METABOLISM OF ARACHIDONIC ACID *via* THE LIPOXYGENASE PATHWAY IN SHEEP UTERUS

THESIS SUBMITTED TO
THE UNIVERSITY OF HYDERABAD
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STATEMENT

I here by state that the work presented in this thesis entitled **"Metabolism of Arachidonic acid** *via* **the Lipoxygenase Pathway in Sheep Uterus"** has been carried out by **me** under the supervision of Dr.P.Reddanna and that this has not been submitted for any degree or diploma of any other University earlier.

14.11.94

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CERTIFICATE

This is to certify that **Mr.Sailesh Surapureddi,** has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the PhD ordinance of this University. I recommend his thesis entitled **"Metabolism of Arachidonic acid** *via* **the Lipoxygenase Pathway in Sheep Uterus"** for submission for the degree of Doctor of Philosophy of this University.

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Abbreviations

AA	arachidonic acid
ALA	α-linolenic acid
BSTFA	bis(Trimethyl silyl) trifluoro acetamide
BF_3	boron tri fluoride
CM-52	carboxy methyl cellulose-52
COX	cyclooxygenase
DEAE	diethyl aminoethyl cellulose
DHA	docosahexaenoic acid
DHGLA	dihomo-γ-linolenic acid
GLA	γ-linolenic acid
GSH	glutathione reduced
GST	glutathione S-transferase
GC	gas chromatography
HPETE	hydroperoxyeicosatetraenoic acid
HETE	hydroxyeicosatetraenoic acid
HRP	horse radish peroxidase
HPLC	high performance liquid chromatography
LA	linoleic acid
LOX	lipoxygenase
LT	leukotrienes
LX	lipoxins
MS	mass spectrometer
Me	methyl ester
NDGA	nordihydroguaiaretic acid
O	ortho
PMP	penta methyl piperidine
PMSF	penta methyl sulfonyl fluoride
PG	prostaglandins
PAGE	poly acrylamide gel electrophoresis
PGI	prostacyclins
RP	reverse phase
SDS	sodium dodecyl sulphate
SP	straight phase
THF	tetra hydrofuran
TFMSA	trifluoromethane sulfonic anhydride

TEA	triethylamine
TMS	trimethyl silyl
TX or Tx	thromboxane
min.	minutes
	inches
µ	micro
L	liters
M	molarity
vol	volume
Enz	enzyme
conc.	concentration
sol	solvent
g	grams
nm	nanometer
hr	hour

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Introduction

1.0.0 INTRODUCTION

Proteins, carbohydrates and lipids apart from being the energy supplicants to the biological system, have an integrated role in transfer of information. Proteins as enzymes and hormones transfer vital inter and intracellular information. Carbohydrates being the backbone of nucleic acids dissipate stored information within and to the progeny in the form of mRNA and DNA. However, the information transfer mechanism of lipids was largely unclear till the late 1970s. The advent of membrane lipid theory in 1925 by Gorter and Grendell has facilitated the understanding of the importance of lipid moiety in biological systems. Traditional classification of lipids into saturated and unsaturated fatty acids depending on the presence of double bonds was followed by the concept of "pre and essential fatty acids" (Burr and Burr, 1929), where the consumption of fat free diets was shown to be the causative factor for the loss of growth, skin texture, tail necrosis and eventual death. These effects were found to be reversible and could be prevented by the addition of unsaturated fatty acids called linoleic acid (LA) to the diets (Burr and Burr, 1930).

The advent of chromatography and labelled isotopes have facilitated the discovery of other major dietary fatty acids such as, α-linolenic acid (ALA), arachidonic acid (AA) apart from LA and their metabolites, which could be easily followed and identified (Mead *et al.*, 1981).

1.1.0 ESSENTIAL FATTY ACIDS- *A contribution of plants*

Animal systems cannot synthesize the fatty acids such as AA and EPA *de novo*. Hence, they depend on two major precursors from plants, linoleic acid (n-6) and linolenic acid (n-3) for the synthesis of various other fatty acids which have 3 to 6 double bonds in their structure.

The relationship among fatty acids are now being evaluated based on their groups or families. The dominant fatty acid families are *n-6* and *n-3* and the lesser known are *n-9* and *n-7*. These fatty acid families are clearly demarcated and are not inter-connected metabolically except for their synthesis (Fig 1).

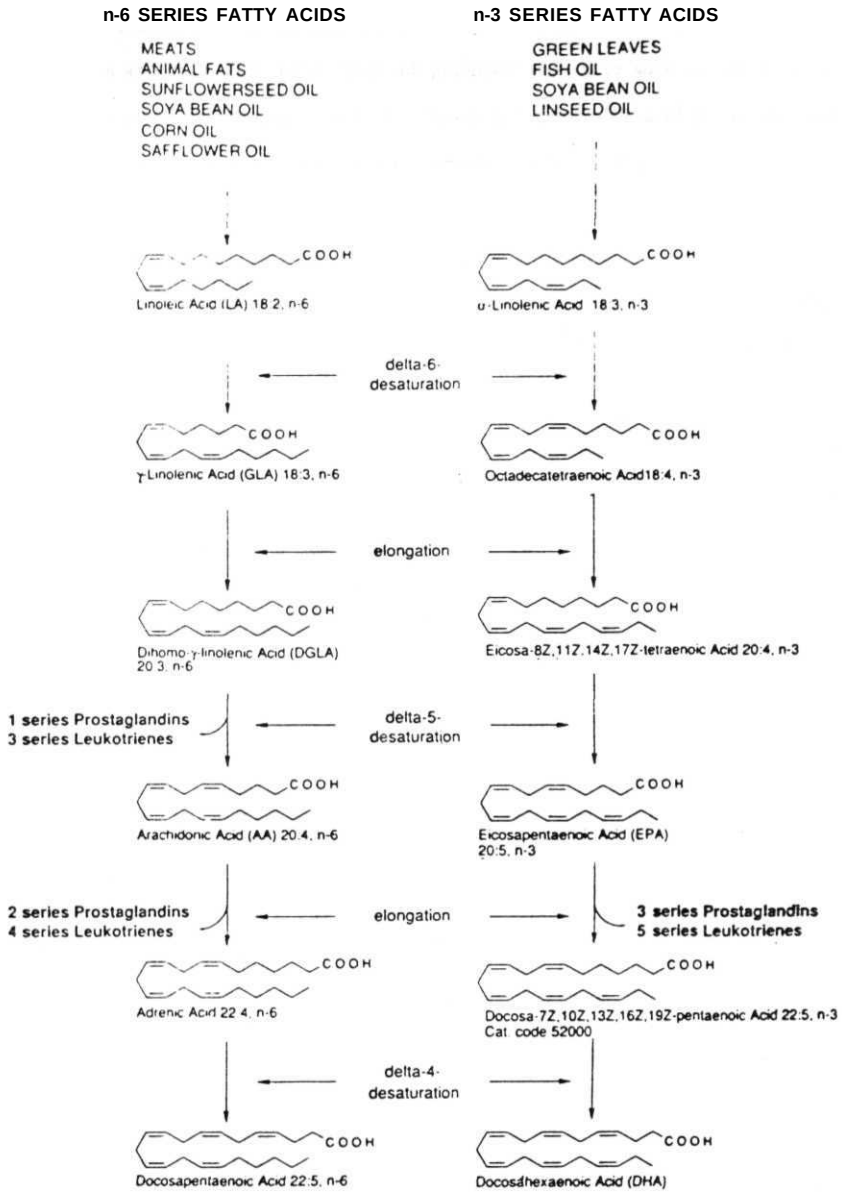


Fig.1: **Metabolic pathways of *(n-3)* and *(n-6)* fatty acids.**

The synthesis of various fatty acids in all the families are brought about by the desaturation and chain elongation from the precursor molecules such as, ALA for *n-3* family, LA for *n-6* family, oleic acid for *n-9* family and palmitoleic acid or vaccenic acid for *n- 7* family by the same enzyme complexes (Holman *et al.*, 1964).

1.2.0 Biological effects of *n-6* and *n-3* fatty acids

Most of the existing diets contain enough 18:2 (n-6) and 18:3 (n-3) PUFAs or their metabolic products to meet the tissue demands. However severe deficiency states have been observed in infants on prolonged intravenous feeding or artificial milk formulations without adequate lipid supplements. Changes in serum fatty acid pattern are characterized by (depletion of n-6 fatty acids and a major increase in the 20:3 (n-9) to 20:4 (n-6) ratio) severe skin rash, loss of hair, loss reproductive capacity and irritability. These symptoms could be reversed rapidly by supplementing the diets with lipid emulsions containing esterified 18:2 (n-6) fatty acid (Cook *et al.*, 1991). Further the deficiency of n-3 fatty acid did not seem to have any particular effect, but proved useful in inhibiting the excess production *of n-6* fatty acid metabolites (Friedman *et al.*, 1981 and Tinco, 1982). Infact epidemiological studies have indicated, that high ratio of *n-3* to *n-6* fatty acids present in the diets of Eskimos could be responsible for low incidence of (heart attacks in the form of) myocardial infarctions, bronchial asthma, psoriasis, diabetes and autoimmune diseases compared to other non-Eskimo populations. The high levels of *n-3* PUFAs appear to compete with *n-6* PUFAs in the formation of eicosanoids and thereby limiting the formation of eicosanoids, which are responsible for the onset of degenerative diseases (Lands, 1986).

1.3.0

Arachidonic **acid cascade**

" The arachidonic acid content of active tissue is high and it is natural to assume some important role for this highly unsaturated long chain fatty acid" (Burr and Burr, 1930).

A statement with vision was immediately followed by an unrelated observation, wherein human semen containing an acid factor was shown to cause either contraction or relaxation of human uterine smooth muscles (Kurzrok and Lieb, 1930). The identification of the acidic factor was mainly done by von **Euler** who coined the name **"Prostaglandin** since it was believed to have originated from the prostate gland (von Euler *et al,* 1934, 1935). The structural aspects of this acidic factor was solved by Bergstrom after nearly 30 years and identified as PGE_1, PGE_2 and $PGF_{2\alpha}$ (Bregstrom *et al,* 1962a and 1962b). The relationship with arachidonic acid, an (n-6) family derived essential fatty acid and the acidic factor of human semen (PGE and F) was established by Bregstrom and van Drop independently couple of years later paving the way for unfurling the arachidonic acid cascade in all biological systems known (Bregstrom *et al,* 1964 and van Drop *et al,* 1964).

Early studies of the oxygenation of unsaturated fatty acids were restricted to two main groups, the synthesis of prostaglandins from arachidonic acid was seen only in animal systems where as the oxygenation of linoleic acid which was being investigated by plant **lipid oxidases** termed later as lipoxidases and lipoxygenases (**Holman** *et al.,* **1951**; **Hamberg** ***et al.,*** 1965, 1967). The common mechanism of oxygenation of unsaturated fatty acids by both plant and animal oxygenases was identified by **Samuelsson** group (Hamberg and Samuelsson 1967a, 1967b) which paved the way for the search and ultimate recognition of lipoxygenases, in animal systems in the form of **12-lipoxygenase** in platelets (Hamberg and Samuelsson, 1974). Since then the operation of arachidonic acid metal olism and various sources for cyclooxygenase and lipoxygenase have been **identif** ed in a variety of animal species in animal kingdom and within different tissues of humans like **polymorphonuclear** leukocytes (PMNL), macrophages, lymphocytes, platelets, lung,

liver, skin and uterus (Goetzel *et al,* 1979; Borgeat *et al,* 1979; Doig *et al,* 1980; Parker *et al.,* 1979, Ziboh *et al.,* 1992, Flatman *et al.,* 1986).

Polyunsaturated fatty acids and their metabolites have been linked with every cellular function in one way or the other and have been known to mimic drug actions (Abbrachio *et al,* 1986). These fatty acids are present in the membrane lipid moiety in the form of phosphoglycerides (Ananda Rao *et al,* 1979; Berlin *et al.,* 1980). The unsaturated fatty acid is attached to the 2nd carbon atom of the glycerol backbone as an ester. While the acylation of glycero-3-phosphate takes place, i.e the transformation of saturated and monoenoic fatty acids to phospholipids, during *de novo* synthesis, arachidonic acid is introduced into phospholipids not by *de novo* synthesis, but by acylation of lysophosphophatides (Hill *et al.,* 1968a; 1968b, Kanoh *et al.,* 1969). In resting tissues free arachidonic acid is in equilibrium with arachidonyl esters and the formation of latter is favoured (Irvine, 1982, Lands *et al.,* 1982). The release of free arachidonic acid from its esters has been amply investigated and attributed to acyltransferases (Kroner *et al.,* 1981, Trotter *et al.,* 1982). The arachidonic acid from phospholipid moiety is released by phospholipases (PLAs) on specific endocrine or paracrine stimuli i.e phospholipase A_2 (PLA_2) when the arachidonic acid is from phosphotidylethanolamine and PLC when the arachidonic acid is from phosphatidylinositol (van den Bosch, 1980). The means of AA release from the membrane phospholipids is summarized in Fig 2. This arachidonic acid is available for further metabolism only on the inhibition of acyl transferases In summary "AA is rapidly liberated from and reesterified into phopholipids, but a fraction of released AA can only be oxygenated" (Oliw *et al,* 1983). The released arachidonic acid is enzymatically oxygenated by 3 main enzymes in animal systems (Fig. 3): 1. cyclooxygenase 2. lipoxygenase and 3. epoxygenase (Cyt P-450 pathway).

1.3.1 Cyclooxygenase pathway

Cyclooxygenase (E.C 1.14.99.1) catal/zes the conversion of released arachidonic acid to prostaglandins. This enzyme, which exists in endoplasmic reticulum of almost all animal tissues, exhibits two different catalytic activities a). one which catalyzes the

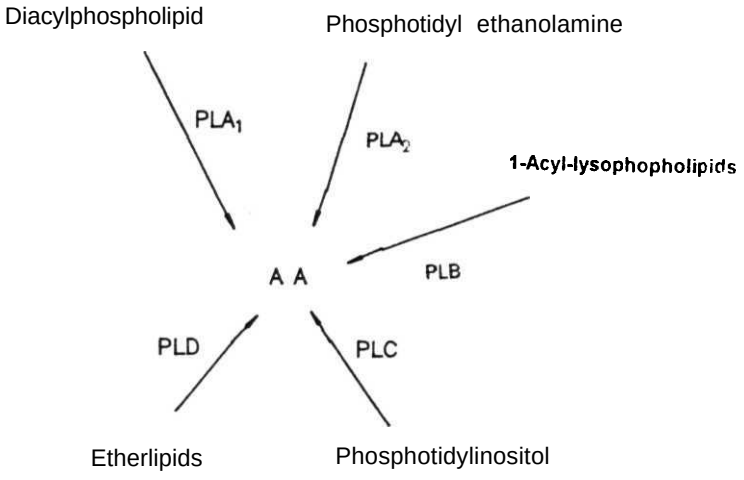


Fig.2: Pathways leading to the release of arachidonic acid from membrane phospholipids by phospholipases.

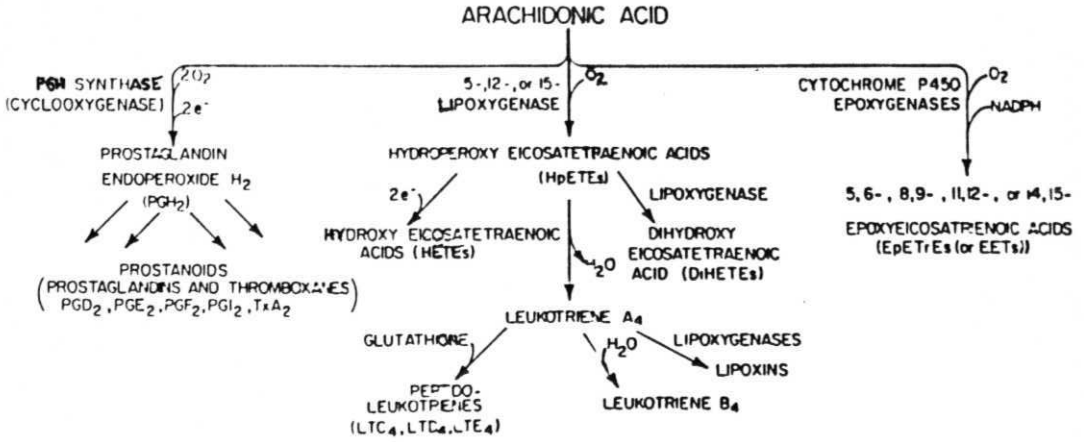


Fig.3: Pathways leading to the formation of eicosanoids from arachidonic acid.

formation of PGG_2 from arachidonic acid by forming a carbon centered radical (bis oxygenase) and b) a peroxidase activity which reduces PGG_2 to PGH_2 These two activities though occur at distant, but interactive, sites within the same protein.

The so formed PGH_2 depending on the cell specificity gets converted into thromboxanes, prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$) and prostacyclin by the action of different enzymes (Smith *et al.*, 1991. Fig 4).

1.3.2 Epoxygenase pathway

Transformation of arachidonic acid via **Cyt P-450** mixed oxidase (Capdevila *et al*, 1982, Laniado-Schwartzman *et al*, 1988; Oliw *et al*, 1982) is also known. These enzymes, also referred to as monooxygenases which are cofactor dependent *viz.* flavoproteins, **P-450** reductases, NADPH/NADP or $NADH/NAD^+$ and also exhibit substrate specificity. The epoxygenase on reacting with arachidonic acid gives two main products: 1) epoxy eicosatrienoic acids (EETs) and 2) hydroxyeicosatetraenoic acids (Fig 5). The enantio selectivity of epoxygenase is specific for the formation of (R) hydroxyl configuration (Capdevila *et al*, 1990) whereas the (S) configuration is known for lipoxygenase derived products of arachidonic acid. The products of both groups are functionally active in biological systems though not well defined.

1.3.3. Lipoxygenase pathway

Lipoxygenases (EC. 1.13.11.__) are a group of enzymes that catalyze the conversion of arachidonic acid to bioactive lipids called hydroperoxyeicosatetraenoic acids (HPETEs). These enzymes have been identified in almost all plants and animals after their discovery nearly four decades ago in plants Depending on their site of oxygen insertion on AA the enzymes are designated as 5-, 8-, 9-, 11- 12- and 15-lipoxygenases. The animal lipoxygenases are classified as 12-lipoxygenase (EC. 1.13.11.31), 15-lipoxygenase (E.C. 1.13.11.33) and 5-lipoxygenase (E.C.1.13.11.34) etc. depending on the position of insertion of molecular oxygen on the arachidonic acid. Based on the site of oxygen

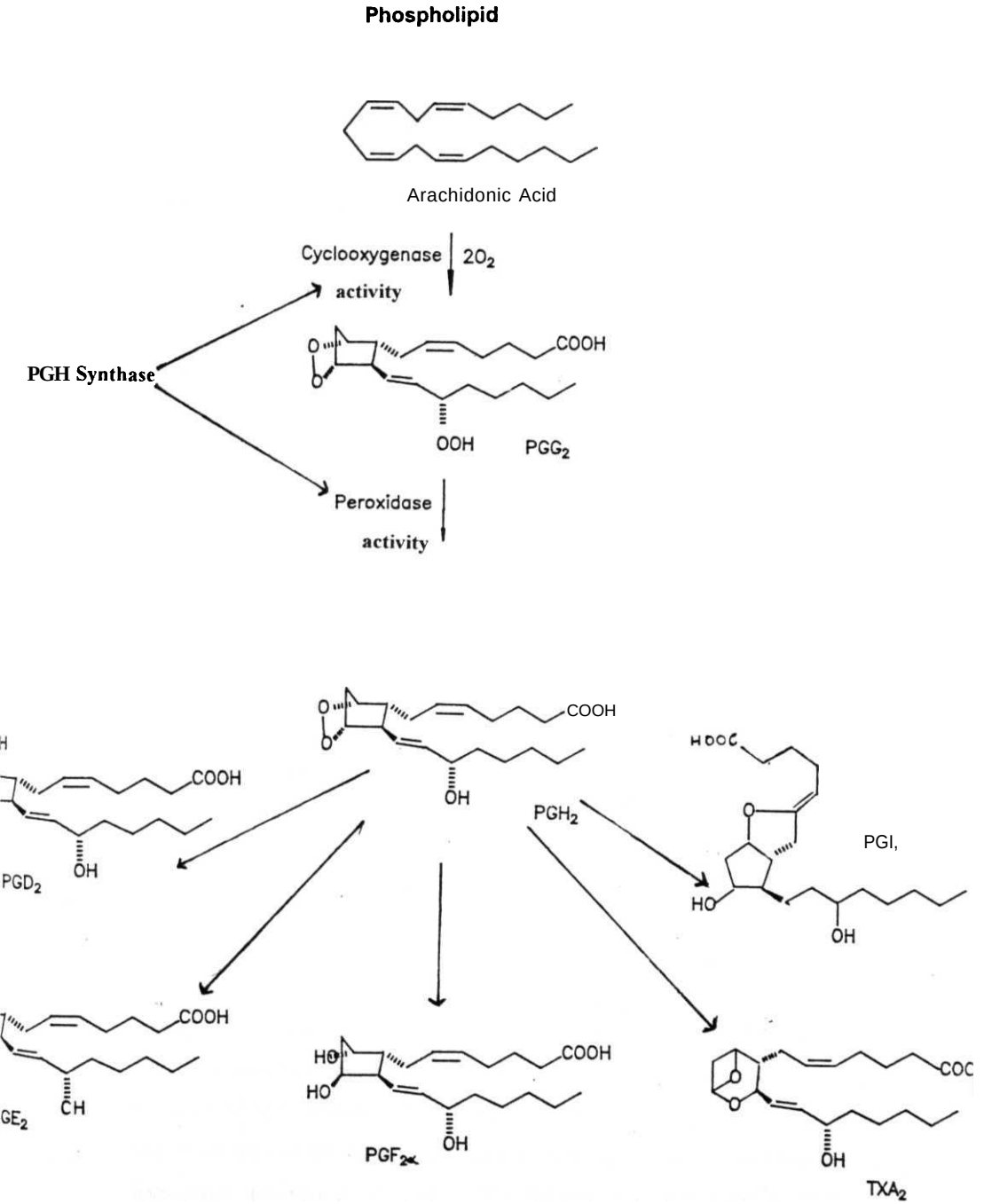


Fig.4: Biosynthetic pathways for the formation of PGs, PGI, and Tx from AA by cyclooxygenase pathway.

insertion into the 20 carbon polyunsaturated fatty acids, the plant lipoxygenases are classified as 15-lipoxygenase (E C. 1.13.11.12) and 5-lipoxygenases.

The primary product of oxygenation of the arachidonic acid *cis, cis* 1,4-pentadiene system is the 1-hydroxy *cis, trans*-2,4-pentadiene product In case of AA, the products formed are hydroperoxyeicosatetraenoic acids (HPETEs). These HPETEs can further be reduced or oxygenated to a variety of compounds. The reduction of peroxy group to an alcohol yields the corresponding hydroxyeicosatetraenoic acids (HETEs). On the other hand, lipoxygenation of an HPETE gives rise to either a dihydroperoxyeicosatetraenoic acid (diHPETE) or may lead to the formation of trihydroxyeicosatetraenoic aciid called as lipoxin (Rokach *et ah,* 1988). Further the HPETEs can be transformed to leukotrienes by dehydration of the peroxy group by adjacent lipoxygenation to yield an epoxy (oxido) fatty acid called leukotriene A_4 (LTA4, Borgeat, P. 1979).

