 hr.

ii) To determine whether there was a stimulation or inhibition of release of ^{3}H-AA from the lipids of Day 7 and Day 15 endometrial tissue in the presence of steroid hormones it was necessary to compare the ^{3}H-AA content of lipids after 48hr incubation in the absence of steroids with the ^{3}H-AA content of lipids after 48hr incubation in the presence of oestradiol or progesterone.

Using either the Student's unpaired 't' test or the modified 't' test for unequal variances as appropriate it was determined that there was no significant increase or decrease in ^{3}H-AA content in any of the lipids following a 48hr incubation in the presence of 10ng/ml oestradiol or 1000ng/ml progesterone (Fig. 4.11 and Fig. 4.12). Similar findings were observed in Day 15 tissue (Fig. 4.13 and Fig. 4.14).

Conclusion

Basal release of ^{3}H-AA occurred from the neutral lipid fraction only ($P<0.05$) after a 48hr incubation period on Day 7. As there was no significant decrease in ^{3}H-AA content in any of the individual components of the neutral lipid fraction it is possible that there was a general release of ^{3}H-AA from all the neutral lipid classes over the 48hr time period. There was no release of ^{3}H-AA from phospholipids on Day 7. However, basal release of ^{3}H-AA occurred from both phosphatidylcholine and phosphatidylethanolamine of Day 15 endometrial tissue was shown to be significant ($P<0.05$) after the 48hr incubation period. The presence of oestradiol (10ng/ml) and progesterone (1000ng/ml) were shown to have no significant effect on the basal release of ^{3}H-AA from either

the neutral lipids or phospholipids of Day 7 and Day 15 endometrial tissue.

Discussion

In all the release experiments carried out in the current section, prelabelling of the neutral lipids and phospholipids of the endometrial tissue with ^{3}H-AA was the prerequisite step. After the 24hr uptake period it was generally observed (in Sections 4(b) and 4(c)) that both Day 7 and Day 15 tissue incorporated similar amounts of ^{3}H-AA, unlike the results reported in Section 2. The use of less ^{3}H-AA per mg of tissue as an economy measure was probably the reason why the significant differences in uptake between Day 7 and Day 15 endometrial tissue were not observed here. However the main purpose of these experiments was to measure ^{3}H -AA release, and uptake of ^{3}H-AA was considered only as a prelabelling procedure.

The results from Section 4(a) indicated that the basal release of ^{3}H-AA from the lipid stores was low over the 24hr incubation period. An accurate assessment of the amount of ^{3}H-AA released was difficult because factors such as (i) the large variation in the initial uptake of ^{3}H-AA (ii) dilution of the incorporated ^{3}H-AA by varying amounts of endogenous fatty acids (Blackwell, Duncombe, Flower, Parsons and Vane, 1977) and (iii) reacylation of released arachidonic acid into other lipid classes were probably involved. The only significant release of ^{3}H-AA occurred from diglycerides and monoglycerides of Day 7 endometrial tissue. The reason for this occurrence is not yet clear but since an apparent rise

of mean total content of ^{3}H-AA in triglyceride coincided with a decrease in the amount of ^{3}H-AA in diglycerides and monoglycerides then it is possible that some diglyceride was converted to triglyceride.

Although there were no other significant changes in ^{3}H-AA content in the lipid classes on either Day 7 or Day 15, it was evident that there was an apparent greater release of arachidonic acid after 3hr of incubation from all lipids of Day 15 compared to Day 7 endometrial tissue. In order to try and observe a significant release of ^{3}H-AA from any lipid of the endometrial tissue, further experiments involving the use of stimuli were carried out.

The calcium ionophore A23187 was used as a stimulus in the experiments reported in Section 4(b). The data observed indicated that 5μg/ml calcium ionophore A23187 was unable to cause a significant release of ^{3}H-AA from any lipid class of the endometrium. Consideration of the action of the calcium ionophore is necessary to explain the results. Calcium ionophore acting to increase the availability of calcium ions to all enzymes within the cell exhibits a non-specific action. So although calcium may have activated phospholipases to release arachidonic acid from phospholipids (Flower and Blackwell, 1976), enzymes involved in reacylation were probably also stimulated. Thus, increases in the activities of acyl-CoA synthetases and acyl-CoA:lyso-phosphatide acyltransferases would results in reincorporation of arachidonic acid into the lipids. The stimulation of enzymes acting in opposition may have obscur ed any effects

of A23187 on release of ^{3}H-AA from the lipid reservoirs.

At this juncture it was decided that another stimulus must be utilised, where monitoring may be facilitated by a greater release of ^{3}H-AA. The use of the steroid hormones oestradiol and progesterone was decided upon because both hormones have been implicated in neutral lipid metabolism (Nicol and Snell, 1955; Snell and Nicol, 1955), and oestradiol has a known effect on protein synthesis (Mueller et al., 1961) and phospholipid synthesis (Aizawa and Mueller, 1961; Stooner and Gorski, 1972) in the rat uterus. Furthermore, Leaver and Seawright (1982) using cultured guinea pig endometrium have demonstrated that 10ng/ml 17 β-oestradiol had the capacity to increase $PGF_{2\alpha}$ output 6-fold in Day 7 endometrial tissue and 2-fold in Day 15 endometrium (from which basal output is 6-fold higher than on Day 7). The administration of 50ng/ml progesterone resulted in the inhibition of output of $PGF_{2\alpha}$ from both Day 7 and Day 15 endometrium. Other investigators (Abel and Baird, 1980: Tsang and Ooi, 1982) have demonstrated a similar response using proliferative and secretory human endometrium. The $PGF_{2\alpha}$ in these experiments must be formed from arachidonic acid available within the tissues studied. The experiments reported in Section 4(c) were carried out to determine if the release of arachidonic acid for $PGF_{2\alpha}$ synthesis from any of the neutral lipid and phospholipid classes of endometrium from guinea pigs on Day 7 and Day 15 of the oestrous cycle was affected by oestradiol or progesterone. Tritiated arachidonic acid content of the lipids were monitored over a 48hr incubation period and compared to controls incubated for

the same time period in the absence of steroids. There was a significant ($P<0.05$) basal release of ^{3}H-AA from phosphatidylcholine and phosphatidylethanolamine of Day 15 endometrial tissue only. It is possible that the increased basal release of arachidonic acid from phospholipids on Day 15 of the oestrous cycle may account for the increased $PGF_{2\alpha}$ output observed at this time. Neither oestradiol (10ng/ml) nor progesterone (1000ng/ml) exhibited a significant effect on the ^{3}H-AA content of any of the lipids on either day. Since oestradiol stimulated $PGF_{2\alpha}$ output and progesterone inhibited $PGF_{2\alpha}$ output (Leaver and Seawright, 1982) it was unlikely that the receptors were already occupied by endogenous steroids.

Since one mole of arachidonic would result in one mole of $PGF_{2\alpha}$ being formed, each gram wet weight of endometrial tissue containing 1mg arachidonate (Leaver and Poyser, 1981) has the capacity in theory to produce 1mg $PGF_{2\alpha}$. The amounts of arachidonic acid in the uterus and the amounts of $PGF_{2\alpha}$ synthesized and released from the endometrium have already been measured by Leaver and Poyser (1981) and Leaver and Seawright (1982) respectively. Therefore it was possible to calculate the percentage of the available arachidonate which is released and converted to $PGF_{2\alpha}$ in Day 7 and Day 15 endometrial tissue.

Table 4.1 Effects of Oestradiol and Progesterone on Arachidonate Release from Day 7 and Day 15 Endometrial Tissue

	Approximate % AA Released and Converted Into $PGF_{2\alpha}$ From 1gm Wet Weight Endometrial Tissue	
Experiment A	Day 7	Day 15
Control	0.8	2.44
Oestradiol (10ng/ml)	2.9	3.7
Experiment B	Day 7	Day 15
Control	0.38	1.85
Progesterone (1000ng/ml)	0.22	0.99

The data in Table 4.1 indicates that a release of 0.22 to 3.7% of the total esterified arachidonic acid in the endometrium would account for the amounts of $PGF_{2\alpha}$ synthesized by the endometrium during the culture period. Since the variation in the initial labelling of endometrial lipids among animals and among tissue samples for the same animal is greater than 3.7%, these tissue culture techniques have proved inadequate to monitor the action of steroids on the release of arachidonic acid.