The most studied lipoxygenases are 5-, 12- and 15-lipoxygenases because of their physiological significance. 5-, 12- and 15-lipoxygenases have been extensively studied in neutrophils (Goetzel *et ah,* 1979), leukocytes (Borgeat *et al.*, 1976, 1979) and platelets and reticulocytes (Bryant *et ah,* 1982 and Hamberg *et ah,* 1974). 15-lipoxygenases have also been studied in plants (Soybean) and host of other leguminoceae species such as Arachis hypogea and Pisum sativum and 5-lipoxygenases in potato tubers (Galliard, 1971) and tulip bulbs (Reddanna *et ah,* 1987. Fig.6).

1.3.4. Leukotrienes

These are the bioactive components of slow reacting substances of anaphylaxis (SRS-A) originally identified in leukocytes. Leukotrienes are mainly the products of 5-lipoxygenase pathway. The arachidonate 5-lipoxygenase catalyzes the formation of 5-HPETE from arachidonic acid 5-HPETE upon action by dehydrase forms an 5,6-epoxide by the loss of water (Borgeat and Samuelsson, 1979). The dehydrase was later identified to be 8-lipoxygenase activity that copurifies with 5-lipoxygenase and hence termed as dual lipoxygenase (Shimizu *et ah,* 1984). The 8-lipoxygenase acts stereospecifically in removing the 10 S hydrogen from 5-HPETE followed by a free radical movement which

takes place in the 1-5 carbon atoms resulting in loss of water and formation of an epoxide (Fig. 7).

The LTA4 formed is converted rapidly both by enzymatic and non-enzymatic reactions into various dioxygenated products. As expected an allylic epoxide LTA4 is highly unstable and undergoes facile nucleophilic substitution in acidic conditions and is stabilized only in alkaline conditions and upon binding to serum albumin (Borgeat *et al,* 1979). The acidic hydrolysis of LTA4 results in 5,12-diHETEs and 5,6-diHETEs with 2 isomers each i.e. 5S,12R-dihydroxy (E.E.E.Z)-6,8,10,14-eicosatetraenoic acid (6-trans LTB_4); 5S, 12S-dihydroxy (E.E.E.Z)-6,8,10,14-eicosatetraenoic acid (6-trans-12 epi LTB_4); 5S,6R-dihydroxy (E.Z.E.Z)-7,9,11,14-eicosatetraenoic acid, 5S,6S- (E.E.E.Z) dihydroxy-7,9,11,14-eicosatetraenoic acid (Borgeat *et al.,* 1979).

The enzymatic hydrolysis of LTA4, on the other hand, occurs in leukocytes (PMNLs) by LTA4 hydrolase, resulting in the formation of LTB_4 (5S,12R-diHETE) in (Z.E.E.Z) configuration (Borgeat *et al,* 1977). However the formation of 5S,12S diHETEs can also be seen in leukocytes by the action of 12-lipoxygenase on 5-HPETEs resulting in 5(S),12(S)-diHETEs with (E.Z.E.Z) configuration. The diHETEs derived from non enzymatic hydrolysis of LTA4 are devoid of biological activities, whereas the enzymatically formed diHETEs are known for their potent biological properties.

LTA4 transformation into the peptido-leukotrienes takes place by another specific enzyme LTC4 synthase which conjugates glutathione (GSH) to the epoxide forming LTC4 which is 5(S),6(R)- 5-hydroxy-6-S-glutathionyl (E.E.Z.Z)-7,9,11,14-eicosatetraenoic acid (Jakschik *et al.,* 1982). LTC4 upon proteolytic cleavage of the peptide moiety of glutathione by γ-glutamyl transpeptidase results in the formation of LTD4 (Orning *et al,* 1980). Dipeptidase acts on LTD4 resulting in further cleavage of the glutathione moiety forming LTE4, which upon further action by γ-glytamyltranspeptidase results in the formation of LTF_4. LTF_4 can also be formed from LTC_4 directly by the action of carboxy peptidase-A (Reddanna *et al,* 1988) LTC_4, LTD_4, LTE_4 and LTF_4 are called as the cysteinyl leukotrienes because of the presence of sulphite (cysteinyl) moiety originally identified in the components of SRS-A (Fig. 8)

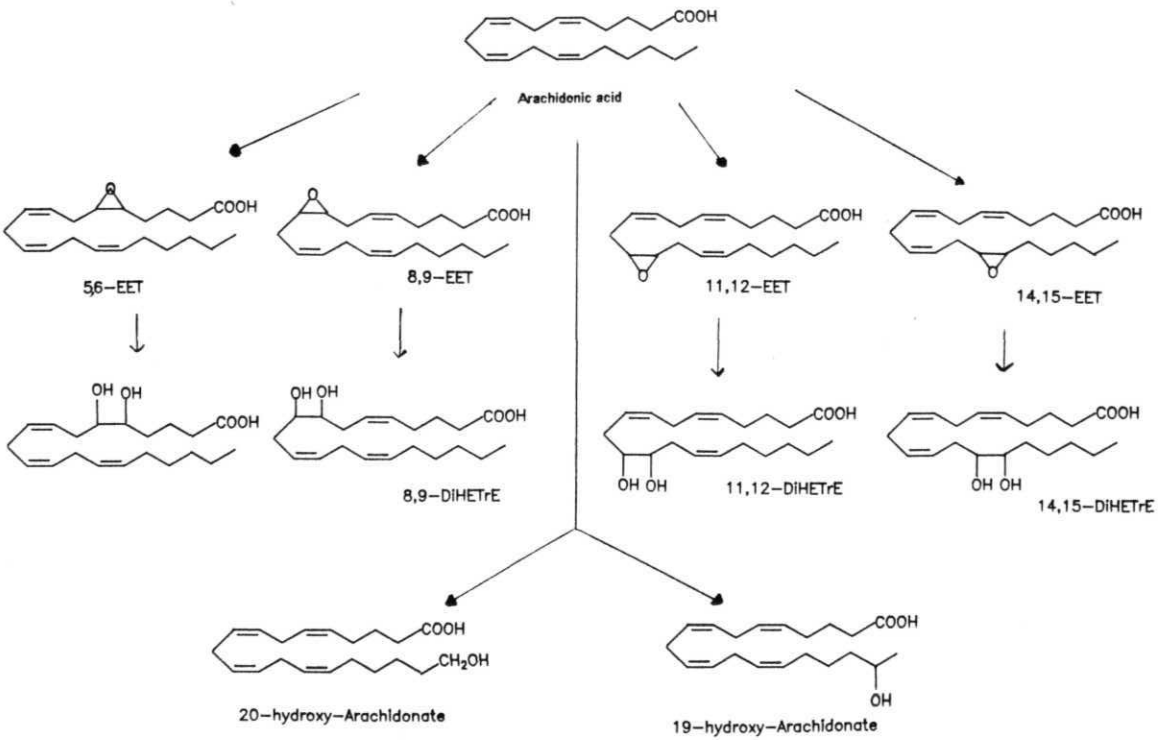


Fig.5: Epoxygenase pathway of the arachidonic acid.

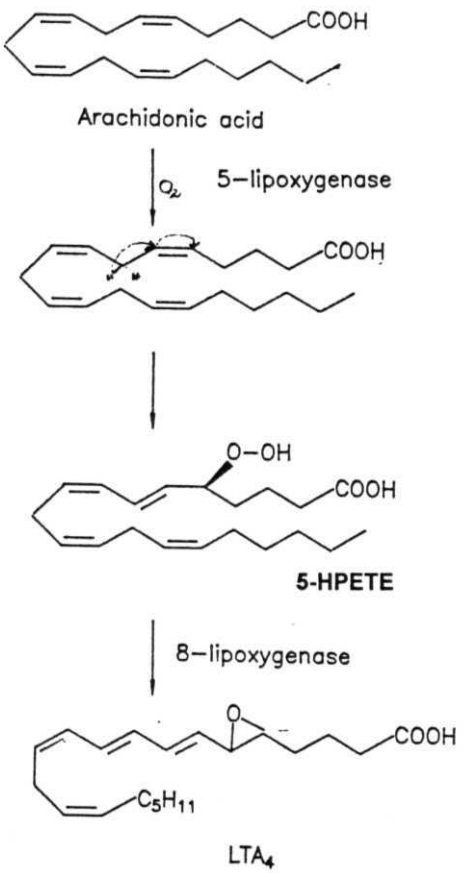


Fig.6: Biosynthetic transformation of AA to LTA_4 through 5-HPETE.

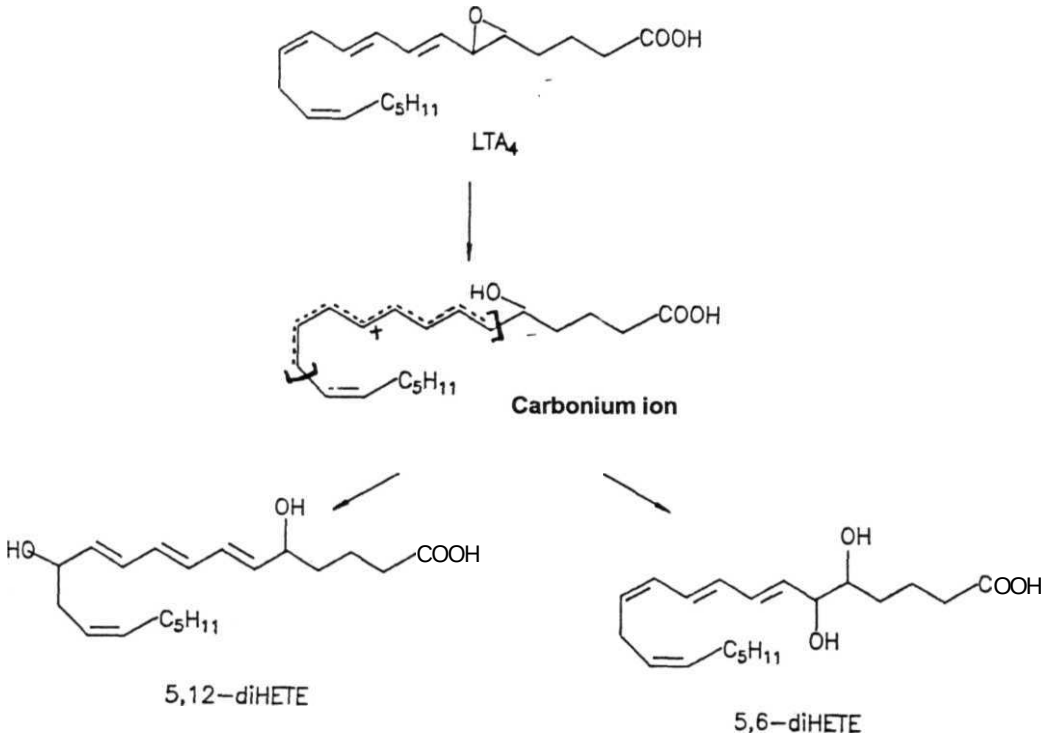


Fig.7: Formation of carbonium ion intermediate resulting from opening of 5,6-epoxide and formation of non-enzymatically hydrolyzed products.

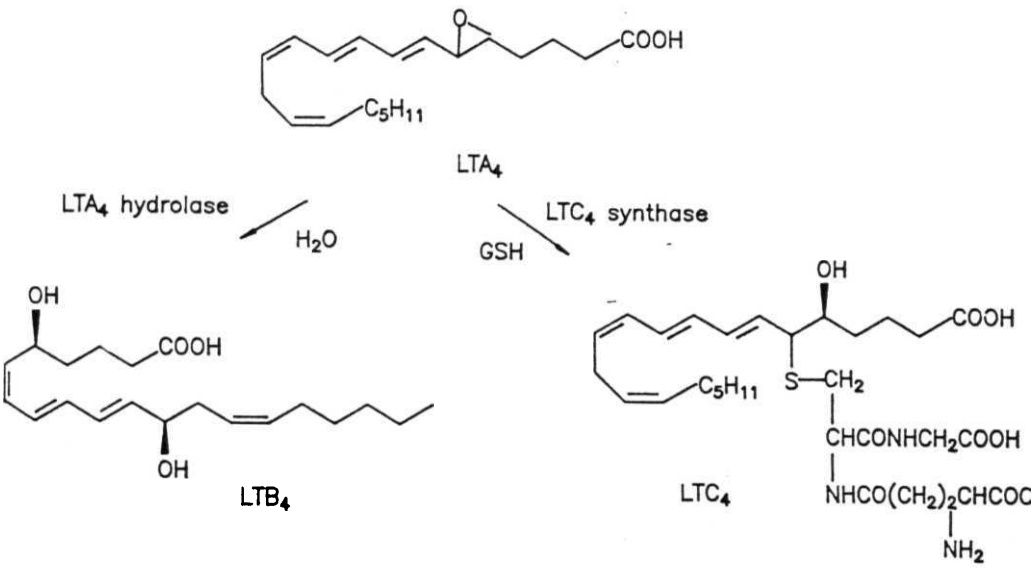


Fig.8: Enzymic transformation of LTA_4 to LTB_4 and LTC4.

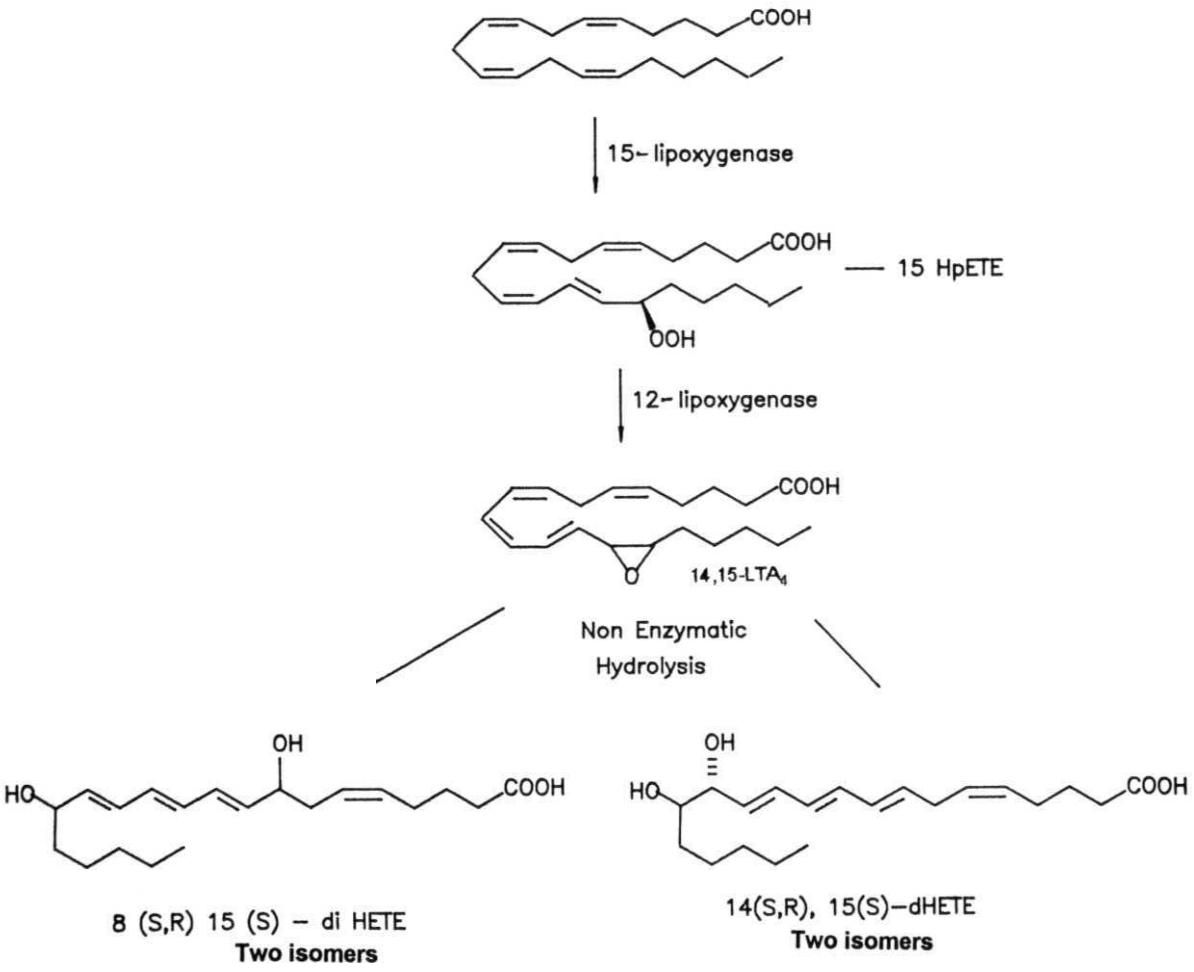


Fig.9: Biosynthetic transformation of 15-HPETE to 14,15-LTA$_4$

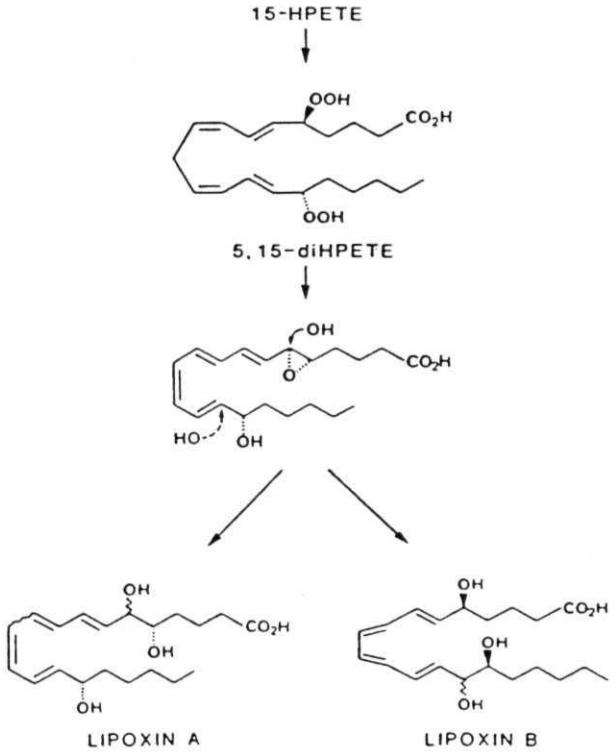


Fig.10:Mechanism of formation of lipoxins.

1.3.5. Other types of Leukotrienes

Apart from 5,6-series of leukotrienes, other types of leukotrienes have also been identified in both plant and animal systems. Potato lipoxygenase which has non-regiospecificity or multiregiospecificity was shown to produce 5,6-LTA_4, 8,9-LTA_4, 11,12-LTA_4, and 14,15-LTA_4 (Whelan *et al*, 1988). A number of studies have shown the operation of 14,15-LTA_4 pathway in animal systems where the leukotrienes synthesis was shown either by 15- or 12-lipoxygenase having the synthase activity with 15-HPETE as the substrate (Mass *et al.*, 1981 and Borgeat and Samuelsson, 1979, Shimizu *et al.*, 1984, Par Westlund *et al.*, 1988 Fig. 9)

Though the formation of leukotrienes is predictable and synthesized using biomimitic methods (Corey *et al.*, 1981), the operation of 8,9- and 11,12-LTA_4 pathways has not been identified in animal systems so far.

As mentioned earlier the compounds, in which the placement of 3-OH groups is in tandem are called as lipoxins This could be achieved by reacting 5,6-LTA_4 with 15-lipoxygenase which results in 3-OH groups at 5, 6 and 15th position resulting in complete tetraene epoxide This was infact observed in human neutrophils when incubated with 15-HPETE (Serhan *et al.*, 1984) The hydrolysis of the tetraene epoxides results in the formation of lipoxin A and lipoxin B. These types of lipoxins could also be generated by the action of 5-lipoxygenase on 14,15-LTA4 (Fig 10).

1.4. Biological effects of Eicosanoids

The term Eicosanoid' is used to denote the group of oxygenated twenty carbon fatty acids (Smith *et al.*, 1989, 1991) including prostanoids, leukotrienes, lipoxins, hydroperoxides etc.

Eicosanoids have been implicated in one way or other to every biological activity (Abbracchio *et al.*, 1986; Borell *et al.*, 1986; and Godfriend *et al.*, 1981). Prostaglandin E series contracts smooth muscles, lowers arterial blood pressure, inhibits gastric secretions, exerts cytoprotection, inhibits platelet aggregation and has many more functions. On the

other hand, prostaglandin F series decreases blood pressure, relaxes smooth muscles of human uterus, acts as a luteolytic hormone. PGD_2, PGI_2 and $PGF_{2\alpha}$ are also known to affect medullary blood flow in kidneys and the osmotic gradient in papilla apart from being the renal vasodilators. PGG_2, and PGH_2 are potent inducers of platelet aggregation, and the effects are mediated by transformation of PGH_2 to TxA_2 and TxB_2 by thromboxane synthase in platelets. Prostaglandins have also been implicated to have physiological roles in temperature regulation, brain circulation and possible effects on neuromodulation. On the other hand the oxygenated metabolites of lipoxygenase pathway namely HPETEs and HETEs, leukotrienes and lipoxins have been found to be associated with different aspects of inflammation. 12-HETE inhibits prostaglandin endoperoxide-induced aggregation (Aharoney *et al*, 1982). 12-HETE can also influence inflammatory responses particularly when PMNLs are involved (Maclouf *et al*, 1982; Wong *et al*, 1985). 5-HPETE the precursor for LTA4 produces various products with bewildering functions. 5-HETE on further oxygenation by 12-lipoxygenase produces 5,12-diHETEs which are potent chemo attractants (Marcus *et al*, 1982). 15-HETE and its further oxygenated products such as 8,15-diHETEs (Shak *et al.*, 1983) have chemokinetic effect with regulation of NK cell activity (Field *et al.*, 1988).

The active components of SRS-A namely LTC_4, LTD4 and LTE4 are responsible for contraction of respiratory tract smooth muscle, vasoconstriction and increased vascular permeability (Lewis *et al*, 1990). LTB4, on the otherhand has important role in inflammatory responses. LTB_4 also induces adherence of neutrophils to the vascular endothelium and stimulates migration of neutrophils into extravascular tissues (Ford Hutchinson, 1990). Signal transduction mechanisms are mediated by LTB4 and its analogues. Apart from this, LTB4 also induces Ca^{2+} influx, activation of phospholipase C and reorganization of cytoskeleton. Leukotrienes, like prostanoids function via a group of receptors coupled to G proteins (Naccache *et al*, 1989). The oxygenated products of epoxygenase pathway have prominent biological actions. They stimulate peptide hormone release from endocrine cells, inhibition of Na^+/K^+-ATPase, mobilization of microsomal

Ca^{2+} in neuronal cells and most importantly inhibition of cyclooxygenase activity (Smith *et al.*, 1991).

1.5.0 Eicosanoids and Reproduction

1.5.1 Cyclooxygenase pathway

The high concentration of prostaglandins present in seminal fluids led to their discovery. Certain prostaglandins were found to be atleast 10^6 fold higher than their counter-parts found in other biological fluids. It is therefore surprising that the physiological roles of prostaglandins in semen are still largely unknown Several actions in the male as well as the female reproductive tract have been implicated for the eicosanoids and inspite of our lack of knowledge of their mechanism of action they were found to be useful tools in obstetrics and gynaecology.

Prostaglandins of the F type contract and E type relax human uterine smooth muscles *in vitro*. Given intravenously they were found to elicit labor-like rhythmic contractions. These prostaglandins were further found to be detected in labor and high concentrations of these were found to be responsible for the hypercontractile nature found in dysmenorrhoea. Prostaglandins were found to be good abortifacient agents with little side effects. $PGF_{2\alpha}$ has been identified as the endogenous luteolytic hormone in many species and administration of this prostaglandin or certain similarly acting analogues is now used frequently to synchronize estr us in domestic animals and thereby to increase the efficiency of artificial insemination experiments.

1.5.2 Lipoxygenase pathway

1.5.2a Male reproduction

Testicular tissue has an unusually high concentration of PUFAs such as arachidonic acid (Johnson, 1970, Neil *et al.*, 1974) Hence, it is natural that these PUFAs might be playing an important role in testicular metabolism. The presence of lipoxygenase like enzyme was discovered by Shahin *et al.* (1978) in the microsomes of rat testis. Later studies from the same laboratory revealed the formation of 15-HPETE and 15-HETE through the

15-lipoxygenase pathway. ETYA, a general inhibitor of AA metabolism and NDGA, an inhibitor of lipoxygenase pathway were shown to inhibit hCG-induced testosterone secretion in rat Leydig cells with no effect of indomethacin (Hamberg, 1976). However the effects of these inhibitors on the testicular steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD) showed a different pattern (Sullivan *et al,* 1987). Indomethacin as well as NDGA both increased the activity level of 3β-HSD in rat testicular slices The effects were more pronounced when the testicular slices were incubated with indomethacin and NDGA. Also Vitamin E, a potent inhibitor of 5-lipoxygenase pathway (Reddanna *et al,* 1985) showed a similar trend on 3β-HSD activity level.

These findings suggest a regulatory role for lipoxygenase products in the biosynthesis of testosterone. 5-HPETE, the product of 5-lipoxygenase pathway enhanced testosterone production stimulated by LH in a dose dependent manner (Abayasekara *et al,* 1987), suggesting that 5-HPETE maybe enhancing steroidogenesis at a point subsequent to cyclic AMP production. LTA4 and LTB4 also were shown to mediate LH-stimulated testosterone production in rat testis Leydig cells (Abayasekara *et al,* 1987).

Besides the role of lipoxygenase products in the steroidogenesis of Leydig cells, they have also been implicated in hCG-mduced inflammatory type of reaction in the testis. Since LTB4 is secreted in Leydig cells (Cooke *et al,* 1986) and is also a potent chemotactic agent to various immune cells, it is proposed that LTB_4 may mediate hCG-induced inflammatory type of reactions.

There is not much information available on the role of lipoxygenase products in male accessory glands though the presence of 15-and 11-type of lipoxygenase activities has been reported (Hamberg *et al,* 1967). Although individual receptors for LTC_4, LTD4 and LTE4 sites were found in these tissues these could not compete with naturally occurring sulfidopeptide leukotrienes suggesting the operation of a different pathway (Krilis *et al.*, 1983).

1.5.3 Female reproduction

1.5.3a Ovary

It is reported that the products of cyclooxygenase pathway especially prostaglandins are obligatory mediators in several events of female reproduction (Goldberg *et ai,* 1975 and Green *et ai,* 1982). The information on the involvement of lipoxygenase metabolites, on the other hand, is limited and is of recent origin. Ovarian follicles from the proestrus rats were shown to transform arachidonic acid to HETEs via the lipoxygenase pathway (Reich *et ai,* 1983). AA metabolism was also reported in human granulosa cells **(Fledman** *et al,* 1986). Their later studies have shown that NDGA blocked ovulation and inhibited hCG-induced ovarian collagenolysis (Reich *et* ***al.,*** 1985). These results suggest the possible involvement of lipoxygenase products in the process of ovulation along with the free radicals generated by the leukotrienes in LH-induced ovulation in mammals **(Laloraya *et*** *ai,* **1988)**

Leukotrienes were also shown to exert pronounced effects on *in vitro* **motility** of hen oviducts (Wechung *et* ***ai,*** 1987). LTC_4 and LTD_4 stimulated the motility of all oviducal parts *in vitro*. LTD4 in the conscious animal induced a short-lasting increase in **uterine** myoelic activity.

1.5.3b **Metabolism of arachidonic acid in uterus:** ***An*** *undiscovered saga.*

Ironically six decades after the discovery of human semen factor and essential fatty acids, the research of **polyunsaturated** fatty acids have shifted from reproductive system to asthma. The high concentration of prostaglandins found in seminal fluid is atleast 10^6 **(Samuelsson** *et ai,* 1975) folds higher than other biological fluids and yet their physiological **role** is unknown, though they have been continuously used as pharmacological tools in obstetrics and gynaecology.

Despite the recognition of the importance of **PUFAs** in uterus and their presence in all age groups, the enzymes that catalyze these PUFAs to eicosanoids were not found. Espey in 1980s hypothesized ovulation to be similar to **inflammation** as both the processes are involved in using eicosanoids (Bonney *et ai,* 1992; Espey, 1980; Roberts *et* ***ai,*** 1976). Prostaglandin synthase was **identified** in late **80's** in female reproductive tract

(Kelly *et al,* 1981). Later prostaglandin and its analogues were thoroughly investigated in both male and female reproductive systems. But unfortunately the studies on uterine lipoxygenase pathway were limited.

The discovery of similarities between lung, skin and uterus ultrastructurally and their resemblances in arachidonic acid metabolism in their mast cells have opened up the lipoxygenase pathway in uterus (Massey *et al,* 1991). Lipoxygenase products were identified in endometrium and were found to be responsible for myometrial contractions (Demers *et al,* 1984, Carraher *et al,* 1983). The lipoxygenase and cyclooxygenase activities were found to be inversely related in endometrium (Pakarasi *et al,* 1985). The 12-HETE was found to be present after labor in uterus, placenta and amnion suggesting that it could traverse or equally be synthesized in, fetoplacental unit whereas 15-HETE was found in chorion only (Mitchell *et al,* 1987). These monohydroperoxides were also found to summon white blood corpuscles as first line of defense mechanism in uterus (Walsh, 1991). Though it is still unclear whether the conversion of arachidonic acid into lipoxygenase products is responsible for cervical ripening (Radestad *et al,* 1990, Heidvall *et al,* 1992), it is clear that lipoxygenase products have a major role in uterine physiology (Cantabrana *et al,* 1991). It was found that when lipoxygenase pathway was inhibited by NDGA, luteolysis was found to be delayed (Hamilton *et al,* 1990) suggesting their importance in normal uterine physiology.

The enzyme that catalyzes the conversion of arachidonic acid to 12-HETE or 15-HETE in uterus was not characterized fully though 12-lipoxygenase activity was found in human uterine cervix (Flatman *et al.*, 1986) and 15-lipoxygenase activity was found to be induced after pregnancy (Li *et al,* 1992). Further the enzyme and its products were found to be localized in the smooth muscles and blood vessels of myometrium by immuno histochemistry and were even found to be present in the nuclear chromatin suggesting genomic as well as non-genomic actions for 15-lipoxygenase and its products. None of the studies, however, concentrated on the purif.cation and characterization of lipoxygenase in uterus.

Apart from this, on leukotriene front, the receptors of LTC_4 were found in uterine smooth muscles and were found to be responsible for uterine contractions (Cheng *et al*, 1985). Further progesterone was found to be an excellent inducing agent for leukotriene synthesis where as estradiol was found to induce prostaglandin production suggesting that prostaglandins and leukotrienes act in coordination with uterine hormones in maintaining the complex uterine physiology (Hahn *et al*, 1985 and Reddanna *et al*, 1990).

A recent review article by Boone (Boone *et al*, 1993) summarizes the arachidonic acid and eicosanoid involvement in functional dynamics of follicular development, ovulation, corpus luteum formation, maintenance of pregnancy i.e. decidualization, trophoblast invasion and implantation.

Scope of the Present Study

The complex physiology associated with the uterine tissue was found to act in synchronization with the products of arachidonic acid of either the cyclooxygenase pathway or the lipoxygenase pathway Of-late the growing evidence suggests that the lipoxygenase catalyzed arachidonic acid metabolites to be responsible for various intra uterine functions, thereby challenging the dogma that PGs are the universal mediators of labor. However these lipoxygenase products and their biological actions have been largely dealt with using inhibitor studies, due to various constraints involved in isolating lipoxygenase and in understanding the lipoxygenation mechanism.

Flatman *et al.* (1986) have reported the operation of 12-lipoxygenase for the first time and its partial purification from human uterine cervix. Li *et al.* (1992) have shown that catalytically active 15-lipoxygenase to be expressed in human uterus after pregnancy.

Apart from these studies no structural and functional aspects of the enzyme were taken up, though the presence of peptido leukotrienes and their receptors were identified in uterine tissue and several physiological functions were attributed to LTC4. No tfforts were made to identify and characterize leukotrienes and their catalyzing enzymes in uterine tissues.

In the light of these lacunae, an attempt is made in the present study to isolate and purify lipoxygenase from sheep uterus and to analyze its involvement in leukotriene biosynthesis. Also the PUFA composition and the endogenous metabolites of lipoxygenase pathway at different phases of sexual cycle were analyzed to understand the physiological role of lipoxygenase pathway in uterus

The specific objectives are:

A: Purification and structural characterization of sheep uterus lipoxygenase

1. To purify the lipoxygenase present in sheep uterus to homogeneity.

2. To study the distinct physical properties of the purified lipoxygenase like mol.wt, isoelectric point etc.

3. To characterize the enzyme by determining the pH dependence, substrate specificity and its kinetic properties.

B: Functional organization of uterine lipoxygenase

1. To correlate the structure and functional properties of sheep uterus lipoxygenase with other known lipoxygenases.

2. To analyze the product profile of the uterine lipoxygenase.

3. To understand the role of uterine lipoxygenase in leukotriene biosynthesis.

C: Physiological role of lipoxygenases **in the uterus**

1. To measure the activity levels of lipoxygenase and PUFA composition of uteri at different phases of estrous cycle.

2. To neasure the endogenous levels of different eicosanoids of lipoxygenase pathway at different phases of estrous cycle.

3 To analyze the peptido-leukotriene biosynthetic potential of sheep uterus.

Methodology

2.1.0.0. Materials

Arachidonic acid (AA), γ-linolenic acid (GLA), docosahexaenoic acid (DHA), di homo-γ-linolenic acid (DHGLA), linoleic acid (LA), eicosatetraynoic acid (ETYA), O-dianisidine, glutathione (GSH), ethylene diamine tetra acetic acid (EDTA), ethylene glycol tetra acetic acid (EGTA), Freund's complete and incomplete adjuvants, horse radish peroxidase (HRP), nordihydroguaiaretic acid (NDGA) and indomethacin were purchased from Sigma Chemical Company, St. Louis, MO, USA

Ion exchangers (anionic and cat ionic) were obtained from Whatman England Inc.

Gel filtration materials such as Sephadex G-100 and G-50 were from Phramacia, Uppsala, Sweden.

Nylon membranes (NYTRAN) for immunoblotting were from Shleicher and Schuell., USA.

Pentamethyl piperidine (PMP), trifluoromethane sulfonic acid anhydride (TFMSA), triethylamine (TEA), bis (Trimethylsilyl) trifluoro acetamide (BSTFA), boron trifluoride (BF_3) were purchased from Aldrich Chemical Company , MO,USA.

HPLC solvents like hexane, tetrahydrofuran (THF), methylene chloride (CH_2Cl_2), methanol, propane 2-ol, and acetic acid were procured from S.D Fine Chemicals India Ltd.,and Spectrochem, India Ltd.

All other chemicals which have not been mentioned here were procured from local chemical companies and were of high quality grade.

Potatoes (white skin/ brown skin) were obtained from local vegetable markets. Red skin varieties were from Potato Research Station, Simla, India.

Sheep uteri were collected from local slaughter houses immediately after sacrificing the sheep.

2.2.0.0. General methodology

2.2.1.0. Lipoxygenase assay

Lipoxygenase activity was measured polarographically in a closed reaction vessel using Clark's oxygen electrode connected to an Yellow Springs oxygen monitor of model YSI-3500. The assay was initiated by the addition of substrate which could be any of the unsaturated fatty acids under discussion and the consumption of oxygen was monitored at room (27°C± 3°C) temperature The typical assay mixture of 3mL consists of reaction buffer (150 mM KH_2PO_4 of pH 6 3 for Potato lipoxygenase or citrate-phosphate pH 5.5 for uterine lipoxygenase), 10-50 µg enzyme protein and 10 uL of ethanolic substrate solution giving a final concentration of 133 µM for potato lipoxygenase and 250 µM for uterine lipoxygenase. Typical polarographic recording of lipoxygenase activity is presented in Fig 11. The maximal slope of the O_2 curve is used for calculation of the enzyme activity as per the following formula (Berkely and Galliard, 1976):

$$\text{umoles of } O_2 \text{ consumed/min} \times \text{mL} = \frac{(Vol_T) \times (O_2/mL) \times \text{slope/min}}{\text{Sensitivity} \times Vol_{Enz}}$$

Where

Vol_T = Total volume of the reaction mixture

O_2/mL =Conc. of O_2 under standard atmospheric conditions (0.23 µmoles/min)

slope/min =Maximal slope generated/min

Vol_{enz} = Volume of the enzyme taken for the reaction mixture in mL.

For pH dependency of the lipoxygenase the buffers employed were from citrate-phosphate for pH range 3.0 to 6.0, 100 mM KH_2PO_4 for pH 6.0 to 7.5 and 50 mM Tris-HC1 for pH 7.5 to 9.0

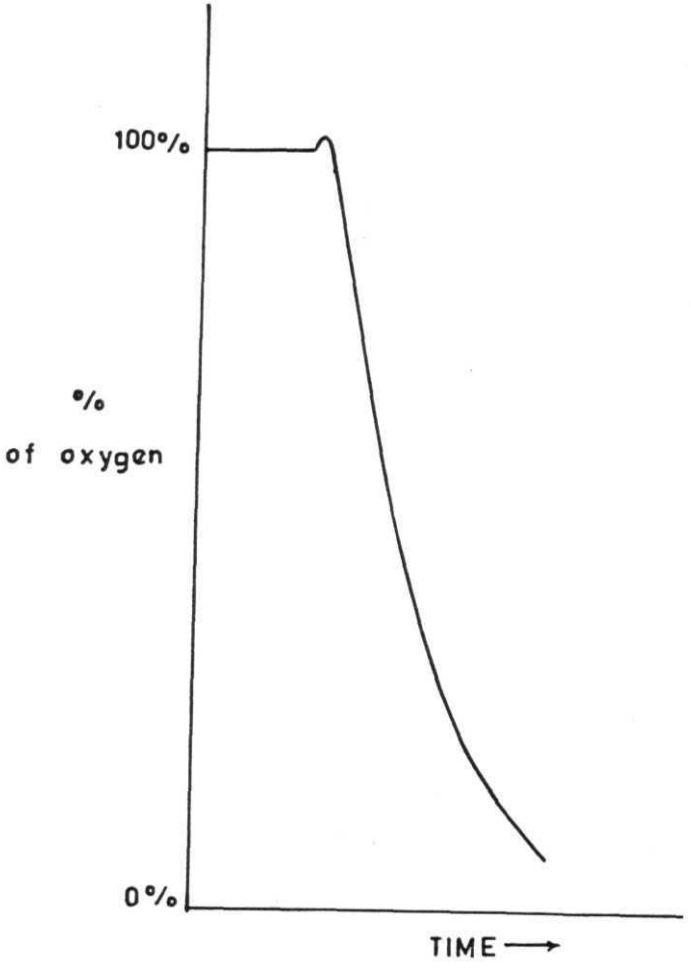


Fig. 11: Typical oxygraphic tracing of assay of lipoxygenase activity. Activity was calculated basing on the rate of O_2 consumption.

To study the substrate dependent kinetics of the lipoxygenase under investigation the substrate concentrations employed were from 0 μM to 400 uM of arachidonic acid For substrate specificity studies, different unsaturated fatty acids and HPETEs were used at constant 250 μM concentration in the reaction medium and the oxygen consumption was measured.

2.2.2.0. Protein determination

Protein concentration in all the chromatographic fractions were estimated by spectrophotometric methods of Warburg and Christian (1941). The UV absorption at 260nm and 280nm was measured on Beckman DU-64 spectrophotometer using 1cm path length quartz cuvette. The concentration of the protein was calculated using the following formula:

mg protein/mL = {(A-280 X 1.55) - (A-260 X 0.76)}n

where n is the dilution factor.

The protein concentration at various stages of purification was determined by the modified method of Lowry where the alkali concentration was increased and the concentration of folin-phenol was reduced to get a better color development specifically at lower concentrations of the protein (Enhanced alkali method-Christa.M.Stoscheck, 1990)

2.2.3.0. Definition of enzyme unit

One unit of the lipoxygenase activity was defined as one umole of O_2 consumed per minute and specific activity as units per mg protein.

2.3.0.0. Electrophoresis: **SDS-PAGE**

Polyacrylamide gel electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate (SDS) according to the method of Lammeli (1970).

Samples containing 1-2 μg of protein in 10 uL of sample buffer consisting of 1% SDS, 5% 2-mercaptoethanol, 0.003% bromophenol blue and 10% sucrose in 0.063 M Tris-HCl pH 6.8 were boiled at 100°C for 3 min to separate the polypeptide chains and

loaded on 4.5 % stacking gel cast with 0.125 M Tris-HCl buffer pH 6.8 and the separating gel consisting of 10% polyacrylamide and bisacrylamide in a ratio of 28:0.74 casted in 0.375 M Tris-HCl, pH 8.8. The proteins were subjected to a current of 120V for 5 hours for optimum separation of the peptide bands in an electrode buffer consisting of 0.25 M Tris, 0.192 M glycine and 0.1% SDS at pH 8.5.

After the completion of the electrophoresis the gel was fixed in 7.5% acetic acid solution and later developed with silver nitrate (Oakley *et al.*, 1980) or coomassie blue staining Standard mol.wt markers of Sigma were simultaneously run and the mol.wt of the interested peptide was calculated using UVP-2000 gel documentation software program

2.3.1.0. Native PAGE

To confirm the native structure of the purified proteins they were electrophoresed on poly-acrylamide gel under non-denaturing conditions (native PAGE). The separating gel was cast on vertical slabs The acrylamide to bisacrylamide ratio was 28:0.74 and were polymerized in Tris-HCl pH 8.8 buffer to give a final acrylamide concentration of 8% in the gel. The stacking gel was polymerized with a final concentration of 4.5% acrylamide with ascorbic acid by photo polymerization instead of the usual peroxy radical polymerization. The protein sample was dissolved in sample buffer consisting of 10% sucrose and 0.003% bromophenol blue in Tris-HCl buffer pH 6.8 and loaded onto the stacking gel. The proteins were separated using 60V at 4°C. After the completion of the run, gel was removed and stained with coomassie blue in methanol and destained with methanol: wateracetic acid (75:20:5). The native mol.wt markers from Sigma (BSA, egg albumin, carbonic anhydrase, α-lactalbumin) were run along with the desired protein simultaneously and the mol.wt was calculated (Bryan, 1977).

2.4.0.0. **Immunotechniques**

The purified lipoxygenase from sheep uterus was concentrated to 400 μg/mL and 0.5 mL of this protein solution was thoroughly emulsified with Freund's complete adjuvant and injected at multiple sites on the dorsal part of New Zealand white male rabbits of 3

months old This was followed by 3 booster doses each of 100 µg protein emulsified with equal volumes of the Freund's incomplete adjuvant for three weeks after the initial fortnight rest Blood was collected from the ear vein 4-7 days after the last injection and was allowed to clot overnight at 4°C. The serum was obtained by centrifuging the blood clot at 2000 X g for 5 min and stored in aliquots at 4°C. The titre of the antibodies (Ab) produced was checked by Ouchterlony double diffusion method in 1% agarose in phosphate buffered saline either on the glass plate or on the petri plates. This serum was used as the primary Ab source for sheep uterine lipoxygenase for further studies.

2.4.0.1. Immunoblotting (Western blots)

Immunoblot analysis was carried out on nylon membranes according to the published procedures of Toubin *et al.* (1975). The cytosolic proteins of sheep uterus separated on SDS-PAGE or the Native PAGE was transferred onto the nylon membranes by the semi dry blotting protocol of LKB. The gels were initially soaked in Buffer A consisting of 0.045 M Tris, 0.192 M glycine and 0.1% SDS in 20% methanolic water. The separated peptides/proteins were transferred with a current of 0.8 mA/cm for 4 hours. The nylon membrane was then rinsed in solution B consisting of 10 mM Tris, 150 mM NaCl and 0.5% Tween-20 for 15 min. Further the nylon membrane was blocked with 5% skimmed milk in solution B for 120 min. The primary Ab was then added to 5% skimmed milk and was allowed to react for 120 min or more depending on the size of the blot and titre of the Ab. The membrane was then washed thoroughly for 10 min X 6 times with solution B, so as to remove non-specific binding of the Ab with the membrane The secondary Ab (commercially procured, which has been produced for the heavy chain of the rabbit IgG and coupled to horse radish peroxidase) was allowed to bind to the primary Ab in 5% skimmed milk in solution B The nylon membrane was again washed with solution B for 10 min X 6 times with solution B and finally transferred into 10 mM Tris containing 150 mM NaCl solution (Sol C).

The membrane was developed with 4-chloro-1-naphthol (4C1N) and freshly prepared H_2O_2 (30%) till blue color appears on the membrane. The reaction was stopped by changing the membrane from solution C to distilled water and later dried for photography.

2.5.0.0. Isoelectric focussing

The Pi of the proteins was determined both by electrofocussing as well as chromatofocussing methods.

2.5.1.0 Electrofocussing

Iso electric focussing gels were prepared in glass tubes according to the published procedures of Farrell (1975), with pH 3-10 range of 2% ampholines. The gel tubes were then pre-electrofocussed using 0 01 M H_3PO_4 solution in the lower tank and 0.02 M NaOH in the upper tank with 300 V for 4 hours and after loading the protein samples the voltage was increased to 600 V for 4 hours and 800 V for 4 hours. The gels were removed from the glass tubing and stained with 0.1% coomassie blue in 50% trichloroacetic acid (TCA) for 30min and destained with 7.5% acetic acid with several changes till clear protein bands appear One reference gel tube which was run without protein sample was cut into 0.5 cms length and each was left overnight in 1 mL of double distilled water in clean test tubes. The pH of the test tubes were checked and calibrated. With this as reference the Pi of the sheep uterine lipoxygenase was calculated.

2.5.2.0. **Chromatofocussing**

Chromatofocussing was carried on HPLC using anion exchange column. The purified protein was loaded onto the anion exchanger column (PA-DEAE) equilibrated with 50 mM sodium acetate and the protein was eluted with 50 mM acetic acid on a linear pH gradient The eluted protein was collected and checked for activity. The retention time of the peak with activity was noted and the elution pH was taken as the Pi of the lipoxygenase under discussion.