A different and perhaps more accurate ap proach for the determination of the lipid source(s) of the prostaglandin precursor is now being considered. The idea stems from investigators utilising single cell preparations and there is little problem where release studies are concerned. For example it can be demonstrated that bradykinin stimulates the

release of arachidonic acid from phosphatidylinositol in mouse fibrosarcoma cells maintained in culture (Bell et al., 1980). The bradykinin is immediately available to every cell and the response is very rapid. For a response to be monitored it is therefore necessary to use cells of one type only so that all the cells respond in a similar fashion and at the same time to a stimulus. Since endometrial tissue is composed of glandular epithelium and stromal cells, current research must centre around the isolation and maintenance of the cell types in monolayer culture. To date the investigators have demonstrated that prostaglandin biosynthesis in human endometrium is principally a function of the stromal cells (Gal, Casey, Johnston and MacDonald, 1982). The next stage will probably involve stimulation of the cells with steroid hormones after prelabelling with ^{3}H-AA to determine the lipid source(s) of arachidonic acid.

GENERAL DISCUSSION

The overall evidence from the literature indicates that $PGF_{2\alpha}$ released during the latter half of the oestrous cycle is the uterine luteolytic hormone in the guinea pig. The release of arachidonic acid from some bound source is necessary for endometrial $PGF_{2\alpha}$ synthesis as this is the rate-limiting step in PG synthesis. However the level of free arachidonic acid available for PG synthesis is influenced by many other factors as well as those which govern arachidonate liberation (reviewed in the General Introduction).

In order to study arachidonic acid metabolism within the uterine cell, the ability to monitor the movements of a compound behaving in an identical fashion to the endogenous arachidonate was needed. The use of tritiated arachidonic acid (^{3}H-AA) fulfilled this requirement. Such a radioactive tracer technique monitored the fatty acid pattern of incorporation within the uterine lipids as well as enabling the products of the cyclooxygenase and probably lipoxygenase to be detected. There is only one theoretical disadvantage to this technique i.e. the uptake and release (or "turnover") of arachidonic acid varies amongst the lipids (Jesse and Cohen, 1976) such that during the short labelling period, the distribution of ^{3}H-AA does not match the distribution of endogenous arachidonic acid among the various lipids. In the present study, neutral lipids and phospholipids were approximately equally labelled with ^{3}H-AA, yet phospholipids contain over 90% of the endogenous arachidonic acid present in the guinea pig uterus (Leaver and Poyser, 1981). Also it is not possible to detect the loss of fatty acids from non-

labelled sources. However, no serious problems were caused by this objection since all the major neutral lipids and phospholipids incorporated ^{3}H-AA (previously described in Section 2). Also the 'turnover' of some of the phospholipids were monitored by specific radiolabelling techniques such as those described in Section 3.

In the guinea pig uterus it has been demonstrated that, although the mass amounts of neutral lipids and phospholipids are equal, 93% of the total arachidonic acid in the uterus is esterified to uterine phospholipids (Leaver and Poyser, 1981). Therefore it is possible that the major contribution of arachidonic acid for PG synthesis in the guinea pig uterus comes from the phospholipids. If this is the case then prolonged synthesis of PGs in vivo would tend to deplete the available substrates and draw upon the reservoirs of arachidonic acid in the lipid stores (Lands and Samuelsson, 1963). A mechanism must exist to replenish fatty acids within these stores. The results presented in Section 2 established that there was an uptake of free ^{3}H-AA into both the neutral lipids and phospholipids of Day 7 and Day 15 guinea pig endometrial tissue incubated in vitro for 24hr in a modified tissue culture system. Analysis of the fatty acid pattern within the lipids of both tissues indicated that triglyceride, phosphatidylcholine and phosphatidyl-ethanolamine incorporated most ^{3}H-AA. Furthermore there was significantly ($P<0.05$) and specifically more ^{3}H-AA incorporated into the phospholipids of Day 15 tissue than into the phospholipids of Day 7 tissue. On both days, the incorporation of ^{3}H-AA was higher into phosphatidylcholine

than into phosphatidylethanolamine, although more endogenous arachidonic acid is found in phosphatidylethanolamine than in phosphatidylcholine (Leaver and Poyser, 1981). In spite of the enhanced rate of incorporation of ^{3}H-AA into the endometrial phospholipids on Day 15 than on Day 7, the amount of endogenous arachidonic acid bound as phosphatidylcholine and phosphatidylethanolamine in guinea pig uterus is lower on Day 15 than on Day 7 (Leaver and Poyser, 1981). This suggests that the release of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine in the guinea pig endometrium is also higher on Day 15 than on Day 7 (Leaver and Poyser, 1981) and that the increased amount of arachidonic acid released possibly contributes to the increase in $PGF_{2\alpha}$ synthesis during the latter half of the oestrous cycle.

The incorporation of the tritiated fatty acid into the phospholipids could have occurred in two ways, namely by the de novo synthesis of fresh phospholipid or by acylation of lysophospholipids by the enzyme acyl-CoA:lysophosphatide acyltransferase. The results presented in Section 3(b) established that phosphatidylcholine, the major phospholipid component of both Day 7 and Day 15 endometrial tissue (see Section 3(a)) was synthesized by de novo synthesis via the 'choline pathway' as well as by stepwise methylation of phosphatidylethanolamine. However, of the two pathways, the choline pathway synthesized significantly more ($P<0.05$) phosphatidylcholine i.e. it is the major pathway for phosphatidylcholine synthesis in the guinea pig endometrium on both Day 7 and Day 15 of the oestrous cycle. The data

presented in Section 3(b) also indicated that there was no significant difference between the amounts of phosphatidylcholine synthesised by the choline pathway of Day 7 and Day 15 tissue. Therefore the increased incorporation of ^{3}H-AA into Day 15 endometrial phosphatidylcholine cannot be explained by an increase in de novo synthesis. Since phosphatidylethanolamine is synthesized de novo in a similar manner to phosphatidylcholine presumably phosphatidylethanolamine synthesis was also not increased between Days 7 and 15 of the cycle. Analysis of the synthesis of phosphatidylinositol also indicated that there was no difference in activities of the de novo synthetic pathway or the phosphatidylinositol cycle between Day 7 and Day 15 endometrial tissue (see Section 3(c)). Consequently the increased incorporation of arachidonic acid into phospholipids on Day 15 is not due to an increase in the biosynthesis of phospholipids. Therefore the increased uptake of ^{3}H-AA into the phospholipid of Day 15 tissue must be due to other mechanisms.

Trotter and Ferber (1981) stated that 'the rapid incorporation of fatty acids into membrane phospholipids appears to be due to a turnover of fatty acyl chains rather than the de novo synthesis of phospholipids'. Furthermore, arachidonic acid is incorporated into phospholipids almost exclusively by thismechanism in rat and guinea pig liver microsomes (Hills and Lands, 1968; Yamashita, Hosaka and Nuna, 1973). An increased uptake of ^{3}H-AA into phospholipids by this route could be due to one or more of 4 reasons, namely:

i) an increase in acyl-CoA:lysophosphatide acyltransferase activity

ii) changes in the amount of lysophospholipid present

iii) an increase in the amount of arachidonyl-CoA present due to an increase in acyl-CoA synthetase activity

iv) an increase in coenzyme A availablity.