2.6.1.0 Morphological identification of estrous stages in uterus

Sheep uteri were classified into four stages with the help of Dr.Seshagiri Rao, Vet. college, Andhra Pradesh Agricultural University (APAU): The sizes of the uteri and ovarian follicules were taken into consideration for determining the stage of the animal

2.6.1.1. Nulliparous stage : The uteri from mature sheep without previous conception were categorized as nulliparous uteri This was determined based on the size of the uteri

2.6.1.2. Follicular stage : Based on the size of the uterine horns and the absence of corpus luteum in the ovary (seen on the follicles as dark spots), the uteri were classified into this category.

2.6.1.3. Luteal stage : The follicles at this stage had black spots with yellowish background indicating the formation of corpus luteum in the ovary.

2.6.1.4. Early and late pregnancy stages : Based on the size of the embryo present in the uterine horns, these stages were identified.

2.7.0.0 Purification of Sheep uterine lipoxygenase

2.7.1.0. Preparation of crude extract

Non pregnant sheep uterus (28-30 g) was homogenized to 20% in 100 mM KH_2PO_4 pH 7.4 buffer containing 1 mM ascorbic acid, 1 mM EDTA, 1 mM EGTA, 2 mM calcium chloride, 2 mM magnesium chloride, 1 mM KG, 10 mM sodium meta bisulfite and 250 mM sucrose. The homogenate was filtered through a two layered cheese cloth and treated with antiproteases, 1 mM PMSF and 1 uM pepstatin A. The crude homogenate was spun at 10,000 X g for 20 min to remove unbroken cells, nuclei and other cell debris. This was further centrifuged at 105,000 X g for 90 min in order to remove the microsomal and ribosomal components of the cell systems and the clear cytosol was used for the enzyme assay and for further purification.

2.7.2.0. Ion-exchange chromatography

DE-52 (15 g) and CM-52 (15 g) were suspended separately in 0.5 M KH_2PO_4 pH 7.0 buffer and the matrix was allowed to settle The supernatant* from the ion-exchangers were decanted carefully without disturbing the matrix. The matrix was further washed in decreasing concentrations of phosphate buffer till the supernatant was particle free in both the ionic media The gels were then prepared to slurry in 100 mM KH_2PO_4 pH 7.0 buffer consisting of 250 mM sucrose (equilibration buffer) and packed in glass columns (6 X 1 inch) separately. The columns were then connected in series and equilibrated with the same buffer as above in cold room at 4°C.

The paniculate free cytosol was passed through the ion exchange columns in series and the flow through was collected. This was checked for activity and protein. The column was washed with 3 vols of buffer (until no protein was eluted) and then eluted with 1 vol of buffer containing 0.4 M NaCl. The fractions were checked for activity and protein. Since most of the activity came in the flow through, washing and elution of the column was discontinued in the later experiments. In the above process only the non ionic proteins elute out in the flow through without being bound. This flow through was further processed for the enzyme.

2.7.3.0. Ammonium sulfate fractionation

The non-ionic sheep uterine cytosol was fractionated by salting out the proteins with ammonium sulfate first upto 0-30% followed by 30-70% and upto 70-100%. The lipoxygenase activity was found to salt out at 70-100% saturation, which was pelleted down by centrifugation. The pellet rich in lipoxygenase was desalted out by dialysis with equilibration buffer. The dialysate was concentrated using reverse osmosis created by sucrose or polyethylene glycol (PEG-5000). The concentrate was centrifuged and later used for further purification.

2.7.4.0. Gel filtration

The 70-100% ammonium sulfate extract was loaded onto a gel filtration column which was packed with 15 g of sephadex G-100, swollen in 50 mM KH_2PO_4. The swollen beads were carefully packed into a slender glass column of 36 X 0.5 inch dimensions. The flow through was collected in 2 mL fractions with approximately 1 mL/3min flow rate. The fractions were checked for activity and protein. The active fractions were pooled and used for further purification.

2.7.5.0. HPLC anion exchange chromatography

The lipoxygenase sample obtained from the previous step was further separated on HPLC using PA-DEAE column (12 X 0.46 cms, 10μ). The column was pre-equilibrated with 50 mM sodium acetate and the protein was loaded onto the column through a Rheodyne injector. The protein was eluted by a linear pH gradient developed with 0-100% acetic acid. The protein peaks were collected individually and checked for activity and protein. The peak(s) with activity were used for protein characterization and to understand the structural and functional organization of the enzyme.

2.7.6.0.RP-HPLC analysis

The HPLC anion exchange purified sheep uterine lipoxygenase was checked for its purity on RP-HPLC analysis by the modified method of Ostlund et al. (1987). The lipoxygenase was loaded on to waters ODS column (25 X 0.46 cms). The solvents employed were 0.1% trifluoroacetic acid (vol/vol) in 35% acetonitrile (solvent A) and 0.1% trifluoroacetic acid (vol/vol) in 85% acetonitrile (solvent B) The lipoxygenase was injected at 100% solvent A, and eluted using a linear gradient from 0% to 30% solvent B over 30 min and later to 100% of B, over 15 min. The effluent was continuously monitored at 280 nm for the detection of the protein.

2.7.7.0. Activity staining

2.7.7.1. Incubation of the **gel with substrate solution**

Active lipoxygenase was stained according to the published procedures of Heydick *et ed.* (1985). The purified uterine lipoxygenase was loaded onto 8% native-PAGE and separated under cold conditions. On completion of the electrophoresis the gel was rinsed immediately with distilled water and soaked in 0.1 M KH_2PO_4, pH 7.0 buffer for 15 min at 4°C. The gel was then incubated with substrate solution which was prepared by mixing one volume of 0.53 M substrate (arachidonic acid) with equal volumes of methanol and 0.53 M KOH to give potassium arachidonate. The substrate solution was then added to 100 vols of the fresh buffer to give a final concentration of 2.5 mM substrate in the solution along with 0.1% sodium cholate. The gel was incubated for exactly 15 min at 4°C. Longer incubations and higher temperatures were avoided to eliminate the formation of secondary metabolites of hydroperoxides. After incubation, the gel was briefly rinsed with the same buffer as before and stained with O-dianisidine-HCl in pH 7.0 potassium phosphate buffer.

2.7.7.2. Preparation of the staining solution and gel staining

O-dianisidine (0.1 g) was dissolved in ether and dry HC1 gas generated by reacting sodium chloride with sulfuric acid was bubbled through the ethereal solution. In this process O-dianisidine on conjugation with HC1 becomes O-dianisidine-HCl and precipitates from the ethereal solution The ether extract was evaporated by flash evaporator and the precipitate was dissolved in ethanol. To this solution 90 vol of hot 0.1 M KH_2PO_4 of pH 6.8 buffer was added. Upon dissolving the O-dianisidine-HCl in aqueous solution, a brown color was developed The solution was cooled slowly and used as the activity staining solution for the lipoxygenase separated on native PAGE.

The staining was stopped by transferring the gel from the staining solution to distilled water and washing repeatedly to clear all the precipitate present above the gel. The color developed by this method was found to be stable for months and the gels can be dried and preserved with no loss of the band intensity till 3 months.

2.8.0.0. LTA_4 synthase activity

The LTA4 synthase activity of the uterine lipoxygenase was determined by reacting the enzyme with 15-HPETE and analyzing the products on reverse phase HPLC using Water's C-18 analytical column (25 X 0.4cms). The solvent system employed was methanol : water : acetic acid (65 : 35 : 0.1), pH adjusted to 6.8 with ammonium hydroxide. The flow rate was 1 mL/min. The effluent was monitored for absorbance at 268 nm for the first 20 min and at 235 nm for the rest of the period. The 14,15-LTA_4 hydrolysis products were identified based on their characteristic ultraviolet spectra and co-chromatography with standard 14,15-LTA_4 hydrolyzed products and GC-MS analysis.

2.9.0.0. GC-MS analysis

All the peaks showing diene and triene spectra were concentrated, evaporated, redissolved in ether and methylated with diazomethane The methyl esters were trimethylsilyated using Supelco Inc. (Bellefonte, PA) derivatization kit. The derivatized compounds were analyzed on Hewlett Packard 5890 series II gas chromatogram coupled to Hewlett Packard 5971 mass spectrometer. The compounds were separated on 15 m fused silica column of 0.2 mm internal diameter with 0.20 μm film thickness. The temperature program employed for the separation of the compounds was 70°C for the initial 3 min and later the temperature was raised to 240°C at 10°C per min. The carrier gas was helium which was at a constant flow of 2 mL per min.

3.0.0.0. Preparation of eicosanoid standards of lipoxygenase pathway

3.1.0.0. Purification **of potato** lipoxygenase

Lipoxygenase from potato tubers was purified for use in the preparation of arachidonic acid hydroperoxides, which were employed as standards. Lipoxygenase from potato tubers was essentially purified as per the method described by Reddanna *et al.* (1990) with slight modifications. Potato lipoxygenase was purified mostly from potatoes obtained from the local markets i.e of white skin variety.

3.1.1.0. Preparation of crude extract

250 g of potatoes were homogenized using the wearing blender at 4°C to obtain 20% crude extract in 100 mM potassium phosphate buffer pH 6.3 containing 2 mM ascorbic acid, 2 mM EDTA and 5 mM sodium metabisulfite. The homogenate was passed through 2 layers of cheese cloth and the extract was spun at 10,000 X g for 20 min at 4°C to remove the cell debris and unbroken cells The clear supernatant was used as the crude enzyme for estimation of lipoxygenase activity.

3.1.2.0. Ammonium sulfate fractionation

After checking the activity of the lipoxygenase and confirming it to have more than 25 units/g (lipoxygenase activity as high as 32 units/g was observed in other preparations) the tissue extract was processed for further purification.

The homogenate was fractionated by using ammonium sulfate, first from 0-15% and later from 15-45% by the slow addition of the salt to the homogenate under continuous stirring at 4°C taking care that no froth was being formed. The salted out proteins were centrifuged at 10,000 X g for 20 min at 4°C and the 15-45% pellet was dissolved in 50 mM KH_2PO_4 pH 6.3 buffer. The concentrate was then dialyzed against 200 volumes of the same buffer for 4 hours and the dialysate was further spun at 10,000 X g for 20 min. The supernatant of the dialysate was used for further purification.

3.1.3.0. Anion exchange chromatography

50 g of Whatman's DE-52 was suspended in 500 mL of 0.5 M KH_2PO_4 pH 6.3 buffer and degassed for 30 min (the gel which settles down was then decanted of the supernatant solution with minimum disturbance to the already settled gel). The gel was then resuspended in 100 mL of 50 mM KH_2PO_4 pH 6.3 buffer called as the **"enzyme buffer".** The gel was made into slurry in the same buffer and poured into glass column (10 X 1 inch) and allowed to equilibrate with the enzyme buffer. After equilibration the sample from the previous step was loaded onto the column. The column was washed with the equilibration buffer continuously till the absorbance of the flow through was below 0.1

A at 280 nm The bound protein was then eluted with either a salt gradient of KCl (0- 0.4 M) or a pH gradient developed by 50 mM sodium phosphate and 50 mM acetic acid The eluted proteins were collected in 2 mL fractions at a flow rate of 1 mL/min. All the fractions collected were checked for activity and protein. The active fractions were pooled and dialyzed against the enzyme buffer and concentrated using reverse osmosis by sucrose or partial lyophilization. The typical anion exchange column chromatography profile of the potato lipoxygenase is presented in Fig 12.

3.1.4.0. HPLC anion exchange chromatography

The dialyzed enzyme from the previous chromatographic step was applied onto Shimpak PA-DEAE (100 X 8 mm) column which has been earlier equilibrated with 50 mM sodium acetate and 50mM acetic acid at 70 : 30 ratio. The bound proteins were eluted by a pH gradient (from 30% acetic acid to 100% acetic acid) developed with the above solvents over a period of 40 min. The flow rate was 1 mL/min and the eluant was monitored at A-280 nm by SPD-6AV detector. The eluted fractions were immediately checked for activity and the active fractions were dialyzed against the enzyme buffer. The resultant protein was further characterized for mol.wt and regiospecificity. The typical chromatogram is presented in Fig. 13.

3.1.5.0. HPLC gel filtration **(Diol-300)**

The protein from the previous step was loaded onto high performance liquid chromatographic gel filtration column (Diol-300) for the determination of mol.wt. The column was equilibrated with 10 mM KH_2PO_4 pH 7.4 buffer containing 0.2 M NaCl and the protein was loaded at a flow rate of 1 mL/min. The eluant was continuously monitored at A-280 nm and the fractions were collected separately and checked for activity. The retention time of the active fraction was noted and basing on the relative retention times of the standard proteins such as soybean lipoxygenase, myosin, serum albumin, egg albumin and carbonic anhydrase, the mol.wt of the potato lipoxygenase was determined

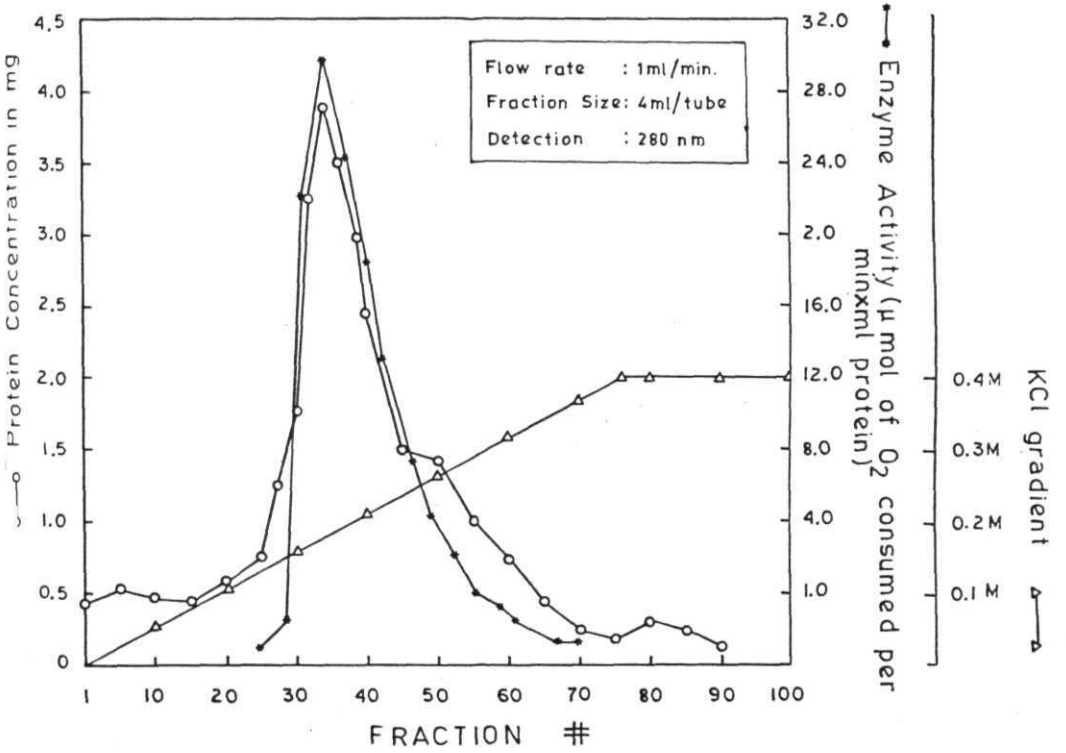


Fig.12: Anion exchange chromatography of Potato lipoxygenase on DEAE-Cellulose (DE-52) column:
15-45% ammonium sulfate fractionated proteins were loaded on 40 mM KH_2PO_4 equilibrated DE-52 column and after washing with the equilibration buffer, the protein was eluted with salt or pH linear gradient. The fractions were checked for activity and protein.

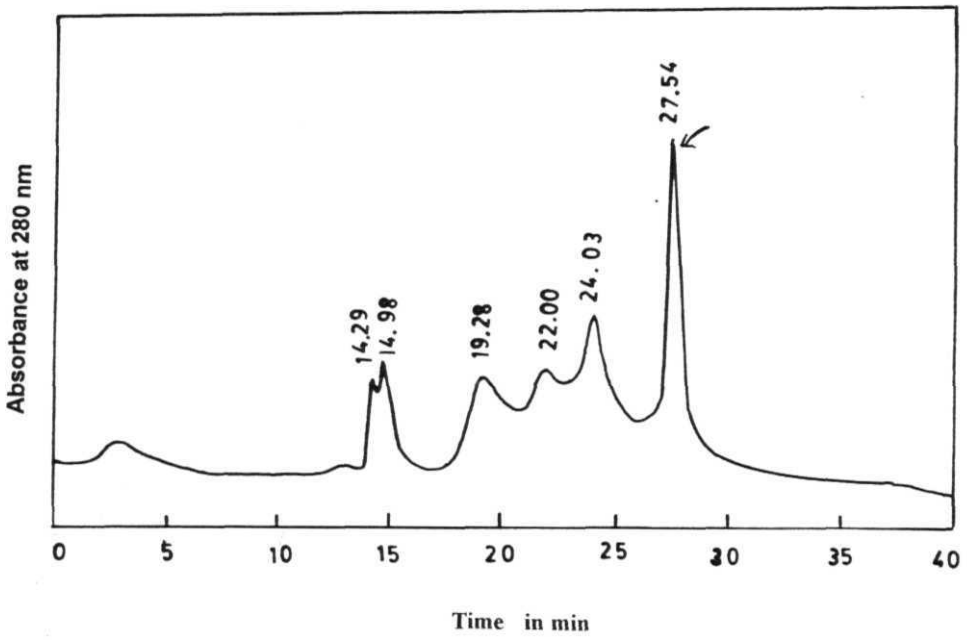


Fig.l3: HPLC profile of potato LOX on HPLC (PA-DEAE) anion exchange column:

DE-52 purifled potato LOX was loaded and eluted with a pH gradient developed with 50 mM sodium acetate and 50 mM acetic acid . The eluted peaks were checked for activity. Peak with RT 27.54 min showed activity on oxygraph.

Fig.l4: SDS-PAGE analysis of HPLC (PA-DEAE) column fractions.
HPLC purified **fractions of Fig 13 when electrophoressed on 10% SDS-PAGE: The arrow shows the active LOX.**

Peak I to 5 are the proteins peaks separated on HPLC PA-DEAE (only showed activity). Peak V was later employed for genearting standard arachidonic acid metabolites. **Peak 5**

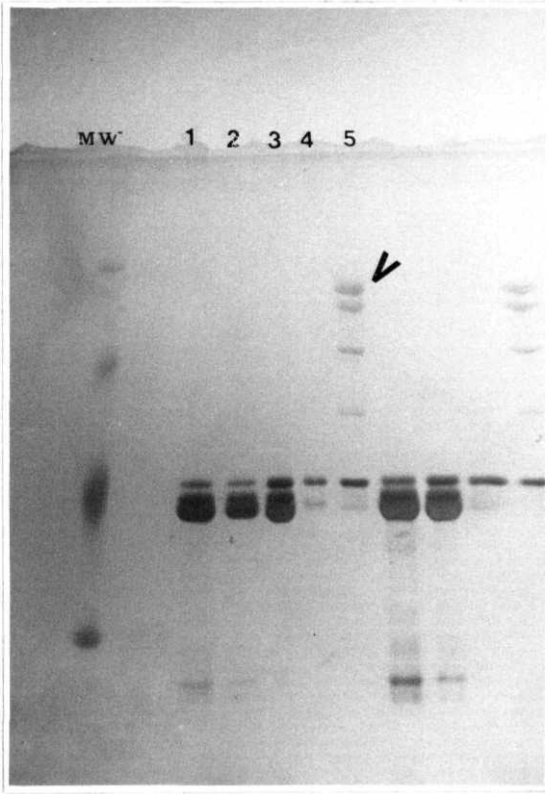

MW
1
2
3
4
5

Table I:
Purification profile of Potato lipoxygenase

Stage	Total Activity (Units)	Total Protein (mg)	Sp.Activity (Units/mg) protein	Yeild	Purification (fold)
Crude extract	3050	7770	0.39	100	1.0
15-45% pellet	2467	1888	1.30	80.1	3.35
Anion exchange chromatography	1642	228	7.20	54.0	18.46
HPLC PA-DEAE	871	47.0	18.5	29	47.30

The purification profile of the potato lipoxygenase was shown in Table I. Fig 14 shows the SDS-PAGE of the purified potato lipoxygenase

As shown in the table I, the lipoxygenase was purified from potatoes to a final specific activity of 18.45 units/mg protein with an over all yield of 29% and 47.3 fold purification.

3.2.0.0.Preparation of Monohydroperoxides

The highly purified potato lipoxygenase thus prepared was employed for the preparation of standard hydroperoxides as per the method described by Reddanna *et al,* (1990) with slight modifications The purified potato lipoxygenase was incubated with 133 uM arachidonic acid at pH 6.3 KH_2PO_4 buffer for 3 min and the reaction was stopped by acidifying the reaction mixture to pH 3.0 with 6 N HC1. The products formed were extracted with equal volumes of hexane : ether (1:1) twice. The organic solvent, separated from the aqueous layer in a separating flask, was passed through anhydrous granular sodium sulfate and subjected to evaporation in a flash evaporator under inert conditions. The residue was dissolved in straight phase HPLC mobile system consisting of hexane : propane 2-ol: acetic acid in 1000: 14: 1 ratio A portion of it was kept aside for the preparation of hydroxy compounds while the rest was loaded onto the straight phase HPLC system through a Rheodyne injector. The compounds were separated on a silica column (CLC-SIL, 25 X 0.46 cms) under isocratic conditions employing HPLC mobile phase at a flow rate of 1 mL/min The eluted solvent from the column was continuously monitored on Shimadzu SPD-6AV UV-VIS detector at 235 nm. The peaks eluted were continuously fed into the C-R4A integrator with visual display and the retention times, concentration of the eluted compounds and the percentages of the peaks were calculated by the integrator with either a single internal standard method or multiple internal standard method.

The individual peaks were collected and checked again for the presence of conjugated diene spectrum (Fig. 15) on Beckman scanning spectrophotometer and the peaks were pooled individually from a number of injections The HPETEs thus generated (Fig. 16) were used as standard HPETEs after identification.