The acyl-CoA:lysophophatide acyltransferase in rat liver has been shown to be fairly specific for arachidonyl-CoA, being eight times more active when arachidonyl-CoA rather than oleoyl-CoA was used as substrate (Yamashita et al., 1973). Furthermore, Okuyama, Yamada and Ikezawa (1975) used rat liver to demonstrate that, although maximum velocities for oleoyl-CoA and arachidonyl-CoA were approximately the same, more arachidonic acid was incorporated than oleate at very low concentrations of the acceptor even when both acyl-CoAs were present at saturating concentrations. This latter study suggested that different enzymes or different sites on a single enzyme existed for the transfer of different acyl-CoAs to lysophospholipids in rat liver (Okuyama et al., 1975). An increase in activity or affinity of the enzyme, or of the sites on one enzyme for arachidonyl-CoA, but not for other acyl-CoAs, towards the end of the oestrous cycle may explain the increased uptake of ^{3}H-AA but not of ^{3}H-OA (tritiated oleic acid) on Day 15 (see Section 2).

Changes in the concentration of lysophospholipid have been shown to affect the rates of incorporation of different fatty

acids into the lysophospholipid in rat liver microsomes (Holub, MacNaughton and Piekarski, 1979). Low concentrations of lysophospholipid were found to favour the incorporation of arachidonic acid and this factor may contribute towards the increase in the uptake of ^{3}H-AA but not of ^{3}H-OA on Day 15.

The amounts of arachidonyl-CoA synthesized by acyl-CoA synthetase must also be considered. Until recently it was assumed that only one acyl-CoA synthetase was present in tissues (see Groot, Scholte and Hülsmann, 1976). However platelets have been found to contain an acyl-CoA synthetase specific for arachidonic acid and a more general acyl-CoA which reacts with a wide range of fatty acids including oleic acid (Wilson, Prescott and Majerus, 1982). The existence of two such enzymes in the guinea pig endometrium with the specific stimulation of arachidonyl-CoA synthetase towards the end of the cycle could then explain the increased uptake of ^{3}H-AA but not of ^{3}H-OA into endometrial phospholipids observed on Day 15.

Finally, an increase in the amount of coenzyme A available to a specific acyl-CoA synthetase may favour the equilibrium towards more arachidonyl-CoA being synthesized, and therefore more arachidonyl-CoA being available for lysophospholipid acylation.

The findings in this thesis indicate therefore that the control of arachidonic acid turnover for PG synthesis involves a specific increase in the incorporation of arachidonic acid into the phospholipids of endometrial tissue, by the acylation of lysophospholipids at the time of

increased endometrial $PGF_{2\alpha}$ synthesis (i.e. after Day 10 of the cycle). Obviously this hypothesis needs studying further; additional experiments which could be carried out must involve the measurement of the amounts of arachidonic acid incorporated into exogenous lysophospholipids by endometrial tissue possibly using a radiolabelling technique. Any increase in the amounts of arachidonic acid incorporated into lysophospholipids towards the end of the oestrous cycle may then confirm the importance of this pathway for the incorporation of arachidonic acid into phospholipids.

The experiments carried out in Section 4 attempted to identify the lipid classes from which the arachidonate is released for PG synthesis by Day 7 and Day 15 endometrial tissue. However, difficulties occurred because only a small fraction of the arachidonate present is required for $PGF_{2\alpha}$ synthesis. In the case of platelets, approximately 10% of the total arachidonate is released following stimulation by thrombin (Rittenhouse-Simmons and Deykin, 1981), though in most other cells the proportion is less. For example mouse fibrosarcoma cells released 3% of cellular arachidonate in response to bradykinin, and human umbilical vein endothelial cells released about 1% when they were stimulated to produce prostacylin (see Majerus, Prescott, Hofmann, Neufeld and Wilson, 1983). In the present study, to determine which cellular lipid pool liberated arachidonic acid, it was necessary to substract two large numbers from each other to get a small difference. The results from Section 4 indicated that the amounts of ^{3}H-AA present in the different lipid pools following labelling for 24hr and then incubated

further for various periods of time up to 24hr were too variable among tissue samples for small differences in arachidonic content (which reflects arachidonic acid release) to be monitored, even in the presence of a releasing agent such as the calcium ionophore A23187 or oestradiol. However, after a 48hr release period, a significant decrease in ^{3}H-AA content of phosphatidylcholine and phosphatidylethanolamine on Day 15 was monitored. It is probable, therefore, that phosphatidylcholine and phosphatidylethanolamine acted as substrates for hydrolysis in order to release arachidonic acid for $PGF_{2\alpha}$ synthesis. To summarise:

i) 90% of endogenous arachidonic acid is present in phospholipids particularly phosphatidylcholine and phosphatidylethanolamine (Leaver and Poyser, 1981)

ii) there is a drop in the endogenous content of arachidonic acid in phosphatidylcholine and phosphatidylethanolamine on Day 15 compared to Day 7 (Leaver and Poyser, 1981)

iii) there is specific uptake of arachidonic acid into phospholipids on Day 15

iv) there is specific release of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine on Day 15.

The above observations indicate that phospholipids (especially phosphatidylcholine and phosphatidylethanolamine) may form the source of arachidonic acid for $PGF_{2\alpha}$ synthesis in the guinea pig endometrium.

Any release of arachidonic acid from the phospholipid stores may involve one or a combination of the following mechanisms

of acylhydrolase activity:

1) phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine as substrates for distinct phospholipase A_2 activities

2) phosphatidylinositol as a substrate for sequential activities of phospholipase C, a diacylglycerol lipase and a monoacylglycerol lipase

3) phosphatidic acid, generated by sequential actions of a phospholipase C on phosphatidylinositol and phosphorylation of the diacylglycerol, may indirectly stimulate phospholipase A_2 attack of phosphatidylcholine and phosphatidylethanolamine by acting as a calcium ionophore

4) sulpholysis, a CoA-mediated transfer of arachidonic acid followed by hydrolysis of another phospholipid by phospholipase A_2 (see General Introduction for more detailed account).

The lack of any mass change in phosphatidylcholine and phosphatidylethanolamine of uterine phospholipids of Day 7 and Day 15 tissue (see Section 3(a)) suggested that deacylation of esterified arachidonate and the generation of lysophospholipid followed by a reacylation process was the possible mechanism of arachidonic acid release in the endometrial cell.

The minor role played by phosphatidylinositol and phosphatidylserine in providing arachidonic acid for PG synthesis is demonstrated by the fact that ^{3}H-AA was

incorporated in very low quantities into the phosphatidylinositol and phosphatidylserine classes over the time period of 24hr. Work carried out by Leaver and Poyser (1981) confirmed that the amount of arachidonic acid bound (μg/g uterus) to phosphatidylinositol and phosphatidylserine was at least 2 to 3 times lower in value than the amount of arachidonic acid bound (μg/g uterus) to phosphatidylcholine.

The data from Section 3(b) indicated that there was no increase in inositol incorporation into phosphatidylinositol on Day 15 i.e. there was no increase in de novo synthesis of phosphatidylinositol or the activity of the phosphatidylinositol cycle. Also there was no change in phosphatidylinositol content of the endometrium between Day 7 and Day 15. Such data indicates that the second and third mechanisms of acylhydrolase activity are of minor importance in the guinea pig endometrium.

The activity of phospholipase A_2 and the enzymes in sulpholysis need to be considered. Phospholipase A_2 activity was detected in both the microsomal fraction and supernatant fraction of guinea pig endometrial homogenates following centrifugation at 100,000 x g for 60 min (Downing and Poyser, 1983). Between 85 to 95% of the phospholipase A_2 activity was dectected in the microsomal fraction. The phospholipase A_2 enzyme in both fractions had a pH optimum of 8. There was a 1.5 to 1.9 fold stimulation in the initial rate of phospholipase A_2 activity between Day 7 and Day 16 of the oestrous cycle (Downing and Poyser, 1983). However phospholipase A_2 activities on both days were more than adequate to provide sufficient arachidonic acid for PG synthesis.

Arachidonic acid release from PG synthesis may be governed by the activation of phospholipase A_2 rather than by the absolute activity of phospholipase A_2.

Sulpholysis involves a CoA-mediated transfer of arachidonic acid from a donor phospholipid to another phospholipid; subsequently this is followed by hydrolysis of the acceptor phospholipid by phospholipase A_2. If the activity of the latter enzyme remains unchanged then increased activity of the enzymes involved in the initial phase of sulpholysis may be responsible for providing arachidonic acid for PG synthesis. The enzymes involved are acyl-CoA synthetase and acyl-CoA:lysophosphatide acyltransferase, the same enzymes responsible for the increased incorporation of ^{3}H-AA into the phospholipids of Day 15 endometrial tissue (see Section 2). Therefore arachidonic acid for PG synthesis may be provided by stimulating the 'turnover' of arachidonic acid at one focal point i.e. by stimulation of acyl-CoA synthetase and acyl-CoA:lysophosphatide acyltransferase to suitably modify preformed phospholipid molecules and adapt their fatty acid composition to the requirements of a particular tissue. Arachidonic acid may be transferred to a phospholipid which is more available for hydrolysis by phospholipase A_2.

It is necessary to determine which phospholipid source is hydrolysed by phospholipase A_2. In rat liver microsomes it was demonstrated that phosphatidylinositol readily donated arachidonic acid to phosphatidylcholine (Irvine and Dawson, 1979). In homogenates of mouse thymocytes it was demonstrated that phosphatidylcholine acted as the

phospholipid donor and lysophosphatidylethanolamine the acceptor substrate for the CoA-mediated transfer of arachidonyl moieties (Trotter and Ferber, 1981). Whether phosphatidylcholine or phosphatidylethanolamine acts as the substrate for phospholipase A_2 action in the guinea pig endometrium is not known. There is twice as much arachidonic acid bound (μg/g uterus) to phosphatidylethanolamine than to phosphatidylcholine but there is significantly more phosphatidylcholine present in the endometrium compared to phosphatidylethanolamine (see Section 3(a)). Either of these phospholipids could act as the source of arachidonic acid for PG synthesis in the guinea pig endometrium.

Broekman, Ward and Marcus (1981) measured the absolute mass of fatty acids liberated in the human platelet in response to thrombin. They showed that the liberation of fatty acids occurred in roughly the proportion expected if phosphatidylethanolamine were the main substrate, as it is when isolated platelet membranes are incubated in vitro (Jesse and Cohen, 1976). However the situation was complicated by the fact that unknown substantial proportions were found metabolised to eicosanoids. Conclusive evidence in favour of arachidonic acid being liberated from a specific phospholipid has yet to be found.

The importance of the fatty acid composition of specific lipids has been questioned by Hassam, Willis, Denton, Stevens and Crawford (1979). They demonstrated that in rabbits fed an essential fatty acid deficient diet, arachidonic acid in the membranes did not fall over a period of 4 to 8 weeks but

PGE_1, PGE_2 and $PGF_{2\alpha}$ were depressed in the brain, liver, lung and eye. These experiments indicated that there would have been increased cellular linoleic acid uptake followed by direct synthesis to PGs via arachidonic acid i.e. a membrane source of PG precursor was not required. However, Schwartzman, Liberman and Raz (1981) perfused unlabelled arachidonic acid into a kidney prelabelled with radioactive arachidonate and demonstrated a release of radioactive PGs. This experiment suggested that arachidonic acid is not converted directly to PGs but becomes esterified into a lipid component of the cell. Therefore the importance of a direct metabolic link between PGs and linoleic acid has not been confirmed.

Evidence from the literature (reviewed in the General Introduction) has implicated the ovarian steroid hormones, oestradiol and progesterone, in the control of arachidonic acid turnover for PG synthesis in the guinea pig endometrium. The hormones may in some way activate the enzymes acyl-CoA synthetase, acyl-CoA:lysophosphatide acyltransferase and phospholipase A_2 so that specific release of arachidonic acid occurs. How this activation occurs is still unknown. Various hypotheses have been suggested such as regulation by specific association with non-enzymatic proteins, cyclic adenosine monophosphate and the availability of calcium ions (see Van den Bosch, 1980). Since most of the enzymes are calcium dependent it may be that control occurs via increasing the free calcium concentration. The calcium ionophore A23187 stimulates $PGF_{2\alpha}$ release from Day 7 and Day 15 guinea pig uterus (Leaver and Seawright, 1982; Poyser and

Brydon, 1983). It has been proposed, therefore, that oestradiol acting on the progesterone-primed uterus increases the free intracellular calcium ion concentration thereby activating phospholipase A_2 to release arachidonic acid for $PGF_{2\alpha}$ synthesis (Downing and Poyser, 1983). It is possible that oestradiol and progesterone have an affect on the lysosomes in which many of the phospholipases are contained (Liggins, Forster, Grieves and Schwartz, 1977). Gustavii (1975) suggested that progesterone stabilises the lysosomes thereby preventing release of phospholipase A_2 in human decidua. Local withdrawal of progesterone then results in the release of hydrolytic enzymes. The in vitro tissue culture of human and guinea pig endometrium demonstrated a similar inhibition of arachidonic acid for PG synthesis by progesterone (Abel and Baird, 1980; Leaver and Seawright, 1981). This mode of control by the ovarian hormones appears attractive except for the fact that 85 to 95% of the phospholipase A_2 in guinea pig endometrium is membrane-bound and is, therefore, not contained in lysosomes. Also lysosomal phospholipase A_2 tends to have maximum activity at pH 4.5 to 5 and not pH 8 as seen for phospholipase A_2 in guinea pig endometrium.

In conclusion, oestradiol and progesterone may act on the guinea pig endometrium to cause an increased 'turnover' of arachidonic acid by stimulating acyl-CoA synthetase and acyl-CoA:lysophosphatide acyltransferase so that one or more specific phospholipids are enriched with the PG precursor. The increase in activity of phospholipase A_2 (Downing and Poyser, 1983) and PG synthetase levels in the guinea pig

endometrium (Poyser, 1979) means that, following activation of phospholipase A_2 by calcium, a greater release of arachidonic acid occurs and a greater proportion of the released arachidonic acid is converted to $PGF_{2\alpha}$ at stages later in the cycle.

To conclude, increased arachidonic acid turnover for increased $PGF_{2\alpha}$ synthesis in the guinea pig endometrium appears to be controlled at two levels namely (i) increased specific acylation by arachidonic acid of lysophospholipids and (ii) increased release of arachidonic acid from phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine, by stimulation of an acylhydrolytic enzyme (e.g. phospholipase A_2). The subsequent increase in the release of arachidonic acid from phospholipids after Day 10 of the cycle may be partly responsible for the increased synthesis of $PGF_{2\alpha}$ in the guinea pig endometrium at this time. The published data (see Poyser, 1981) has clearly shown that $PGF_{2\alpha}$ released from the uterus is responsible for the luteolytic action of the uterus, so the mechanisms controlling arachidonic acid turnover in the endometrium may have an important and necessary physiological function.

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ARACHIDONIC ACID UPTAKE INTO AND RELEASE FROM GUINEA-PIG ENDOMETRIUM IN VITRO ON DAYS 7 AND 15 OF THE OESTROUS CYCLE

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ABSTRACT

Endometrium from guinea-pigs on Days 7 and 15 of the oestrous cycle (days of low and high endometrial prostaglandin $F_{2\alpha}$ production, respectively) was maintained in tissue culture for periods up to 24 h (uptake experiments) or 48 h (release experiments). Tritiated arachidonic acid (^{3}H-AA) was incorporated into endometrial phospholipids and neutral lipids in a time-dependent manner. After 24 h of culture, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the major phospholipids, and triglyceride (TG) was the major neutral lipid which had incorporated ^{3}H-AA. PC, PE and phosphatidylserine/phosphatidylinositol (PS/PI) incorporated significantly more ^{3}H-AA on Day 15 than on Day 7. TG also incorporated more ^{3}H-AA on Day 15 than on Day 7, but the increase was not statistically significant. Tritiated oleic acid (^{3}H-OA) was incorporated into endometrial phospholipids and neutral lipids in a time-dependent manner. No increase in uptake of ^{3}H-OA occurred on Day 15 compared to Day 7. There appears to be a specific stimulation of the mechanisms involved in the uptake of arachidonic acid into guinea-pig endometrium (particularly into the phospholipids) at the end of the oestrous cycle.