Large amounts of 15-HPETE was prepared by reacting Sigma grade soybean lipoxygenase at pH 9.0 with 50 mM Tris-HCl buffer with 150 μM arachidonic acid. The products formed were separated on straight phase HPLC system and used as standard 15-HPETE (Fig.17)

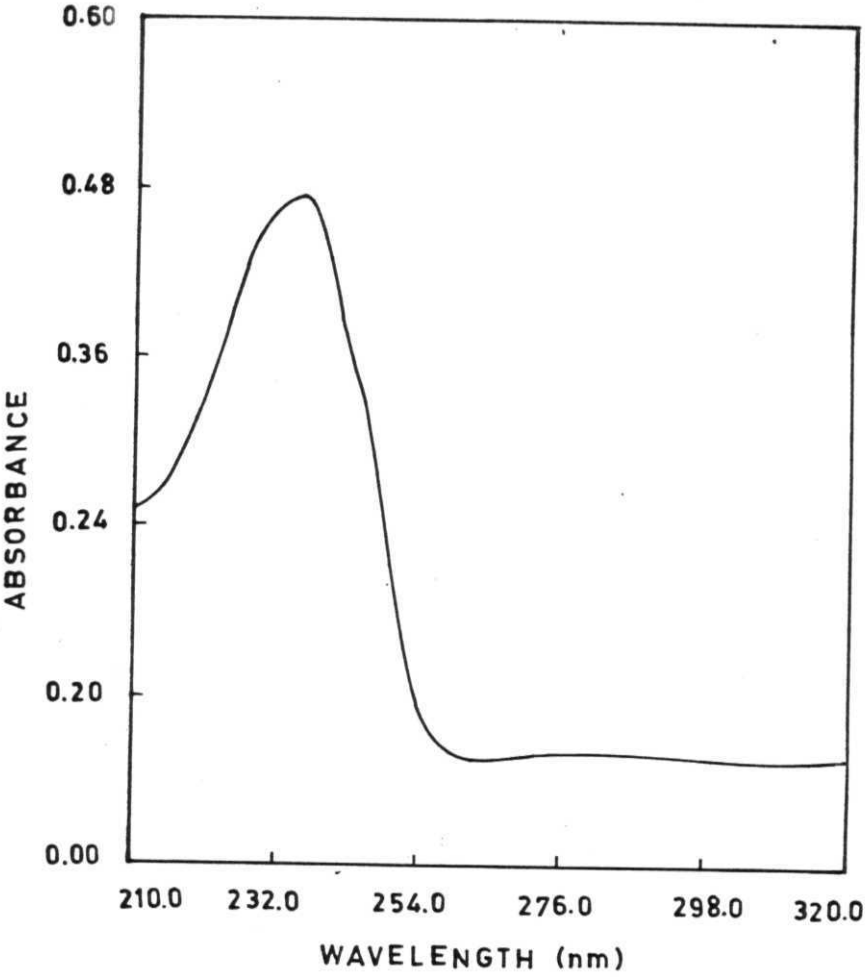
3.2.1.0. Hydroxyeicosatetraenoic acids (HETEs)

The hydroperoxides (HPETEs) generated by employing potato as well as soybean lipoxygenases were reduced with sodium borohydride as follows:

The HPETEs in hexane :ether (50:50) were evaporated to dryness and redissolved in 100% methanol. To the methanolic compounds excess of sodium borohydride (1:3 molar ratio) was added under nitrogen at 4°C. After 10-30 sec the excess borohydride was hydrolyzed by adding 50 μL of acetic acid. The methanol was evaporated again and the compounds were redissolved in appropriate HPLC solvent system for further separation The typical chromatogram of HETEs separated on straight phase HPLC is shown in Fig 18.

3.2.2.0. Preparation of dihydroperoxides

The standard dihydroperoxides required for the present study i.e 8,15-di H(p)ETEs were prepared using the multiple dioxygenation of soybean lipoxygenase under slightly acidic reaction conditions (Axelrod *et al.*, 1974). Arachidonic acid was allowed to react with excess soybean lipoxygenase at pH 6.8 for longer durations i.e for more than 10 min. The reaction was monitored on a scanning spectrophotometer and the formation of the diHPETEs was confirmed based on the increase in absorbance at 235 nm on UV/VIS scanning spectrophotometer. These compounds were extracted into the organic solvent by the addition of equal volumes of hexane : ether (1 : 1) to the reaction mixture shaking well



Scan Speed: 500 nm/min.

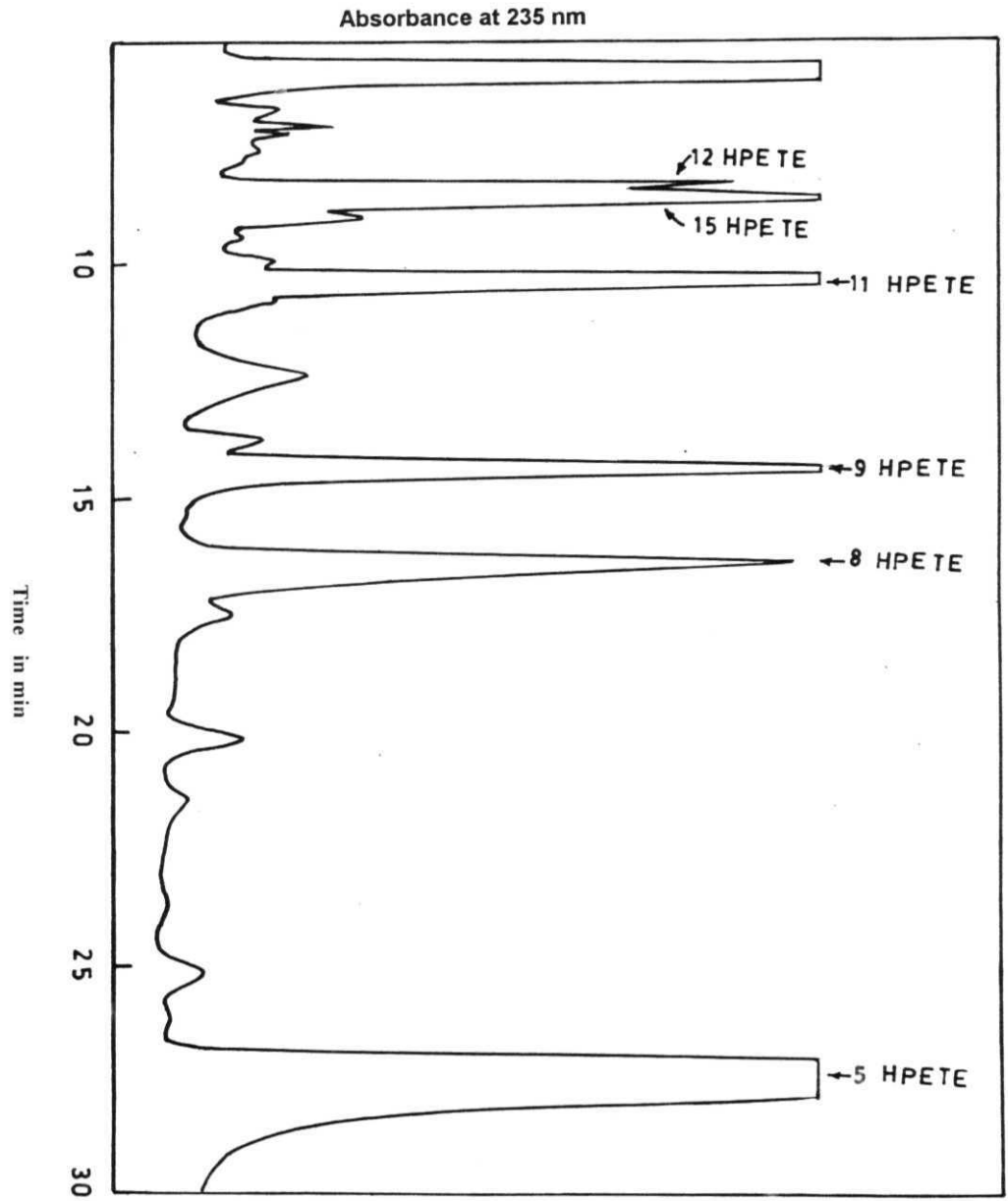
PEAK λ	PICK Abs
282.0	0.083
280.0	0.082
278.0	0.082
276.0	0.084
235.0	0.477

POINT λ	PICK Abs
235 0	0.477

Fig.15: UV/VIS scanning spectrum of conjugated diene, HPETE on scanning spectrophotometer.

. 16: **Separation** of standard IIPETEs generated by reacting 133 |iM AA with potato lipoxygenase on SP-HPLC .
The products were extracted with hexane : ether and evaporated to dryness. The residue was redissolved in SP-HPLC solvent system and analyzed in the following conditions.

Column	:	**CLC-SIL**
Solvent	:	Hexane:propane 2-ol:acetic **acid** (1000:16:1)
Flow rate	:	**1mL/min**
Detection	:	235nm


Absorbance at 235 nm
12 HPETE
15 HPETE
11 HPETE
9 HPETE
8 HPETE
5 HPETE
10
15
20
25
30
Time in min

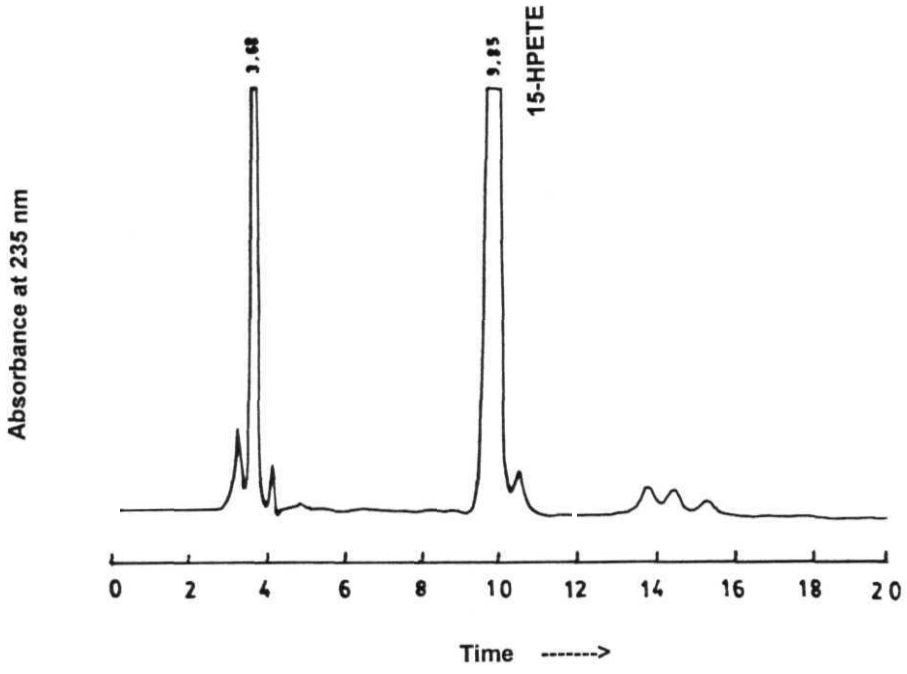


Fig.l7:Separation of 15-HPETE generated by soybean LOX on SP-HPLC. The products were extracted with hexane : ether and evaporated to dryness. The residue was redissolved in SP-HPLC solvent system and analyzed in the following conditions.

Column	:	CLC-S1L
Solv nt	:	**Hexane:propane** 2-ol:acetic acid (1000:12:1)
Flow rate	:	**1mL/min**
Detection	:	235nm

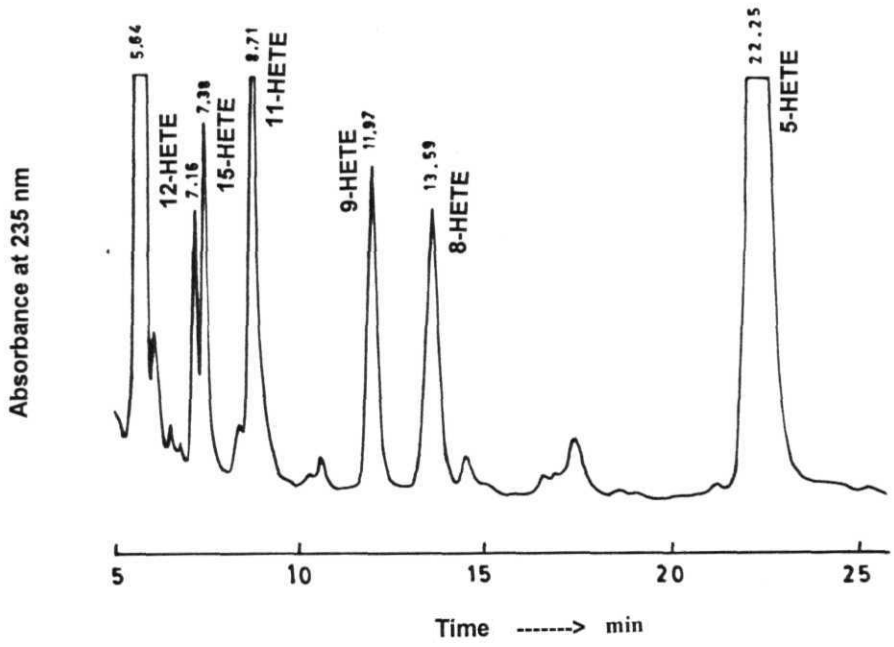


Fig.18: SP-HPLC product profile of HETEs generated from reaction of potato LOX with AA. The HPETEs were reduced to HETEs by **sodium** boroliydridc in mctlinnol under **nitrogen** and after evaporation was redissolved in **SP-HPLC** solvent system.

Column	:	**CLC-SIL**
Solvent	:	**Hexane:propane 2-ol:acetic** acid (1000:22:1)
Flow rate	:	**1mL/min**
Detection	:	235nm

and then separated in a separating flask The organic solvent was then evaporated and the residue was dissolved in reverse phase HPLC solvent system consisting of methanol:water: acetic acid in a ratio of 65: 35: 0.1, pH adjusted to 6.8 with ammonium hydroxide. The diHETES were prepared by reducing diHPETE formed with sodium borohydride as described earlier and separating them on reverse phase HPLC. DiHPETEs and DiHETEs were separated on a CLC-ODS (25 X 0.46 cms) column with the detection at 268 nm for the first 15 min and at 235 nm for the rest of the period. The peaks were collected individually and checked for the spectral characteristics on scanning spectrophotometer. The formation of 8,15-Di HPETE (*X* 268 nm), 5,15 diHPETE (*X* 242 nm) and 15 HPETE (*X* 235 nm) along with traces of other monohydroperoxides such as 15-, 8-, and 5-HPETEs were seen (Fig. 19). The pattern of elution and their spectral characteristics were taken into consideration for the identification of diH(P)ETEs. The typical spectra of 8,15-diHPETE and 5,15-HPETE are presented in Fig. 20 and 21.

The same procedure was used to generate other di-hydroperoxides by reacting them with either soybean lipoxygenase or potato lipoxygenase as follows:

8 HPETE + soybean LOX——————8,15 diHPETE

5 HPETE + soybean LOX——————5,15 diHPETE

9 HPETE + soybean LOX——————9,15 diHPETE

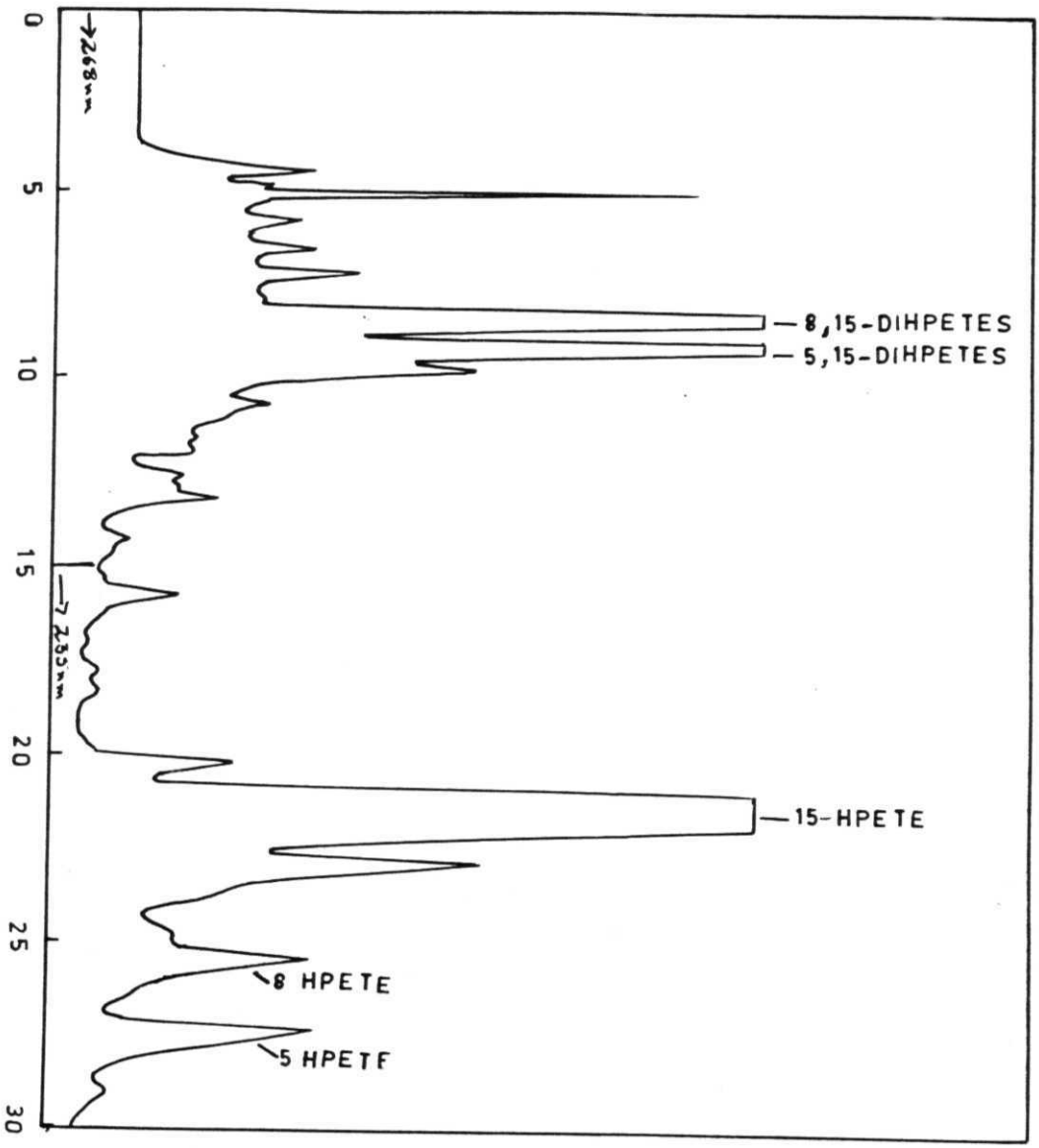
12 HPETE + 5-LOX——————5,12 diHPETE

3.3.0.0. Preparation of **5,6- and 14,15-LTA$_4$** Me

5,6-LTA$_4$ Me was prepared according to the modified procedures of Cori *et al.*, (1987) and Mei Chang *et al.* (1987) 5-HPETE synthesized using potato lipoxygenase was methylated with diazomethane. The dried 5-HPETE was dissolved in anhydrous methylene chloride and tetrahydrofuran (THF) in 50 : 50 ratio. The contents were cooled to -78°C and with rapid stirring pentamethyl piperidine (PMP) was added. After 15 min. of vigorous shaking trifluoromethanesulfonic anhydride (TFMSA) was added These were allowed to react for 2 to 3 hours and the reaction was later terminated by the addition of 2 mL of

Fig.19: RP-HPLC analysis of **HETE**s and diHETEs generated by soy **bean lipoxygenase** with arachidonic acid as the substrate at **pH** 6.8.
AA was incubated with soybean LOX in acidic conditions and the products were extracted into **he xane:** ether. The products were reduced with sodium borohydride in methanol under nitrogen and after evaporation were dissolved in RP-HPLC solvent system.

Column	:	**CLC-ODS**
Solvent	:	**Methanol:water:acetic** acid **(65:35:0.1) pH** 6.8
Flow rate	:	**1mL/min**
Detection	:	**268nm** and **235nm**


0
5
10
15
20
25
30
→268nm
→233nm
8,15-DIHPETES
5,15-DIHPETES
15-HPETE
8 HPETE
5 HPETE

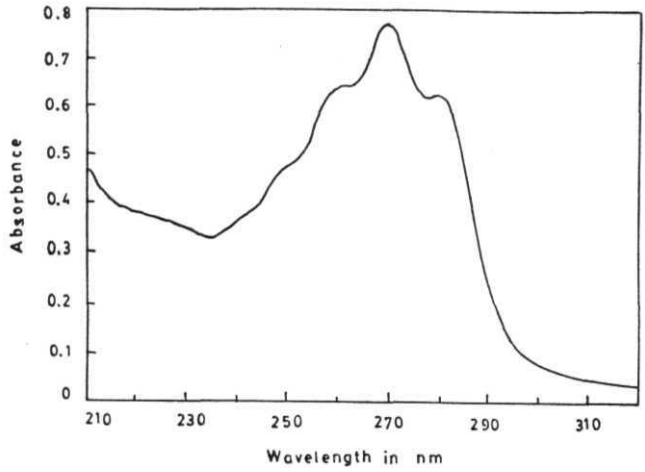


Fig. 20. UV/VIS scanning spectrum of 8,15-diHPETEs

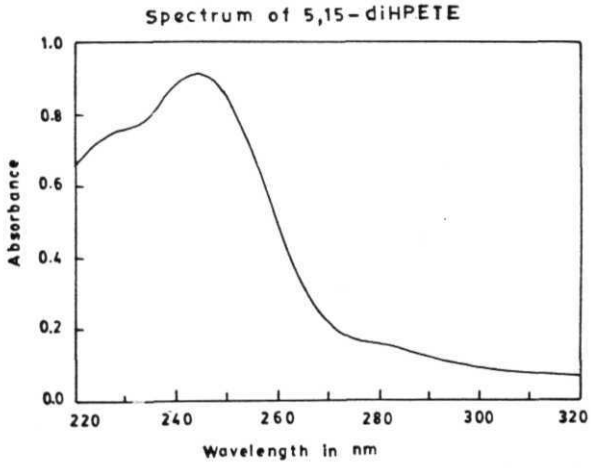


Fig. 21. UV/VIS scanning spectrum of 5,15-diHPETEs

triethylamine (TEA) and the temperature was slowly raised to -10°C The contents were then diluted with hexane : ether : TEA (50: 50: 1) followed by neutralized brine. The organic layer was separated, dried and evaporated under nitrogen and redissolved in a 10 mL of hexane : TEA (99: 1). The concentrate was passed through silica cartridges equilibrated with hexane: TEA (99: 1) and the flow through obtained was checked for LTA4 Me and its yields.

Similarly 14,15-LTA_4 Me was prepared by using the same method except that 15-HPETE was used instead of 5-HPETE as the precursor fatty acid hydroperoxide

3.4.0.0. Preparation of LTC_4

14,15-LTA_4 Me and 5,6-LTA_4 Me synthesized as described above were used for the preparation of 14,15-LTC_4 and 5,6-LTC_4 respectively. LTA4 Me dissolved in methanol was reacted with glutathione (reduced) (3 eq.) in the presence of triethylamine for 30 min. 2 mL of 1 M K_2CO_3 solution was added to the reaction mixture and the reaction was continued for 2 hours in dark. The reaction was terminated by the addition of 6 N HC1 and the contents were then diluted 10 folds with water and passed through a C-18 cartridge. The adsorbed LTC4 was eluted from the cartridge with methanol and was concentrated. LTC4 thus prepared was purified on HPLC using a C-18 column (CLC-ODS, 25 X 0.46 cms) and a solvent system of methanol : water: acetic acid (72:28:0.1), pH adjusted to 6.0 with ammonium hydroxide at a flow rate of 1 mL/min. The effluent was monitored for absorbance at 280 nm .

3.5.0.0. Hydrolysis of LTA_4 methyl ester to LTA_4 free acid

The methyl esters of epoxides were hydrolyzed according to the modified procedures of Fitzpatrick *et al.*,(1982).

Around 10 Abs of 14,15-LTA_4 Me or 5,6-LTA_4 Me was dissolved in 1 mL of 30 mM lithium hydroxide in tetrahydrofuran : water in 1:3 ratio vol/vol. The lithium hydroxide in the above solvent was allowed to react with the methyl esters under inert

nitrogen in dark for 15 hours at room temperature (27°C). The solvent was then removed by lyophilization. The residue containing the lithium salt of the LTA_4 was dissolved in 100% ethanol to give a final concentration of 2 mM. The ester free lithium salt of LTA4S can temporarily be stored at -20°C but in most of the cases they were used immediately.

3.5.1.0. Hydrolysis of LTA_4 to diHETEs

The above described LTA4 free acid was dissolved in 1 mL methanol and to it 10 mL of Tris-HCl buffer of pH 8.0 was added. After 10 min the mixture was acidified with 2 mL of 6 N HC1. The contents were further incubated at room temperature for 60 min Then water was added in sufficient quantity and passed through C-18 cartridges The bound diHETEs were eluted with 1 mL methanol twice. The eluted contents were evaporated and redissolved in reverse phase HPLC solvent system and analyzed on CLC-ODS column (25 X 0.46 cms) with the effluent being monitored at 268 nm as described earlier for diH(p)ETEs.

3.6.0.0. LTC4 synthase assay

LTC4 synthase activity was assayed in uterine microsomal membranes i.e 105,000 X g pellet.

LTC_4 synthase assay was performed according to the procedure of Yoshimoto *et al.* (1985). Typical assay of LTC_4 synthase consists of 50 mM HEPEs buffer of pH 7 6 with 5 mM glutathione (reduced, GSH) and 100 µL of enzyme protein. The assay was initiated after adding all the above three ingredients and keeping it for 10 min at room temperature by the addition of 20 µM lithium salt of LTA4 Me The reaction was allowed to proceed for 10 min at room temperature and terminated by the addition of ice cold (-20°C) methanol containing 0.1% acetic acid. The samples were left at -20°C for 60 min and centrifuged at 10,000 X g. The supernatant was collected and appropriate volume of water was added to it to make it equivalent to the solvent system of reverse phase HPLC system. The reaction products were then separated on CLC-ODS column (25 X 0.46 cms), the effluent being monitored at 280 nm The corresponding peaks were

collected and co-chromatographed with standard LTC4 Me. The identified individual peaks were checked for their typical UV spectra on scanning spectrophotometer

3.7.0.0. Extraction of endogenous leukotrienes

Endogenous leukotrienes from sheep uterus cytosol were extracted as per the procedure described by Huwyler and Gut (1990). Sheep uterine cytosol was mixed with three volumes of iso-propanol and the pH was adjusted to 3.0 with 5 N formic acid. To the mixture 3 vols of CH_2Cl_2 was added and vigorously mixed The aqueous and organic phases were separated by centrifuging the mixture at 10,000 X g for 10 min. The aqueous and interphase layers were carefully removed and 100 uL of distilled water was added to the organic phase under vortexing The sample was then recentrifuged at 10,000 X g for 2 min and the supernatant was concentrated to 50 uL. The concentrate was diluted with 50 uL of HPLC eluant (methanol : water : acetic acid 72:28:0.1., containing 50μM EDTA and pH adjusted to 6.0) and analyzed by reverse phase HPLC on a C-18 CLC-ODS column (25 X 0.46 cms) at a flow rate of 1 mL/min. The effluent was monitored for absorbance at 280 nm. The peaks were collected and their ultraviolet spectra recorded Standard LTs were co-chromatographed with the recovered peaks for identification (Rodney *et al.*, 1981).

3.8.0.0. Extraction of endogenous mono and dihydroxides (HETEs **and** diHETES)

The uterine homogenate (10,000 X g) supernatant was acidified to pH 3.0 with 6 N HC1 and to it equal volumes of hexane:ether (1:1) was added and mixed thoroughly. The organic solvent was separated from the aqueous solvent after thorough mixing in a separating funnel. The organic solvent was later evaporated to dryness and redissolved in appropriate HPLC solvent system i.e either the straight phase solvent system for the separation of mono-hydro xides as described earlier or the reverse phase solvent system for the analysis of di-hydro — xides. The eluted peaks were identified by their retention time, characteristic spectra and further on co-chromatography with standard mono and di hydroxides.

3.9.0.0. Polyunsaturated fatty acid analysis

The cytosolic and microsomal fractions of sheep uteri at different stages of estrous cycle were mixed with equal volumes of methanol : chloroform (3:1 ratio vol/vol) and thoroughly vortexed. The organic layer was separated from the aqueous by centrifugation at 3000 X g for 10 min. The organic extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in boron trifluoride (BF3) in methanol and was allowed to react with the fatty acids present in the residue at 90°C in sand bath for 60 min. in screw capped tubes. After cooling the tubes, the residue was dissolved in HPLC grade n-hexane and the contents were analyzed by gas chromatography. The standard PUFAs were also methylated as per the above procedure and injected onto GC (model Tracor-540) connected with a glass column of 2 m long consisting of Chromosorb.W support and a liquid phase of 10% Silar 10C The mesh size of the column was 80-100 microns The separation conditions for PUFAs was achieved on a temperature program starting at 160°C to 240°C at a rate of 3°C/min over a period of 30 min The carrier gas employed was nitrogen. The data was analyzed on a Nelson integrator on real time basis. Based on the retention times of the standard PUFAs, unsaturated fatty acids present in the samples were identified.

Chapter-1

4.1.0.0. Introduction

Lipoxygenases have been known to enzyme chemists as oxidizing enzymes from early 1930s (Andre *et al,* 1932). Since then lipoxygenases have been identified in a variety of plant and animal sources. Most of the existing plant lipoxygenases are of 15-type except potato and tulip bulbs, where they have been found to be of 5-type (Kiran kumar *et al,* 1991; Reddanna *et al* ., 1988 and Galliard *et al,* 1971). 15-lipoxygenase from plants have been purified from soybean, rice, pea, Phaseolus vulgaris, Vicia sativa and pepper (Theorell *et al,* 1947, Kermasha *et al,* 1986, Andrianarison *et al,* 1992; Mingnezmosquera *et al,* 1993). Out of these, soybean lipoxygenase has been well documented and found to contain 4 distinct isozymes with reference to substrate specificity (Axelrod.,1974). However potato 5-lipoxygenase has been purified by many groups because of its similarity to mammalian leukocyte 5-lipoxygenase in catalyzing the operation of 5,6-LTA_4 pathway (Galliard., 1971; Shimizu *et al,* 1984, 1986, Sekiya *et al,* 1977; Multiez *et al.,* 1987, and Reddanna *et al.,* 1990).

Animal lipoxygenases, on the other hand, have been identified in a variety of tissues. Most of the existing mammalian lipoxygenases can be classified into 3 main types i.e 12-type, 15-type and 5-type. 12-lipoxygenase has been purified and characterized from platelets, neutrophils and mast cells of skin and lung (Nugteren, 1975, Goetzl *et al,* 1979; Roberts *et al,* 1979) whereas 15-lipoxygenase has been studied from reticulocytes, leukocytes and lung (Bryant *et al,* 1982; Borgeat *et al,* 1979, Hamberg *et al,* 1980) 5-lipoxygenase has been well documented in PMNLs, eosinophils, lymphocytes and RBL cells (Goetzl *et al,* 1979, Jorg *et al,* 1982, Morris *et al,* 1980, Parker *et al,* 1979). Though 8-type, 9-type and 11-type of lipoxygenases have been identified in neutrophils and vesicular glands (Goetzl *et al,* 1979, Hamberg *et al,* 1967), none of them have been purified and characterized. Lipoxygenases have been identified in reproductive systems also (Shahin *et al,* 1979, Panduranga Reddy *et al,* 1992, Flatman *et al,* 1986, Li *et al,* 1992). The physiological importance of these lipoxygenase products compelled scientists from various branches to undertake the study of the enzyme and its catalytic activities to gain a better understanding of the mechanism of leukotriene biosynthesis and its

physiological role. Human uterine cervix and uterus in pregnancy were found to contain lipoxygenase activity (Saeed and Mitchell, 1983). The lipoxygenase from human uterine cervix was reported to be of microsomal origin (Flatman *et al.*, 1986) whereas in the pregnant uterus, lipoxygenase was found to be present in the cytosol and nuclear chromatin (Li *et al.*, 1992). The cervix lipoxygenase was found to catalyze the conversion of arachidonic acid to 12-HPETE, whereas the lipoxygenase from uterus in pregnant stage was found to catalyze the formation of 15-HPETE The purified cervix lipoxygenase was found to be devoid of peroxidase activity and further found to be inhibited by NDGA and ETYA, the known lipoxygenase inhibitors The optimum pH and dependence of the enzyme on cofactors such as ATP and Ca^{2+} are comparable with other mammalian lipoxygenases and was attributed to the dimerization of the enzyme. Purification of lipoxygenase has been a frustrating experience to many biochemists. The statement "...attempts to isolate the enzyme for further characterization has been disappointing because of the inherent instability of the protein" - (Rouzer and Samuelsson. 1985 p- 6040) amply supports this aspect. The experience of the investigator in purifying lipoxygenase from uterus is no different. This chapter focuses on the purification of one such lipoxygenase from sheep uterus and on its characteristic features.

4.2.0.0. Results

Traditionally lipoxygenases of animal systems have been reported to be from cytosolic fraction i.e 105,000 X g supernatant. But lipoxygenase from human uterine cervix was shown to be of microsomal origin (Flatman *et al,* 1986). 15-lipoxygenase was identified in human uterine cytosol by Li *et al.* (1992). In the present study on sheep uterus most of the lipoxygenase activity was confined to cytosolic fraction and no appreciable or detectable levels were found in microsomes. The lipoxygenase activity in the cytosolic fraction was found to be abundant and comparable to the levels found in plants. Hence most of the enzyme assays were performed on oxygraph (Fig. 11). As high as 10.7 units/g wet tissue was observed in the cytosolic fraction where one unit refers to one µmole of

O_2 consumed/min. This appears to be one of the richest sources for lipoxygenase reported so far in animal systems.

4.2.1.0. Purification of lipoxygenase

A typical purification profile of sheep uterus lipoxygenase is presented in Table II. Sheep uterus cytosol was obtained by centrifuging the 20% homogenate initially at 10,000 X g for 20 min and later re-centrifuging the supernatant at 105,000 X g for 90 min. The total activity of the lipoxygenase present in the microsomal free cytosol did not alter from the 10,000 X g extract but the specific activity increased to 1.23 from 0.96 units/mg protein. The particulate free cytosol thus obtained was passed through ion-exchangers connected in series i.e the DE-52 anion exchanger outlet was connected to the inlet of the CM-52 cation exchanger. Both the ion exchangers were equilibrated with potassium phosphate buffer pH 7.0. Most of the lipoxygenase activity was eluted in the flow through of the ion-exchangers. The total activity increased to 450 units at this stage and the specific activity to 5.2. The flow through obtained from the ion exchangers was subjected to ammonium sulfate fractionation i.e the non-ionic proteins present in the flow through were salted out by adding ammonium sulfate first from 0-30% saturation, then from 30-70% and later from 70-100%. The lipoxygenase was found to salt out at 70-100% saturation and this was pelleted down by centrifugation. The pellet was desalted by dialysis and re-centrifuged. The supernatant was found to contain 380 units of total activity with a specific activity of 9.2. The enzyme sample was later concentrated by using sucrose in reverse osmosis. The concentrate was then separated by gel filtration technique using sephadex G-100 column equilibrated with the enzyme buffer. The flow through was collected in 2 mL fractions and checked for activity and protein concentration (Fig 22). Though 27 mg out of 41 mg of protein was obtained in this step the total activity obtained was 280 units with loss of 100 units. The fractions containing active enzyme were pooled and further purified on HPLC using anion exchange chromatography (PA-DEAE 10 µ column 12 X 0.46 cms). The column was equilibrated with 50 mM sodium acetate. After loading the protein sample, the enzyme was eluted with a linear pH gradient starting from

Table II:

Purification profile of Sheep uterus lipoxygenase

Stage	Total Activity (Units)	Total Protein (mg)	Sp.Activity (Units/mg) protein	Yeild	Purification (Told)
Crude extract	300	312.5	0.96	100	1.0
105,000 Xg Supt.	300	243.9	1.23	100	1.28
Ion Exclusion Chromatography	450	86.0	52	150	54
70-100% pellet	380	41.0	9.2	126.6	9.68
Sephadex G-100	280	27.0	10.3	93.3	10.73
HPLC PA-DEAE	128	91	14.0	42.66	14.60

50 mM sodium acetate to 50 mM acetic acid over a period of 35 min (Fig 23). The enzyme activity was recovered as a single peak with RT 4.6 min. The lipoxygenase thus obtained showed a specific activity of 14.06 units/mg protein with an overall yield of 40% and 14.6 fold purification.

The purity of the so obtained sheep uterus lipoxygenase was rechecked on RP-HPLC using waters μ Bondapak C-18 column (Fig. 24). As shown in the figure a single major peak with retention time of 23.98 min was obtained.

4.2.2.0. General properties of the enzyme

The purified lipoxygenase when electrophoressed on 12% SDS-PAGE showed only one major band at 66 kDa (Fig 25, lane D). The same protein when loaded onto HPLC gel filtration column (Diol-300 24 X 0 46 cms) eluted as a 66 kDa protein with retention time of 6.96 min (Fig. 26). The purified protein when subjected to electrophoresis under native conditions resolved into two bands with mol.wts of 66 kDa and 132 kDa, the concentration being much higher in the former (Fig 27, lane F). The presence of a single band on SDS-PAGE and two bands on native PAGE indicate a possible monomer-dimer relationship for the enzyme. Similar to BSA, the protein showed 132 kDa (dimeric form) and 66 kDa (monomeric form) protein bands on native PAGE supporting such a possibility (Fig 27, lane D). Thus further showing the peak seen in Fig 26 as a lipoxygenase with a mol.wt of 66 kDa. The activity staining of the native gel by O-dianisidine color reaction showed the dimeric form to be active and not the monomeric form (Fig. 29, lane B). The enzyme was found to be inhibited by 10 uM NDGA (95% inhibition) and 10 uM ETYA (80% inhibition) and not by 100 uM indomethacin (data not shown).

The purified sheep uterine lipoxygenase with a mol.wt of 66 kDa and a specific activity of 14.06 μmoles/minXmg, the turn over no. of the enzyme was 9.28 X 10^2 min^{-1} when calculated using the following formula

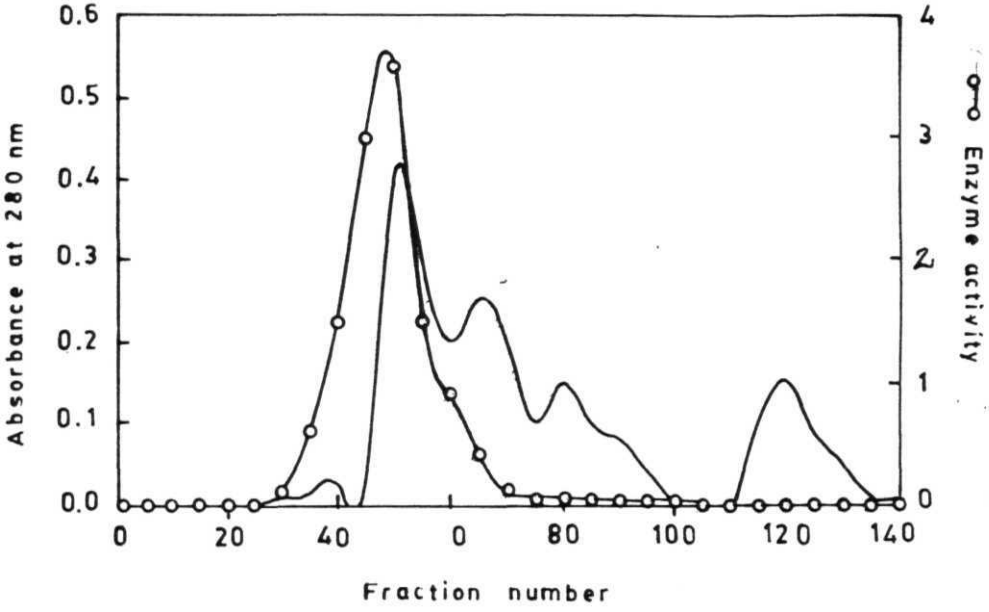


Fig. **22.Separation** of uterus LOX on gel fllteration (sephadex **G-100)** open column chromatography :
LOX obtained after ion-exclusion chromatography was fractionated with ammonium sulfate at 70-100% saturation. This fraction was desalted and concentrated. The concentrate was loaded on equilibrated sephadex **G-100** and the flow through was collected in fractions. The fractions were checked for activity and protein.

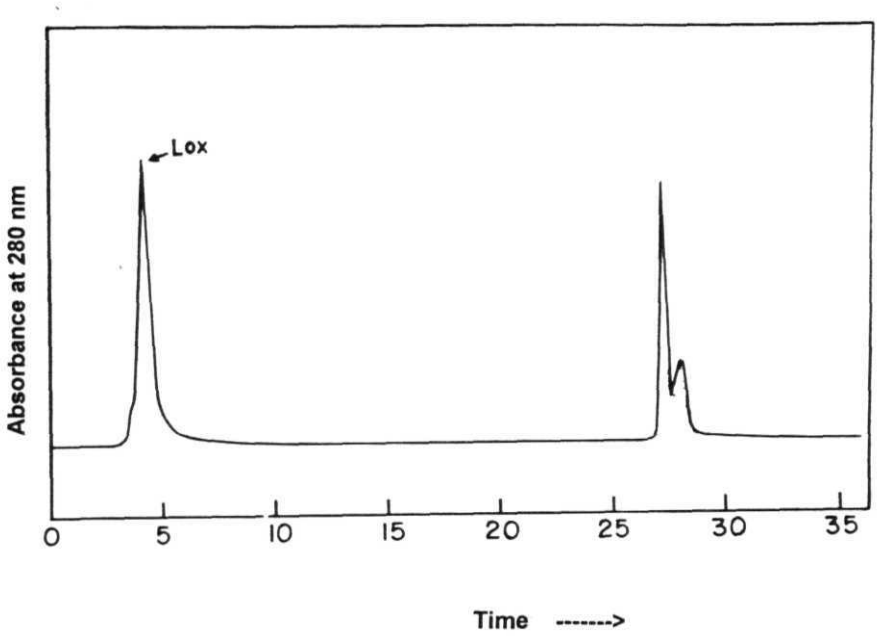

Lox
Absorbance at 280 nm
0
5
10
15
20
25
30
35
Time ------->
Fig.23:liPLC anion exchange chromatography of sheep uterine LOX.
G-100 column fractions were desalted and loaded on HPLC, PA-DEAE, anionic exchanger. The fractions were collected and assayed for activity.
Column : PA-DEAE
Solvent : 50mM Sodium acetate (A) and
50mM acetic acid (B).
Gradient : 100% A to 100% B
Flow rate : 1mL/min
Detection : 280nm

Fig.24:RP-HPLC analysis of purified sheep uterus LOX

Column	:	Waters μ bondapak
Solvent	:	0.1% TFA in 85% Acetonitrile
		0.1% TFA in 30% Acetonitrile
Gradient	:	Step
Flow rate	:	1mL/min
Detection	:	280nm

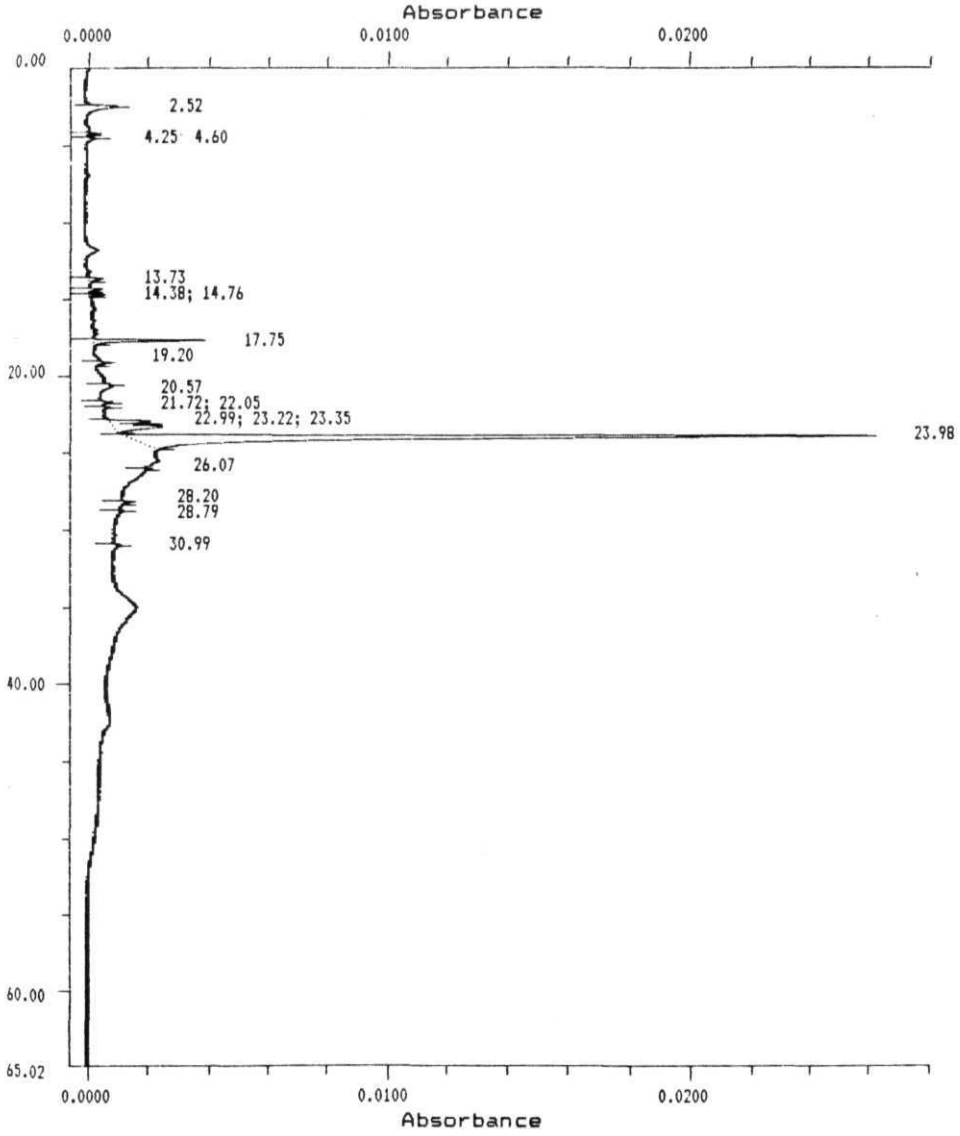
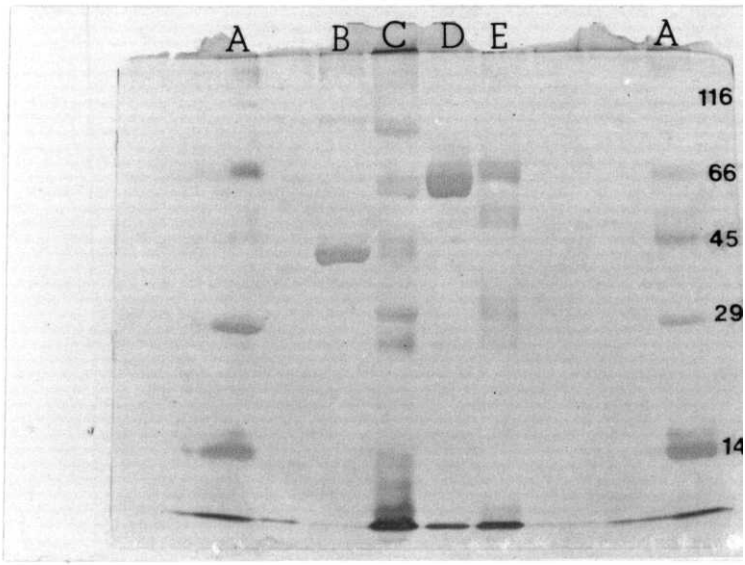

Absorbance
0.0000
0.0100
0.0200
0.00
2.52
4.25
4.60
13.73
14.38; 14.76
17.75
19.20
20.00
20.57
21.72; 22.05
22.99; 23.22; 23.35
23.98
26.07
28.20
28.79
30.99
40.00
60.00
65.02
0.0000
0.0100
0.0200
Absorbance

Fig.2S:SDS-PAGE analysis of sheep uterus LOX on 12% acrylamide gel electrophoresis.
Purified sheep uterine LOX with standard mol. wt markers and known plant LOXs were electrophoressed on 12% gel.