There was little apparent release of ^{3}H-AA from any endometrial lipid class, except diglyceride (DG) and monoglyceride (MG), on Day 7. In contrast, there was an apparent 50 to 80% decrease in the ^{3}H-AA content of several endometrial lipid classes, particularly PC, PE and TG, on Day 15. Overall, the uptake and release studies suggest that PC, PE and possibly TG form the source of free arachidonic acid for $PGF_{2\alpha}$ synthesis by the guinea-pig endometrium.

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INTRODUCTION

There is much evidence that prostaglandin (PG) $F_{2\alpha}$ secreted by the uterus is responsible for controlling the length of the oestrous cycle in guinea-pigs (1). Output of $PGF_{2\alpha}$ from the uterus in vivo is 10- to 20-fold higher on Day 15 than on Day 7 of the oestrous cycle (2, 3). Such a difference in output probably cannot be explained solely by the 2- to 3-fold increase in PG synthetase levels which occurs in the uterus (4); this suggests that the activity of the cyclo-oxygenase enzyme is limited by the amount of substrate available. Previous studies (5) have shown that only low levels (1 μg/g tissue) of free arachidonic acid are present in the guinea-pig uterus, whilst much larger amounts (1 mg/g tissue) of arachidonic acid are bound to uterine lipids, predominantly phospholipids and in particular phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Consequently, arachidonic acid has to be released from some bound source in the uterus for conversion into $PGF_{2\alpha}$. In this study, the turnover of the PG precursor in guinea-pig endometrium has been investigated by monitoring tritiated arachidonic acid (^{3}H-AA) uptake into and release from the various lipid classes. Measurements were made on Days 7 and 15 of the oestrous cycle as these are, respectively, days of low and high $PGF_{2\alpha}$ synthesis by and release from the guinea-pig uterus (2, 3, 4). The uptake of tritiated oleic acid (^{3}H-OA) into guinea-pig endometrial lipids on Days 7 and 15 was also monitored and was compared to the uptake of ^{3}H-AA.

METHODS

Sources of Materials. Arachidonic acid (99% pure and checked by thin-layer chromatography), L-α-phosphatidylethanolamine Type III, L-α-phosphatidyl-L-serine, L-α-phosphatidylinositol Grade III, L-α-phosphatidylcholine Type VII E, cholesterol oleate, sphingomyelin, monoglyceride standard (33% monopalmitin, 33% monostearin, 33% monolein), diglyceride standard (33% dipalmitin, 33% diolein, 33% distearin), and triglyceride standard (25% tristearin, 25% trilinolein, 25% triolein, 25% trilinolein) were all purchased from Sigma Chemical Co. Ltd., Poole, England; tritiated arachidonic acid (sp. act. 112 Ci/mmol) and tritiated oleic acid (Sp. act. 8.2 Ci/mmol) were purchased from Amersham International, Amersham, England; Unisil silicic acid (100 to 200 mesh) was purchased from Clarkson Chemical Co. Inc., Williamsport, USA; thin-layer chromatography plates (0.25 mm silica gel) were purchased from Merck, Darmstadt, Germany; vented petri-dishes were purchased from Sterilin, Ltd., Teddington, England; Medium 199 (plus Earles salts), glutamine, kanamycin and amphotericin B were purchased from Flow Laboratories, Irvine, Scotland.

Preparation of the sodium salts of ^{3}H-AA and ^{3}H-OA. Sufficient of the radioactive fatty acid for each investigation was dissolved in 5 ml ethanol, which contained phenolphthalein as indicator. Ethanolic sodium hydroxide (0.1 M) was slowly added until the solution just changed colour from clear to purple. The solution was then evaporated to dryness at 35°C on a rotary evaporator, and was dissolved in an appropriate volume of culture medium.

Animal Studies. Thirty-two virgin guinea-pigs, weighing between 550 and 850 g, were examined daily and a vaginal smear taken when the vaginal membrane was open. The first day of the oestrous cycle was taken as the day before the post-ovulatory influx of leucocytes when cornification was maximal. All animals had exhibited at least two normal cycles immediately before being used. Each guinea-pig was killed by stunning and incising the neck, either on Day 7 or Day 15 of the cycle. The uterus was rapidly removed and was placed in culture medium. Under sterile conditions at room temperature, the endometrium was dissected from the myometrium by cutting away 1 mm by 2 mm portions of endometrium. The tissue was then allocated to one of the following experiments and was cultured by a method based on that of Baker and Neal (6). Histological examination has shown that guinea-pig endometrium remains viable during tissue culture (7).

Experiment 1 - Uptake Studies

a) Arachidonic acid (AA). Approximately 20 mg endometrial tissue was placed onto sterile lens paper which lay across a sterile, stainless steel grid in a vented petri dish. The dish contained 4 ml Medium 199 (plus Earles salts) and glutamine (1%), amphotericin B (2.5 μg/ml), kanamycin (50 μg/ml) and 10 μCi ^{3}H-AA (as the sodium salt). This arrangement enabled the tissue to remain above the culture medium, but still allowed the medium (containing ^{3}H-AA) to be readily available to the endometrial tissue by capillary action through the lens paper. Six dishes containing endometrial tissue were prepared from the uterus of each guinea-pig. The dishes were placed in modified Kilner jars and were incubated at 37°C, in the presence of a 1:1 mixture of air/carbon dioxide (95%:5%) and oxygen/carbon dioxide (95%:5%) at a pressure of 10 lb/in^{2}, for 3, 9 or 24 h. Two samples were cultured for each time period. Following incubation, the tissue from each dish was washed in fresh medium, blotted dry, weighed and homogenised in 15 ml chloroform and methanol (2:1). Each extract was subjected to silicic acid column chromatography followed by thin-layer chromatography (tlc). Radioactivity in each lipid extract before column chromatography, and in each lipid class after both chromatographic procedures was measured by liquid scintillation counting. Endometrial tissue from five guinea-pigs was studied on both days.

b) Oleic acid (OA). Exp. 1a was repeated but using 10 μCi ^{3}H-OA (as the sodium salt) instead of ^{3}H-AA. Duplicate samples of endometrium from six guinea-pigs on both Days 7 and 15 were cultured for 3, 9 or 24 h, extracted and subjected to silicic acid column chromatography. One of the duplicate samples from four guinea-pigs and both duplicate samples from two guinea-pigs on each day were further analysed by tlc.

Experiment 2 - Release studies.

All portions of endometrial tissue from a guinea-pig were placed in a single petri dish, containing 4 ml Medium 199 (plus Earles salts and supplements) and 62.5 μCi ^{3}H-AA (as the sodium salt), and were cultured for 24 h, under the same conditions as in Exp. 1a, in order to "label" the different lipid classes. The endometrial tissue was then equally distributed among 6 further petri dishes, containing 4 ml Medium

199 (plus Earles salts, supplements and 0.1% bovine serum albumin), and two glass bottles, each containing 5 ml chloroform and methanol (2:1). The last two samples were stored at -20°C, while the remaining samples were incubated as in Exp. 1a for 0.5, 3 or 24 h (two samples for each period of time). The endometrial tissue samples were then analysed as in Exp. 1a.

Details of silicic acid column chromatography.

Each chloroform and methanol extract of endometrial tissue was taken to dryness on a rotary evaporator at 35°C, resuspended in 5 ml diethyl ether, and applied to a glass column (1 cm x 10 cm) containing 4 g silicic acid. Each column was eluted in succession by 100 ml diethyl ether (which elutes neutral lipids and free ^{3}H-AA), 100 ml diethyl ether and methanol (4:1) (which elutes cyclo-oxygenase and lipoxygenase products), and 100 ml methanol (which elutes phospholipids). A comparison of the results before and after column chromatography indicated that 80% or more of the radioactivity was recovered. All results were corrected for recovery.

Details of thin-layer chromatography (tlc).

a) Neutral lipids were separated into individual lipid classes by tlc using a solvent system of petroleum spirit (b.p. 40° - 60°C), diethyl ether and acetic acid (75:25:1). Triglyceride (TG), diglyceride (DG), monoglyceride (MG), cholesterol ester (cho) and AA standards (25 µg of each) were run simultaneously on each tlc plate. Lipids were visualized by exposure to iodine. Areas on the plate corresponding to the R_F values of standard lipids were scraped off the tlc plates and were placed into scintillation vials. Diethyl ether (1 ml) was added to each scintillation vial to extract the neutral lipids, followed by 10 ml scintillation fluid. Results before and after this separation procedure showed that between 50% and 90% of the radioactivity placed on the tlc plates was recovered. All results are corrected for recovery.

b) Phospholipids were separated by tlc using a solvent system of methyl acetate, propan-1-ol, chloroform, methanol and 0.25% aqueous solution of KCl (25:25:25:10:9). Standards (25 µg) of PC, PE, phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (Sph) were run on each tlc plate. Lipids were visualized by iodine vapour and areas corresponding to the R_F values of the standard lipids were scraped from the tlc plates and placed into scintillation vials. PS and PI ran very close together on the tlc plate and were therefore not separated for counting purposes. Methanol (1 ml) was added to extract the phospholipids followed by 10 ml scintillation fluid. The recovery of radioactivity from the tlc plates ranged from 60% to 90%. All results are corrected for recovery.

Statistical tests.

Appropriate data was analysed by the Student's "t" test.

RESULTS

Experiment 1 - Uptake Studies.

a) Arachidonic acid (AA). The total amount of ^{3}H-AA taken up into Day 7 and Day 15 guinea-pig endometrial tissue increased with time, and there was 2 to 3 times more radioactivity incorporated after 24 h than after 3 h (Fig. 1). Day 15 tissue incorporated more ^{3}H-AA than Day 7 tissue (significantly different ($P < 0.05$) at 3 h).

More ^{3}H-AA was incorporated into neutral lipids than into phospholipids at 3 h on both Days 7 and 15 (Fig. 1). By 24 h, however, there was no difference in the incorporation of ^{3}H-AA between the two lipid pools on Day 7, and more ^{3}H-AA was incorporated into phospholipids than into neutral lipids on Day 15. The uptake of ^{3}H-AA into neutral lipids and phospholipids was greater on Day 15 than on Day 7 (significantly different ($P < 0.05$ and < 0.01, respectively) for phospholipids at 9 h and 24 h). There were relatively low levels of free ^{3}H-AA in endometrial tissue at 3, 9 and 24 h on both Days 7 and 15 (Fig. 1).

Incorporation of ^{3}H-AA into the different neutral lipid classes was in the order of MG > DG > TG $\gg$ cho at 3 h and TG > MG = DG $\gg$ cho at 24 h, on both Days 7 and 15 (Fig. 2). The incorporation of ^{3}H-AA into MG and DG at 3 h and 9 h was significantly greater ($P < 0.05$) on Day 15 than on Day 7. At 24 h, more ^{3}H-AA was apparently incorporated into TG, MG and DG on Day 15 than on Day 7, but none of the differences between the two days reached statistical significance. Incorporation of ^{3}H-AA into phospholipids was in the order of PC > PE > PS/PI > Sph on both Days 7 and 15 (Fig. 2). More ^{3}H-AA was incorporated into all phospholipid classes on Day 15 than on Day 7 (significantly different ($P < 0.05$) for PC, PE and PS/PI at 24 h).

b) Oleic acid (OA). The total amount of ^{3}H-OA taken up into Day 7 and Day 15 guinea-pig endometrial tissue increased with time, and there was twice as much radioactivity incorporated after 24 h than after 3 h (Fig. 3). However, Day 15 tissue incorporated significantly less ($P < 0.05$) ^{3}H-OA than Day 7 tissue at 3 h and 24 h. Generally more ^{3}H-OA was incorporated into neutral lipids than into phospholipids, the difference being least marked for the two lipid classes in Day 7 tissue at 24 h. There was a relatively high level of free ^{3}H-OA in both Day 7 and Day 15 tissue at the three times studied. Incorporation of ^{3}H-OA into neutral lipids at 3 h and into phospholipids at 24 h was significantly lower ($P < 0.05$) on Day 15 than on Day 7 (Fig. 3).

Of the ^{3}H-OA incorporated into neutral lipids, generally more ^{3}H-OA was incorporated into either TG or DG than in MG at 3, 9 and 24 h on both days (Fig. 4). However, incorporation of ^{3}H-OA into TG and DG at 3 h was lower on Day 15 than on Day 7 (statistically significant ($P < 0.05$) for TG). Also significantly less ($P < 0.05$) ^{3}H-OA was incorporated into MG on Day 15 than on Day 7 at 24 h. Uptake of ^{3}H-OA into cho was low at all times studied, although uptake on Day 7 was generally higher than on Day 15 (significantly different ($P < 0.05$) at 24 h). Incorporation of ^{3}H-OA into phospholipids was in the order of

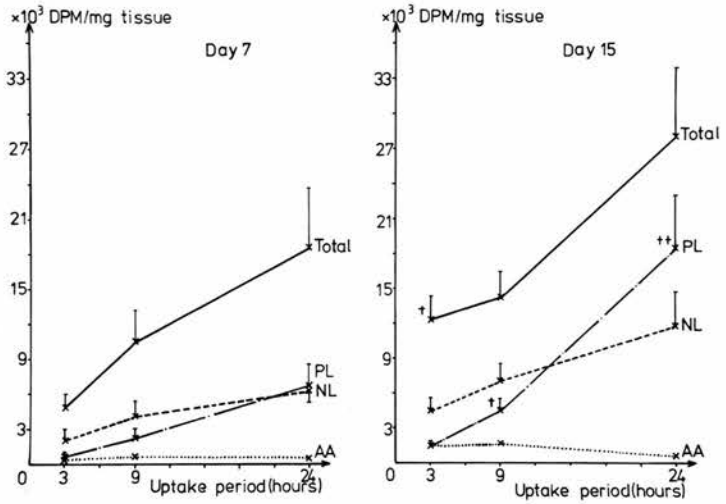

Figure 1. Mean (± s.e.m., n = 10) total uptake of ^{3}H-arachidonic acid into Day 7 and Day 15 guinea-pig endometrium during a 24 h culture period, and its distribution among phospholipids (PL), neutral lipids (NL) and free ^{3}H-arachidonic acid (AA) (Standard errors for AA are too small to include).
† (P < 0.05) †† (P < 0.01) Significantly higher than corresponding Day 7 value.

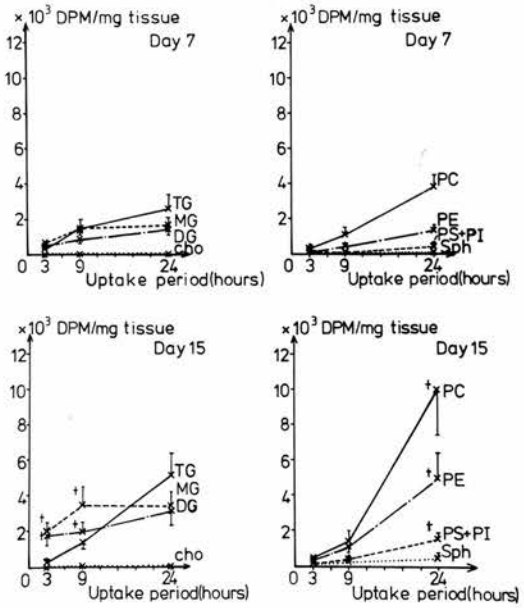

Figure 2. Mean (± s.e.m., n = 10) uptake of ^{3}H-arachidonic acid into triglyceride (TG), monoglyceride (MG), diglyceride (DG), cholesterol ester (cho), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine + phosphatidylinositol (PS + PI) and sphingomyelin (Sph) in Day 7 and Day 15 guinea-pig endometrium during a 24 h culture period (Standard errors for cho, and for Sph on Day 7 are too small to include).
† Significantly higher (P < 0.05) than corresponding Day 7 value.