A	:	Mol.Wt. markers
B	:	DE-52 purified potato LOX
C	:	Soybean LOX (commercial)
D	:	Purified sheep uterus LOX
E	:	Degraded sheep uterus LOX


A
B
C
D
E
A
116
66
45
29
14

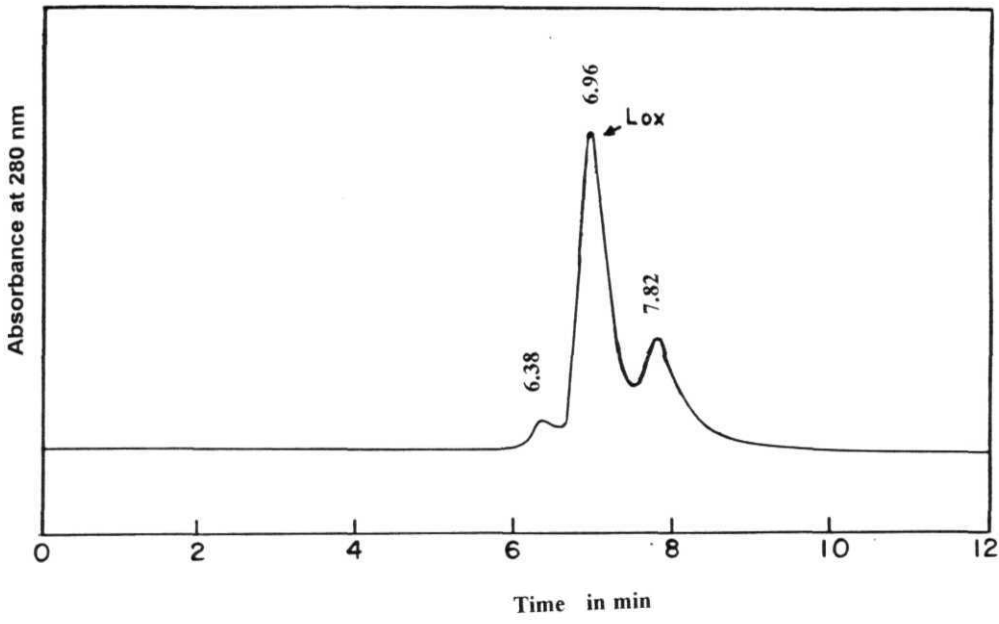


Fig.26: Mol.Wt determination of uterine LOX on HPLC gel filtration (Diol-300). HPLC, PA-DEAE purified LOX was analyzed for its mol.wt determination on Diol-300 column. The solvent system employed was 10 **mM KH2PO4** ωιτ 0.2 M NaCI. The mol.wt was determined with RT and comparing it with RTs of standard **mol.wt.** proteins.

Fig.27:Native PAGE of purified sheep uterus LOX on 8% acrylamide gel electrophoresis.

A	:	α-lactalbumin
B	:	Carbonic anhydrase
C	:	Egg albumin
D	:	BSA
E	:	Mixture of all the markers
F	:	Sheep uterus LOX

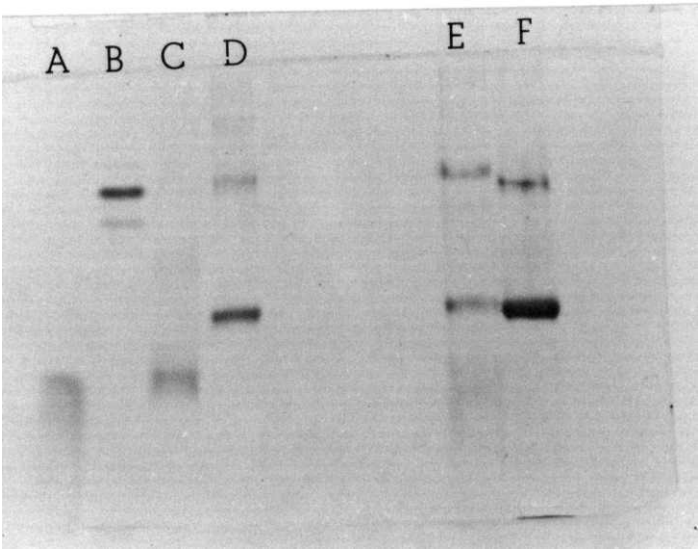

A
B
C
D
E
F

Fig. 28. Activity staining of uterine LOX:

a) Uterine LOX separated on native PAGE and stained with **coomassie** blue

b) Activity staining of uterine LOX separated on native PAGE, the gel was incubated with potassium arachidonate and stained with O-dianisidine.

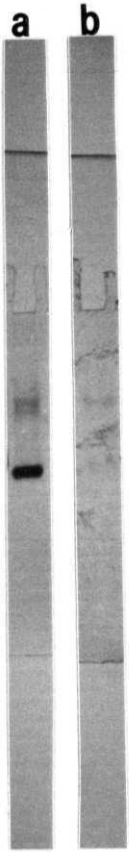

a
b

Fig.29. Western blot analysis of sheep uterus LOX separated on SDS-PAGE cross reacted with Abs raised to purified uterine LOX:
A and *B* are two different concentrations of purifled uterine LOX separated on SDS-PAGE and cross reacted with its own antibodies. Arrow indicates the position of uterine LOX (66 kDa) peptide. The low molecular wt. peptide could be the degraded LOX protein usually seen with LOXs.

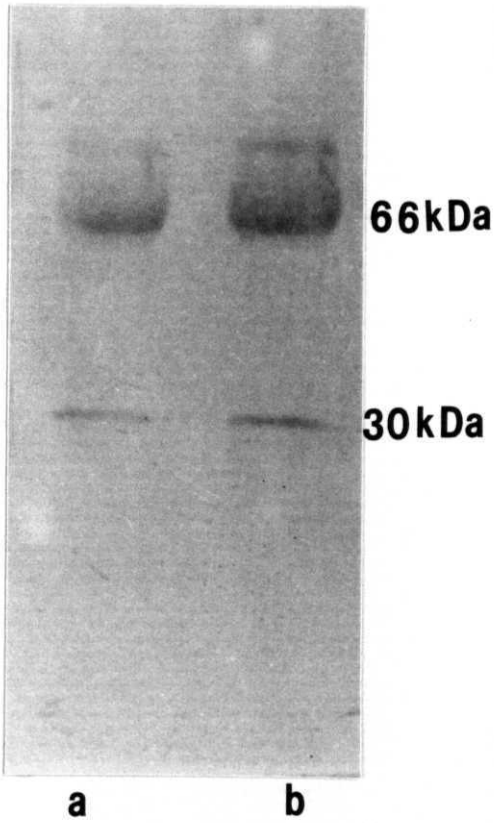
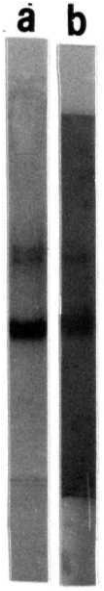

66kDa
30kDa
a
b

Fig. 30. Western blot analysis of sheep uterus LOX separated on native-PAGE cross reacted with Abs raised to purified uterine LOX:
a) Purified uterus LOX separated on native PAGE and stained with coomassie blue.
b) Purified uterus LOX separated on native PAGE and cross reacted with antibodies raised against purified uterus LOX.


a
b

$$K_p = \frac{Vmax}{[E]_t} = \frac{\mu moles(S\text{---}P)min^{-1} \times mL^{-1}}{\mu moles\ (E)\ mL^{-1}} = min^{-1}$$

K_p = turnover number
S = Substrate
P = Product
$[E]_t$ = Enzyme

The catalytic cycle of the enzyme was found to be 1.07 X 10^{-3} min, which was calculated using the following formula:

$$\text{catalytic cycle time} = \frac{1}{\text{turn over no}} = \text{min}$$

4.2.3.0. Western blot analysis

The polyclonal antibodies raised to the purified sheep uterus Hpoxygenase showed cross reactivity to the major 66 kDa peptide (Fig. 29). It also showed cross reactivity to a 30 kDa peptide. The 30 kDa peptide could be the degraded product of the 66 kDa peptide, an usual pattern observed with most of the Hpoxygenase purified (Funk *et al.*, 1990; Solane *et al*, 1992). The western blot analysis of the native gel showed cross reactivity to both the monomeric and the dimeric forms suggesting both of them to be immunologically reactive, though the former is catalytically inactive (Fig. 30).

The polyclonal antibodies raised to the purified sheep uterine Hpoxygenase did not cross react with any other known plant Hpoxygenase* Also the polyclonal antibodies raised against uterus Hpoxygenase did not cross react with uterine homogenates from rat and rabbit suggesting it to be an unique Hpoxygenase under discussion with several unusual features (data not shown).

4.2.4.0. Effect of pH

The effect of pH on the purified sheep uterine lipoxygenase was checked with arachidonic acid as the substrate at 250 μM final concentration. The maximal uptake of oxygen was found to be around pH 5.5 and the activity towards both sides of it decreased dramatically (Fig 31). This narrow range of pH optima, unlike other plant and animal lipoxygenases, suggests its uniqueness.

4.2.5.0. Substrate specificities

In order to understand substrate specificities of the purified lipoxygenase, activity was measured with different substrates at 250 μM concentration and pH 5.5 citrate phosphate buffer.

The following are the fatty acid substrates checked for activity

1. Linoleic acid (LA, 18:2, n-6)
2. Arachidonic acid (AA, 20:4, n-6)
3. Gamma linolenic acid (GLA, 18:3, n-6)
4. Dihomo gamma linolenic acid (DHGLA, 20:3, n-6)
5. Eicosapentaenoic acid (EPA, 20:5, n-3)
6. Docosa hexaenoic acid (DHA, 22:6, n-6)
7. 15-hydroperoxy eicosatetraenoic acid (15-HPETE)

Among the substrates checked, GLA showed the highest activity followed by DHA, 15-HPETE, EPA and AA (Fig. 32). DHGLA and LA were found to be poor substrates for uterine lipoxygenase.

4.2.6.0. Substrate dependency with arachidonic acid

To determine the substrate dependency of the purified lipoxygenase, its activity was measured at different concentrations starting from 0 μM to 400 μM. The volume of ethanol, the carrier of the substrate was maintained at 10 μL in a reaction volume of 3 mL. Fig 33 shows the typical substrate dependent kinetics of uterine lipoxygenase

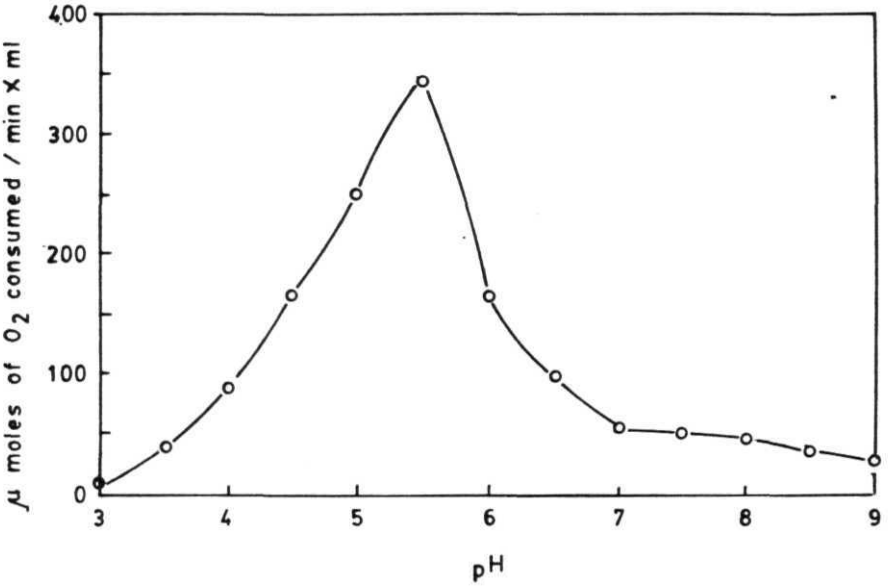


Fig.31: Effect of pH on sheep uterus LOX activity.

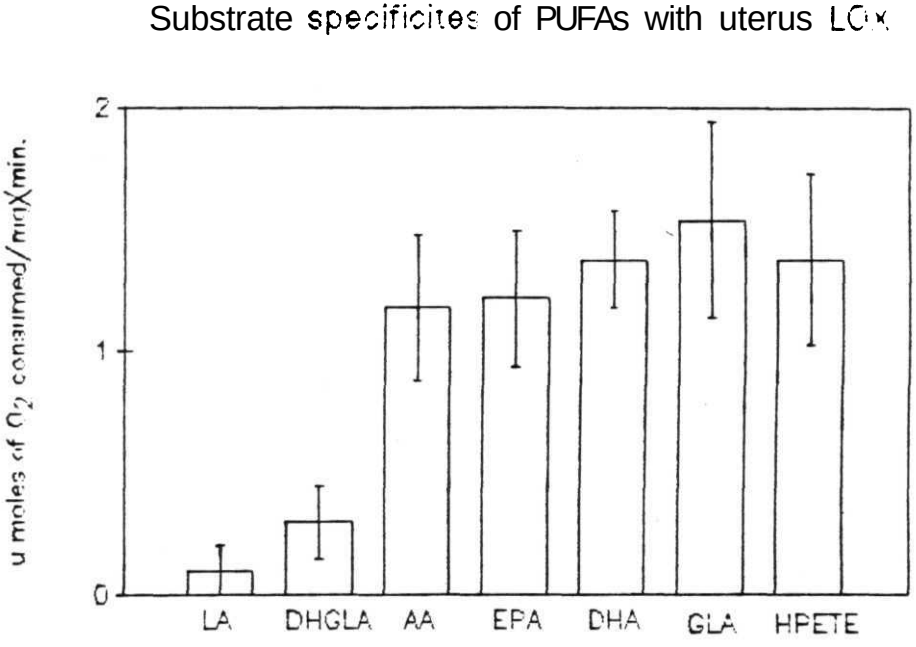


Fig.32:Substrate specificity of uterus LOX as assayed on oxygrapii at 250μM conc.

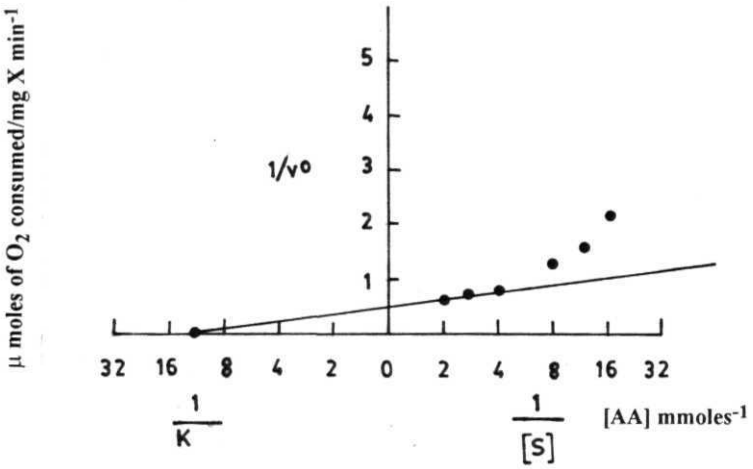


Fig.33:Substrate dependency of uterus LOX with AA

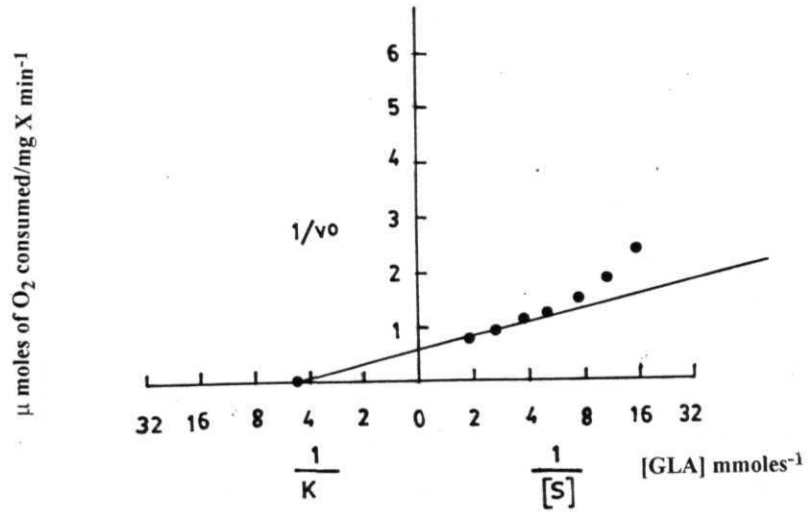


Fig.34:Substrate dependency of uterus LOX with GLA

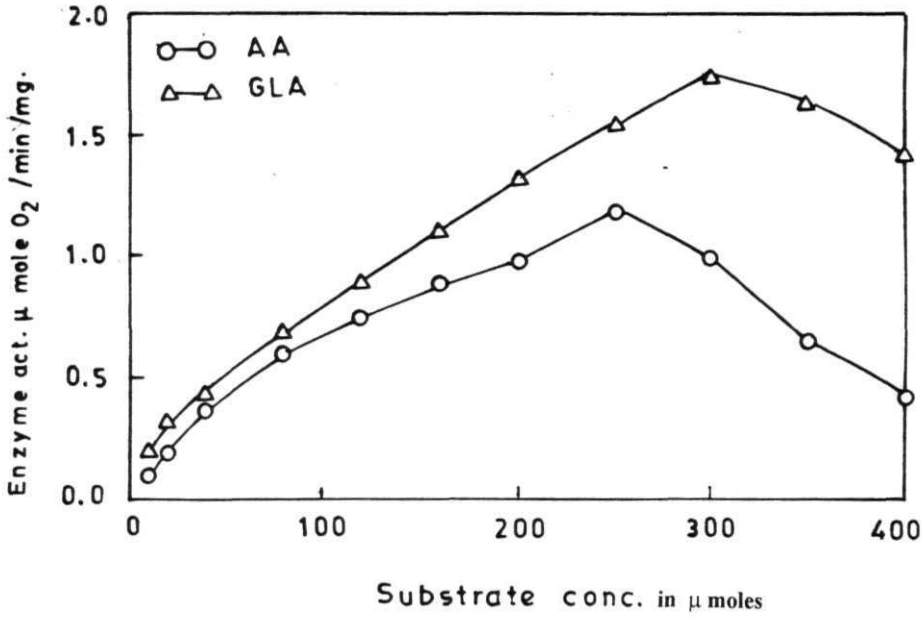


Fig.35: Substrate dependecy curve of uterine LOX with AA and GLA as **the** Substrates

activity with AA. Similarly the effect of various concentrations of GLA on lipoxygenase activity was studied (Fig 34)

Apparent Km values calculated were found to be 180 μM for arachidonic acid and 98 μM for γ-linolenic acid. The maximum velocity (Vmax) calculated for arachidonic acid were 1.94 units/mg protein and 1.91 units/mg protein for GLA (Fig. 35)

4.2.7.0. Iso electric point

The non binding of the sheep uterus lipoxygenase to either of the ion exchangers and recovery of lipoxygenase activity in the flow through fractions from the HPLC anion exchange column suggests it to be a neutral protein with a Pi around pH 7.0 (Fig. 22). The purified lipoxygenase on electrofocussing on tube gels using 3-10 ampholine gradient also focussed around pH 6.8, suggesting the Pi of the lipoxygenase present in sheep uterus to be around pH 7.0, thereby having neutral or non-ionic properties.

4.3.0.0 Discussion

Lipoxygenases have been isolated and purified from many sources in animal systems. These lipoxygenases catalyze the conversion of arachidonic acid to HPETEs and further to leukotrienes. The mast cells of the lung, skin and uterus were found to be similar as far as the product profile of arachidonic acid was considered and their involvement in inflammatory reactions. The lipoxygenase that catalyzes these conversions have not been fully characterized though 5-lipoxygenase and its $5,6\text{-LTA}_4$ synthase activity in lung and 12-lipoxygenase and its metabolites in skin were identified.

Lipoxygenase activity was identified in human cervix (Saeed and Mitchell, 1983) and was classified as 12-lipoxygenase by Flatman et al. (1986). 15-lipoxygenase protein and its products were also recently reported in pregnant human uterus (Li et al., 1992). Purification of this lipoxygenase from uterus was not fully explored though Flatman et al. (1986) have reported a detailed purification procedure. The abundant lipoxygenase activity found in sheep uterus and its unusual characteristics warranted further research to clear the ambiguity of the type of lipoxygenase present in uterus.

In the purification of lipoxygenase from sheep uterus, several unusual steps had to be taken into the conventional protein purification procedures to obtain better enzyme yields. Primarily the extraction buffer or the homogenating buffer had to be modified to suit or increase the stability of the lipoxygenase present in the uterus. Ascorbic acid present in the buffer acts as an antioxidant for the enzyme and prevents the large amount of fatty acids present in the uterine tissue from further oxidations in the homogenate itself. Sodium meta bisulfite, a known histidine protease inhibitor, protects the histidine moiety of the lipoxygenase from proteolytic cleavage. EDTA and EGTA being metallo-protease inhibitors or metal chelators protect the non-heme moiety of the lipoxygenase. Further, though the reason is not known, the presence of Mg^{2+} and K^{+} ions increase the stability and catalytic expression of the uterine lipoxygenase. The presence of calcium has been a debated topic in the scientific circles for lipoxygenase activity. In this study it was found that the presence of calcium is necessary for the purification of the enzyme and its stability but not having a role in the expression of its catalytic activity as seen in the case of rabbit reticulocyte 15-lipoxygenase (Narumiya et al., 1981). The other lipoxygenases like 12- and 5- lipoxygenases, however, were found to be calcium dependent for expression of the activitiy (Ochi et al., 1982; Hamasaki et al., 1984; Jakschik et al., 1980).

The purification of lipoxygenase resulted in specific activity of 14.06 units/mg protein with an overall yield of 42%. This is the highest specific activity reported so far for any animal lipoxygenase. The purification fold also indicates that lipoxygenase is one of the abundant proteins in the uterus.

The purified sheep uterus lipoxygenase was found to be 66 kDa as seen in the SDS-PAGE and on gel filtration using Diol-300 column. This mol.wt is similar to the other 12- and 15-lipoxygenases (Yokoyoma et al, 1986; Solane et al.,1990) reported. However, 5-lipoxygenase was reported to have slightly higher mol.wt i.e 72 kDa (Ueda et al., 1988; Kaneko et al., 1987). Further the purified lipoxygenase was found to be independent of membrane associated factors and activating proteins as seen in case of 5-lipoxygenase and 5,6-LTA_4 synthase (Rouzer et al., 1985;Dixon et al., 1990).