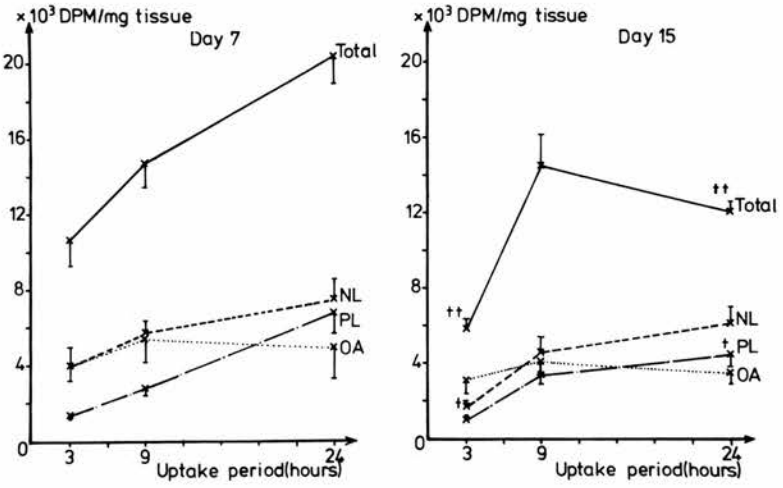

Figure 3. Mean (± s.e.m., n = 12) total uptake of ^{3}H-oleic acid into Day 7 and Day 15 guinea-pig endometrium during a 24 h culture period, and its distribution among phospholipids (PL), neutral lipids (NL) and free ^{3}H-oleic acid (OA).
† ($P < 0.05$) †† ($P < 0.01$) Significantly lower than corresponding Day 7 value.

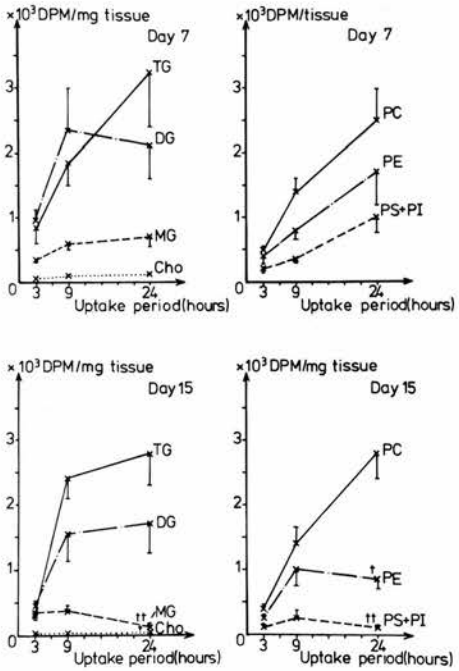

Figure 4. Mean (± s.e.m., n = 8) uptake of ^{3}H-oleic acid into triglyceride (TG) monoglyceride (MG), diglyceride (DG), cholesterol ester (cho), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine + phosphatidylinositol (PS + PI) in Day 7 and Day 15 guinea-pig endometrium during a 24 h culture period.
† ($P < 0.05$) †† ($P < 0.01$) Significantly lower than corresponding Day 7 value.

PC > PE > PS/PI (Sph was not studied). Significantly less ($P < 0.05$) ^{3}H-OA was incorporated into PE and PS/PI, but not into PC, on Day 15 than on Day 7 at 24 h (Fig. 4).

Experiment 2 - Release Studies.

There was no apparent release of ^{3}H-AA from any lipid class in Day 7 endometrial tissue over the time period studied, except for a significant decrease ($P < 0.05$) in the amount of ^{3}H-AA bound to DG and MG at 24 h compared to 0 h (Figs. 5 and 6). On Day 15, there was a general 50 to 80% decrease in the ^{3}H-AA content of all lipid classes over the release period, particularly at 3 h (Figs. 5 and 6). However, none of the values at 0.5, 3 and 24 h were significantly different from the corresponding value at 0 h, due to the wide scatter of results.

DISCUSSION

Phospholipids and neutral lipids of guinea-pig endometrium incorporated ^{3}H-AA and ^{3}H-OA in increasing amounts with time on both Days 7 and 15 of the oestrous cycle. Of the neutral lipids, ^{3}H-AA and ^{3}H-OA were incorporated predominantly into MG, DG and TG. Only small quantities of both fatty acids were esterified to cholesterol. ^{3}H-AA and ^{3}H-OA were incorporated into phospholipids in the order of PC > PE > PS/PI. It is not possible to compare the amounts of ^{3}H-AA and ^{3}H-OA incorporated into the different lipids classes because the quantity of ^{3}H-OA used was much greater than the quantity of ^{3}H-AA used (10 μCi of both tritiated compounds was used but the specific activity of ^{3}H-AA was 14 times higher than that of ^{3}H-OA). However, at 24 h, particularly on Day 15, more of the ^{3}H-OA was incorporated into neutral lipids while more of the ^{3}H-AA was incorporated into phospholipids. In vivo, there is a 10- to 15-fold higher incorporation of endogenous oleic acid than of endogenous arachidonic acid into TG, while the incorporation of both acids into phospholipids is similar (5). These observations indicate that there are differences in the incorporation and relative distribution of arachidonic acid and oleic acid among the different lipid classes in guinea-pig endometrium.

More ^{3}H-AA was taken up into endometrial tissue on Day 15 than on Day 7. Although more ^{3}H-AA went into both neutral lipids and phospholipids, a higher proportion of the increased amount of ^{3}H-AA taken up was incorporated into phospholipids than into neutral lipids. In contrast, the uptake of ^{3}H-OA into guinea-pig endometrium did not increase between Days 7 and 15. In fact, uptake of ^{3}H-OA was lower on Day 15 than on Day 7 at 3 h (due to a lower incorporation into neutral lipids) and at 24 H (due to a lower incorporation into phospholipids, particularly PE and PS/PI).

Levels of free ^{3}H-OA in guinea-pig endometrium were much higher than the levels of free ^{3}H-AA presumably due, at least in part, to the conversion of ^{3}H-AA into cyclo-oxygenase and lipoxygenase products. Approximately 10% of the ^{3}H-AA taken up into endometrial tissue was metabolized in this way. 12-L-Hydroxy-5,8,10,14-eicosatetranoic acid (12-HETE) is incorporated into TG in much greater quantities than into phospholipids of mouse thyroid gland (8). In the present study, it is

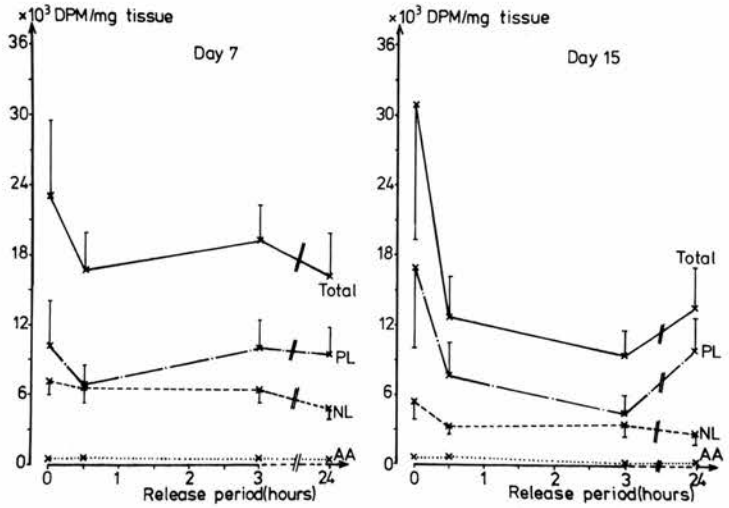

Figure 5. Mean (± s.e.m., n = 10) total content of ^{3}H-arachidonic acid in Day 7 and Day 15 guinea-pig endometrium, and its distribution among phospholipids (PL), neutral lipids (NL) and free ^{3}H-arachidonic acid (AA) during a 24 h release period following a 24 h uptake period (Standard errors for AA are too small to include).

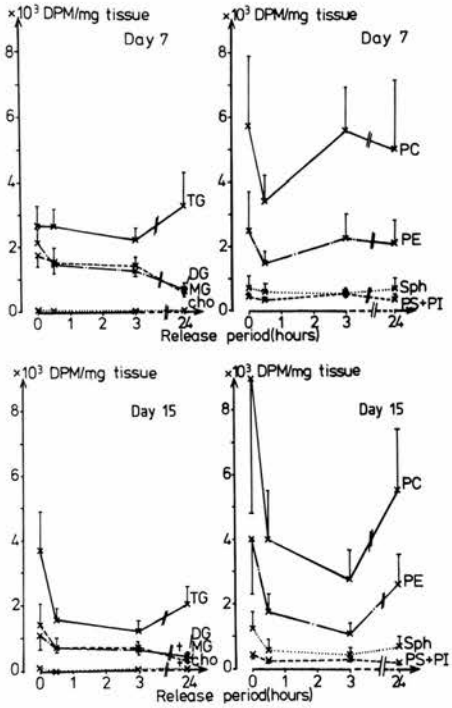

Figure 6. Mean (± s.e.m., n = 10) total content of ^{3}H-arachidonic acid in triglyceride (TG), monoglyceride (MG), diglyceride (DG), cholesterol ester (cho), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylethanolamine (PE), phosphatidylserine + phosphatidylinositol (PS + PI) and sphingomyelin (Sph) in Day 7 and Day 15 guinea-pig endometrium during a 24 h release period following a 24 uptake period (Standard errors for cho are too small to include).
† Significantly lower ($P < 0.05$) than corresponding Day 7 value.

not known whether the radioactivity in neutral lipids is solely ^{3}H-AA, or whether a small proportion of the radioactivity consists also of ^{3}H-12-HETE which has been synthesized from ^{3}H-AA by lipoxygenase enzymes in the guinea-pig endometrium.

Incorporation of the tritiated fatty acids into phospholipids could have occurred in two ways, namely by the de novo synthesis of fresh phospholipid or by acylation of lysophospholipids by the enzyme, acyl-CoA:lysophosphatide acyltransferase. The latter process is the most probable since Trotter and Ferber (9) state that "the rapid incorporation of fatty acids into membrane phospholipids appears to be due to a turnover of fatty acyl chains rather than the de novo synthesis of phospholipids". In addition, arachidonic acid appears to be incorporated into phospholipids exclusively by this mechanism (10, 11).

Increased uptake of ^{3}H-AA into phospholipids on Day 15 could be due to at least three reasons, namely (i) an increase in acyl-CoA:lysophosphatide acyltransferase activity, (ii) changes in the amount of lysophospholipid present, (iii) an increase in the amount of arachidonyl-CoA present due to an increase in acyl-CoA synthetase activity. However, if one or more of these mechanisms were operative, the uptake of ^{3}H-OA would be expected to increase also. This was found not to be the case. Consequently, further reasons must be sought to explain the specific increase in uptake of ^{3}H-AA.

The acyl-CoA:lysophosphatide acyltransferase in rat liver is fairly specific for arachidonyl-CoA, being eight times more active when arachidonyl-CoA rather than oleyl-CoA is used as substrate (11). Furthermore, a subsequent study suggests that different enzymes or different sites on a single enzyme exist for the transfer of different acyl-CoAs to lysophospholipids in rat liver (12). Consequently, if different enzymes or different sites on the same enzyme similarly exist in guinea-pig endometrium, an increase in activity of the enzyme or of the sites on one enzyme specific for arachidonyl-CoA towards the end of the oestrous cycle may explain the increased uptake of ^{3}H-AA, but not of ^{3}H-OA, on Day 15. In addition, it has been found that the concentration of lysophospholipid can affect the rates of incorporation of different fatty acids into the lysophospholipid, with very low concentrations favouring the incorporation of arachidonic acid (13). Consequently, changes in lysophospholipid concentration may also lead to an increase in the uptake of ^{3}H-AA, but not of ^{3}H-OA, on Day 15.

Until recently, it has been assumed that only one acyl-CoA synthetase is present in tissues (see 14). However, platelets have been found to contain an acyl-CoA synthetase specific for arachidonic acid, and a more general acyl-CoA which reacts with a wide range of fatty acids, including oleic acid (15). If two such enzymes exist in guinea-pig endometrium, a specific increase in arachidonyl-CoA synthetase activity towards the end of the oestrous cycle could also explain the increased uptake of ^{3}H-AA, but not of ^{3}H-OA, observed on Day 15. Further study is obviously required to establish the precise reasons for the increased uptake of ^{3}H-AA into guinea-pig endometrium on Day 15.

The incorporation of ^{3}H-AA was higher into PC than into PE, although more endogenous arachidonic acid is found in PE than in PC (5). Furthermore, in spite of the enhanced rate of incorporation of ^{3}H-AA into endometrial phospholipids on Day 15 than on Day 7, the amount of endogenous arachidonic acid bound as PC and PE in guinea-pig uterus is lower on Day 15 than on Day 7 (5). This suggests that the release of arachidonic acid from PC and PE in guinea-pig endometrium is also higher on Day 15 than on Day 7. To determine the viability of this hypothesis, a series of experiments to determine the release of ^{3}H-AA from the different endometrial lipid classes was carried out. There was little apparent release of ^{3}H-AA from any lipid class within the endometrium on Day 7, except possibly from DG and MG at 24 h. However, whether the significant decrease in the amount of ^{3}H-AA bound as DG and MG represents release from these lipid classes or conversion of these intermediate lipids into other lipid classes, remains uncertain. On Day 15, there was an apparent release of ^{3}H-AA from most lipid classes, particularly from PC, PE and TG at 3 h. Due to the wide scatter of the results, no statistically significant differences were obtained. The wide scatters were probably due to several reasons, including (i) a large variation in the initial uptake of ^{3}H-AA, (ii) dilution of incorporated ^{3}H-AA by varying amounts of endogenous fatty acids, (iii) re-acylation of released ^{3}H-AA into other lipid classes. Nevertheless, the apparent higher release of arachidonic acid on Day 15 than on Day 7 may be partly responsible for the higher output of $PGF_{2\alpha}$ from guinea-pig endometrium, maintained in tissue culture, on Day 15 than on Day 7 (8).

In conclusion, the present studies indicate that there is enhanced turnover of arachidonic acid in phospholipids and, to a lesser extent, in neutral lipids in guinea-pig endometrium on Day 15 compared to Day 7 of the oestrous cycle. Our studies have not shown there to be any one specific lipid pool in the guinea-pig endometrium which releases arachidonic acid for conversion into $PGF_{2\alpha}$, the uterine luteolytic hormone. However, PC, PE and possibly TG may form the main pools of arachidonic acid for PG synthesis. Further tissue culture experiments involving pre-labelling with ^{3}H-AA then applying an appropriate stimulus known to increase endometrial $PGF_{2\alpha}$ production (such as oestradiol or A23187) may provide further information about the source(s) of arachidonic acid for $PGF_{2\alpha}$ synthesis by the guinea-pig endometrium.

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