The purified protein on native PAGE, however, showed two bands with mol.wts 132 and 66 kDa indicating possible dimer and monomer relationship. It is interesting to note that only the dimeric form showed activity as evidenced by the activity staining of the protein separated on native PAGE. The fact that both the protein bands are immunologically cross reactive to the antibodies raised against the purified uterus lipoxygenase suggests that both the proteins are different forms of the same lipoxygenase. Similarly uterine cervix lipoxygenase was reported to be a dimer (Flatman et al., 1986). Reticulate 15-lipoxygenase was reported to be inactive in dimeric form (Hohne. M and Andree. H, 1980). The present study thus conclusively demostrates the presence of lipoxygenase in dimeric form with catalytic activity, as against other reports of dimeric lipoxygenases shown to be immunologically reactive, but catalytically inactive.

Though most of the existing lipoxygenases are acidic, some lipoxygenases are known to be basic in nature as seen in case of 15-lipoxygenase from leukocytes, PMNL lipoxygenase from rabbit peritoneum (Izumi et al., 1991; Shuh et al., 1981) and human uterine cervix lipoxygenase (Flatman et al., 1986). The purified uterus lipoxygenase, on the other hand, is neutral.

The optimum pH of the uterine lipoxygenase was found to be in narrow acidic range unlike other mammalian enzymes with a broad pH range. The Km values for arachidonic acid and GLA appear to be on the higher side compared to leukocyte lipoxygenases (180 and 80 μM). However, high Km values have been reported for testicular lipoxygenase (Shahin et al., 1978, Grossman et al., 1979) suggesting that this could be an usual feature with all reproductive tissues. The unsaturated fatty acid levels were also found to be very high in reproductive tissues as seen in the present study and also reported by others (Bell et al., 1980).

Sheep uterine lipoxygenase was also found to have a higher affinity towards GLA as shown by other mammalian lipoxygenases (Rapport et al.,1984; Yamamoto, 1992). The purified lipoxygenase was inhibited both by NDGA and ETYA, the classical inhibitors of lipoxygenase pathway and found to be not affected by the presence of indomethacin, suggesting the enzyme under discussion to be lipoxygenase. Further the

activity staining of the lipoxygenase under native conditions on the gel itself indicates the uptake of oxygen and formation of HPETEs due to lipoxygenase activity and not due to the action of any other heme containing cytochrome P-450 mediated monooxygenases. These monooxygenases, however, are membrane bound fractions unlike the cytosolic nature of the present enzyme.

Although, lipoxygenase was purified from sheep uterus to apparent homogeneity as proved by the SDS-PAGE, HPLC Diol-300 analysis and RP-HPLC analysis, it showed minor impurities. The minor peak at 6.38 min on diol-300 column could be the dimeric form and the peak at 7.82 min could be the degraded low mol.wt form of the uterine lipoxygenase.

The fact that the antibodies raised against sheep uterus lipoxygenase were not cross reacting with other known lipoxygenases, suggests that the enzyme could be of a new class with different antigenic recognition sites.

It would be interesting to study the product profile of such an abundant lipoxygenase and its participation in various physiological functions of uterus. Hence further studies in this direction were taken up and the data is presented in the next chapter.

Chapter-2

S.O.O.O.Introduction

Most of the mammalian lipoxygenases are functionally similar and were even found to share some structural similarities in the conserved regions of their amino acid sequences. Platelet 12-lipoxygenase was found to have more than 80% similarity with the 12-lipoxygenase of porcine leukocytes and the reticulocyte 15-lipoxygenase and up to 62% with human 5-lipoxygenase These lipoxygenases were even found to be similar up to 40% with their counter parts of plant kingdom such as soybean and pea lipoxygenases (Funk *et al,* 1990). Most of the lipoxygenases exhibit overall hydrophobicity which aids in attraction and attachment of the substrate fatty acid (Ponder *et al,* 1987). The conserved sequences of all the lipoxygenases share many acidic and basic residues including the putative iron binding domain (Funk *et al,* 1989). These conserved sequences are fundamental blocks responsible for the structure-function properties of the enzyme Out of these conserved sequences, methionine was found to be the primary determinant for the positional specificity of the lipoxygenases (Solane *et al,* 1990).

The replacement of amino acid methionine with valine in 15-lipoxygenase was found to oxygenate at 15- and 12- positions of arachidonic acid equally (Solane *et al,* 1991). This methionine was found to be monooxygenated by aerobic inactivation of the lipoxygenase (Rapport *et al,* 1984). The oxygenation of methionine at the active center and the reduction of the putative iron bound to the histidine moiety, from Fe^{3+} to Fe^{2+} oxygenate the substrate that has been attracted and attached to the enzyme by the hydrophobic methionine and valine (Degroot *et al.,* 1975).

Changes in the conserved amino acid sequence of the lipoxygenase alters the position of the 1,4-butadiene systems of the substrate from C-13 to C-10 changing the substrate specificity. Such changes in oxidation leads to the formation of dual lipoxygenation capacity seen in case of 5-lipoxygenase of PMNLs, 12-lipoxygenase of platelets or 15-lipoxygenase of reticulocytes. (Rouzer *et al,* 1986; Murray *et al,* 1988, Brash *et al,* 1989).

The primary oxygenation of arachidonic acid with lipoxygenase involves an antarafacial relationship between the hydrogen abstraction and reorientation of the

molecular oxygen (Corey *et al.*, 1983). This antarafacial relationship is seen only in enzymatic conversions and were not found to be present in case of diHETEs formed by the hydrolysis of the epoxide *viz* the formation of 5,12- and 8,15-diHETEs (Walstra *et al.*, 1987). The mechanism of hydrogen abstraction from the primary oxygenated metabolite of arachidonic acid, as in the case of 8-lipoxygenase with 5-HPETE, was found to be analogous with 12-lipoxygenase in 15-HPETE for the formation of epoxide, leukotrienes (Ueda *et al.*, 1988, Mass *et al*, 1983). Dual lipoxygenation of the enzyme gives it an intrinsic ability to catalyze the formation of leukotrienes or it bestows the epoxide synthase ability as seen in cases of 5-, 12- and 15-lipoxygenases. Leukotrienes are formed from the transformation of arachidonic acid through a hydroperoxide intermediate, 5-HPETE in the case of 5,6-LTA_4 and 15-HPETE in case of 14,15-LTA_4 pathway. The hydroperoxy intermediate is acted upon by a dehydrase to form the 5,6 epoxide or 14,15 epoxide derivative. This dehydrase was later shown to be 8-lipoxygenase activity in case of 5,6-LTA4 pathway and 12-lipoxygenase activity in case of 14,15-LTA_4 pathway. These enzymes act at the C-10 position of the arachidonic acid in both the cases. Due to the conjugated diene system in the intermediate (5-HPETE or 15-HPETE) a 1-5 free radical movement results in LTA4 formation. This mechanism is found to be common in both the lipoxygenase pathways (Fig 7 and 10).

5,6-LTA_4 synthase was first demonstrated by the dual regiospecificity of the 5-lipoxygenase of both potato and PMNLs (Shimizu *et al*, 1984 and Rouzer *et al.*, 1985). However later studies have shown the operation of 14,15-LTA_4 pathway in leukocytes by Mass *et al.* (1981) and Lundberg *et al.* (1981). The operation of 11,12-LTA_4 pathway was also demonstrated by Westlund *et al.* (1988). The formation of 5,6-LTA_4, 8,9-LTA_4, 11,12-LTA_4 and 14,15-LTA_4 by the potato lipoxygenase was demonstrated by Reddanna *et al.* (1988).

The operation of 14,15-LTA_4 pathway was found to be catalyzed both by the synthase activities of leukocyte 12-lipoxygenase (Yokoyoma *et al.*, 1986) and reticulocyte 15-lipoxygenase (Bryant *et al.*, 1985). The operation of this leukotriene pathway was also

found to be temperature dependent and oxygen sensitive (anaerobic) i.e it gives various products at different reaction temperatures (Bryant *et al.*, 1985).

In this chapter the regiospecificity of the purified sheep uterus lipoxygenase with various PUFA substrates and reaction conditions such as optimum pH are presented. Also the involvement of uterine lipoxygenase in the synthesis of leukotrienes was analyzed.

5.1.0.0. Results

5.1.1.0. Regiospecificity of sheep uterine lipoxygenase

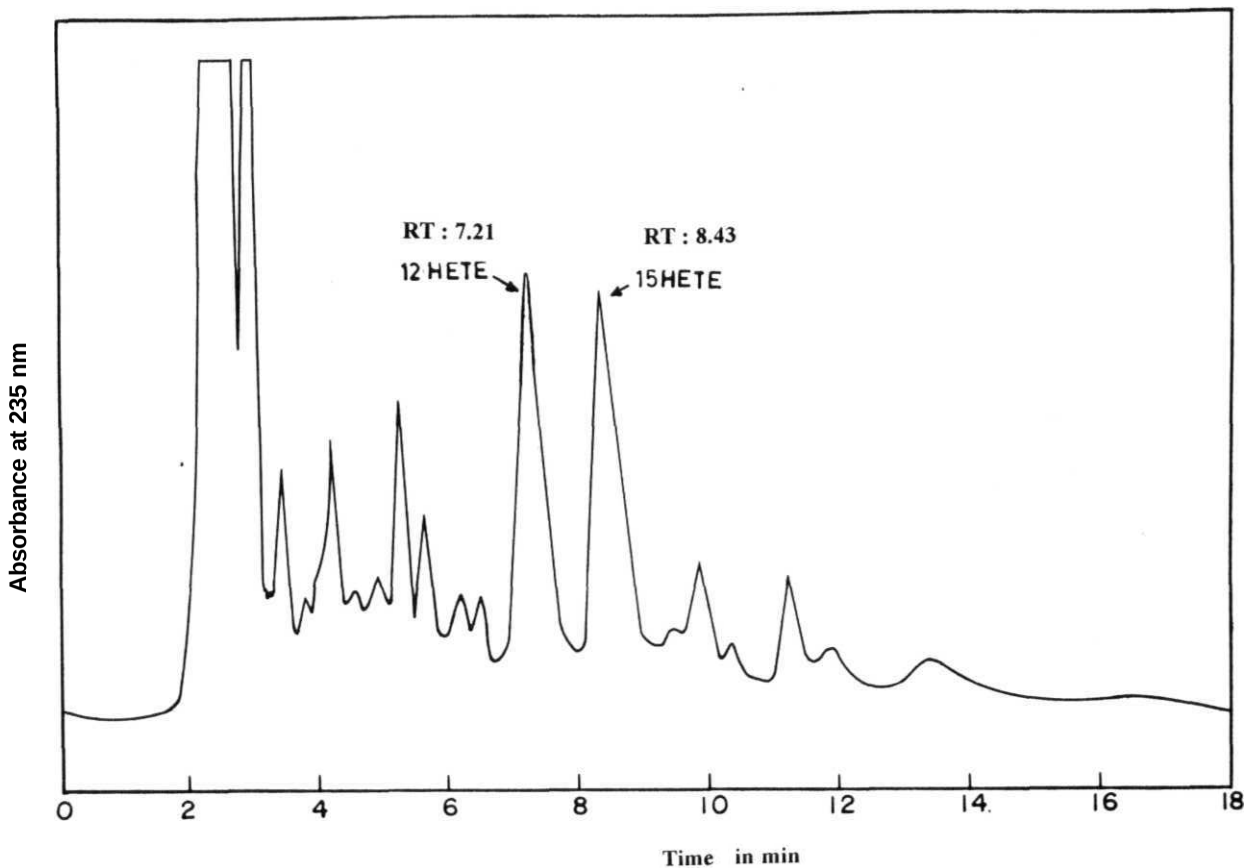
Sheep uterus lipoxygenase was incubated with arachidonic acid at pH 5.5 in citrate-phosphate buffer and the compounds were extracted into hexane: ether (1:1). The products were reduced with sodium borohydride under inert cold conditions and the reduced products were analyzed on SP-HPLC system. The separation of the oxidized metabolites is shown in Fig. 36. The major peaks with retention times 7.21 min and 8.43 min gave a typical conjugated diene spectra (Fig. 15) on the UV/VIS scanning spectrophotometer. The peak with retention time 7.21 min when injected with standard 12-HETE coeluted as a single peak The peak with retention time of 8.43 min coeluted with authentic 15-HETE suggesting that the two peaks are 12- and 15-HETEs. The positional specificty of the uterine dual lipoxygenase is shown in Fig. 37.

5.1.2.0. GC-MS analysis

The two major peaks of Fig. 36 with retention times 7.21 min and 8.43 min were collected from a number of runs and pooled. These were concentrated individually and methylated with diazomethane. The methyl esters after purification on straight phase HPLC, were trimethylsilylated using BSTFA . The trimethylsilylated derivatives were then analyzed on Hewlett Packard GC/MS. The fragmentation pattern of the peak 7.21 min is shown in Fig. 38 and the fragmentation pattern of peak 8.43 min is shown in Fig. 39. The peak with RT 7.21 min gave fragmentations at M/Z 391, 375, 295, 173 and 73 as expected for 12-HETE (Hamberg et al., 1974a). Peak with retention time 8.43 min was identified by the M^+ 406 and the fragmentation pattern of M/Z 391, 335, 225, 173 and 73

Fig.36:SP-HPLC analysis of AA products generated with uterine lipoxygenase. AA was incubated with uterine LOX and the products were extracted into **hexane** : ether, the organic extract was evaporated and **redissolved** in **SP-** ll PI.(' solvent system. The analysis conditions employed for separation were:

Column	:	**CLC-SIL**
Solvent	:	Hexane:propane 2-ol:acetic acid (1000:14:1)
Flow rate	:	**1mL/min**
Detection	:	235nm


RT : 7.21
12 HETE
RT : 8.43
15HETE
Absorbance at 235 nm
0
2
4
6
8
10
12
14
16
18
Time in min

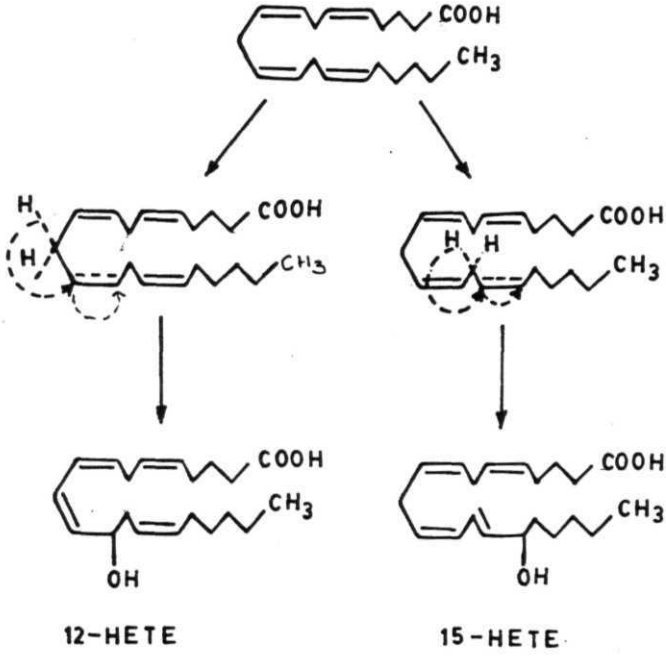
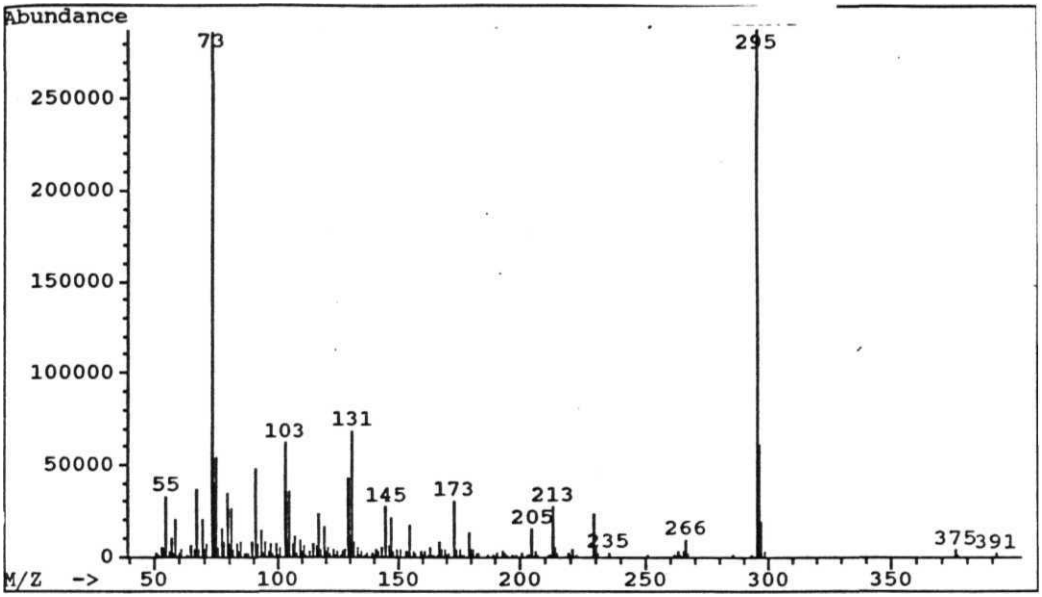
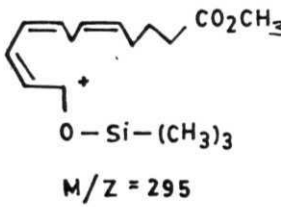


Fig.37:Scheme showing the possible carbon atoms of A A that can be oxygenated with uterine lipoxygenase.





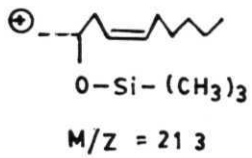
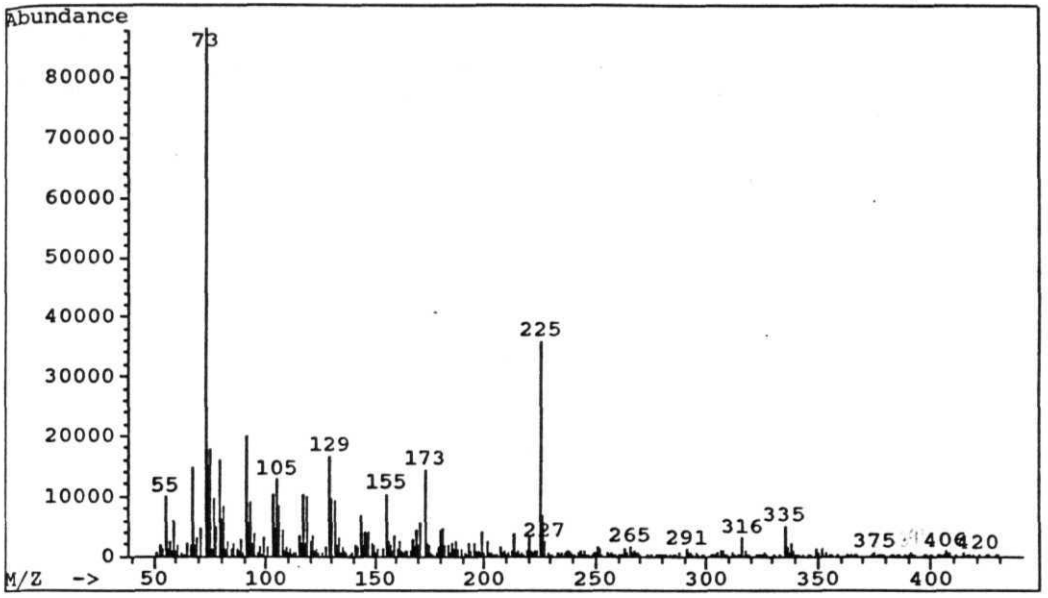
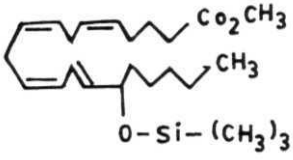
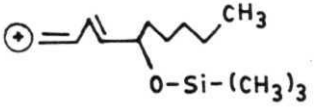


Fig.38:GC/MS analysis of Peak I (RT 7.21min) of Fig. 36.

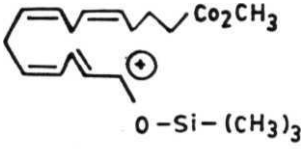




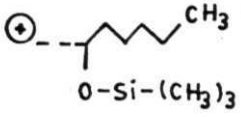
$M^{+} = 406$



M/Z = 225



M/Z = 335



M/Z = 173

Fig.39:GC/MS analysis of Peak II (RT 8.43min) of Fig. 36.

as 15-HETE (Hamberg et al., 1974b) The fragmentation analysis of both the peaks were compared with standards from the data bank and then confirmed as 12-HETE (peak with RT 7.21 min) and 15-HETE (peak with RT 8.43 min).

5.1.3.0.Effect of pH

The effect of pH on the regiospecificity of the uterus lipoxygenase was also checked and the HPLC profiles of the reduced products at different pH are presented in Figs. 40, 41, and 42. As shown in the chromatogram 15-HETE was formed in major quantity compared to 12-HETE at highly acidic conditions such as pH 3.5 (Fig. 40). However under alkaline conditions i.e at pH 9.0 the relative concentration of 12-HETE was higher than 15-HETE (Fig. 42) At its optimum pH i.e at 5.5 both the HETEs were formed in equal proportions (Fig 41). On either side of the optimum pH the total oxygenated products formed were decreased coinciding with decreased activities of the enzyme.

5.1.4.0. Uterus lipoxygenase with y-linolenic acid

The regiospecificity of the uterus lipoxygenase was also studied with y-linolenic acid (GLA) as the substrate which has three double bonds GLA was incubated with sheep uterus lipoxygenase at pH 5.5 in citrate-phosphate buffer and the products were extracted into hexane : ether (1:1) and reduced with sodium borohydride. The reduced products were analyzed on SP-HPLC system as described in the methodology The HPLC separation of the y-linolenic acid products is shown in Fig. 43. Two major peaks were obtained with retention times 8.73 min (peak I) and 10.37 min (peak II). These peaks were found to contain absorption max of 234 nm on UV/VIS spectral analysis. Peak I with retention time of 8.73 min co-eluted with 13-hydroxy octadecatrienoic acid (13-HOTrE). The peak II with retention time of 10.37 min was identified as 10-HOTrE based on the retention time and co-elation with standard 10-HOTrE. . The possible carbon atoms that could be oxidized by the lipoxygenase action on γ-linolenic acid is shown in Fig. 44.

Fig.40:SP-HPLC analysis of **A** A products generated with uterine lipoxygenase at **pH** 3.5.
AA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-HPLC solvent system. The analysis conditions employed for separation **w** ere.

Column	:	**CLC-SIL**
Solvent	:	**Hexane:propane** 2-ol:acetic acid (1000:14:1)
Flow rate	:	**1mL/min**
Detection	:	235nm

Fig.41:SP-IIPLC analysis of AA products generated with uterine lipoxygenase at **pH** 6.0
AA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-HPLC solvent system. The analysis conditions employed for separation **were.**

Column	:	CLC-SIL
Solvent	:	Iiexane:propane 2-ol:acetic acid (1000:14:1)
Flow rate	:	lmL/min
Detection	:	23Snm

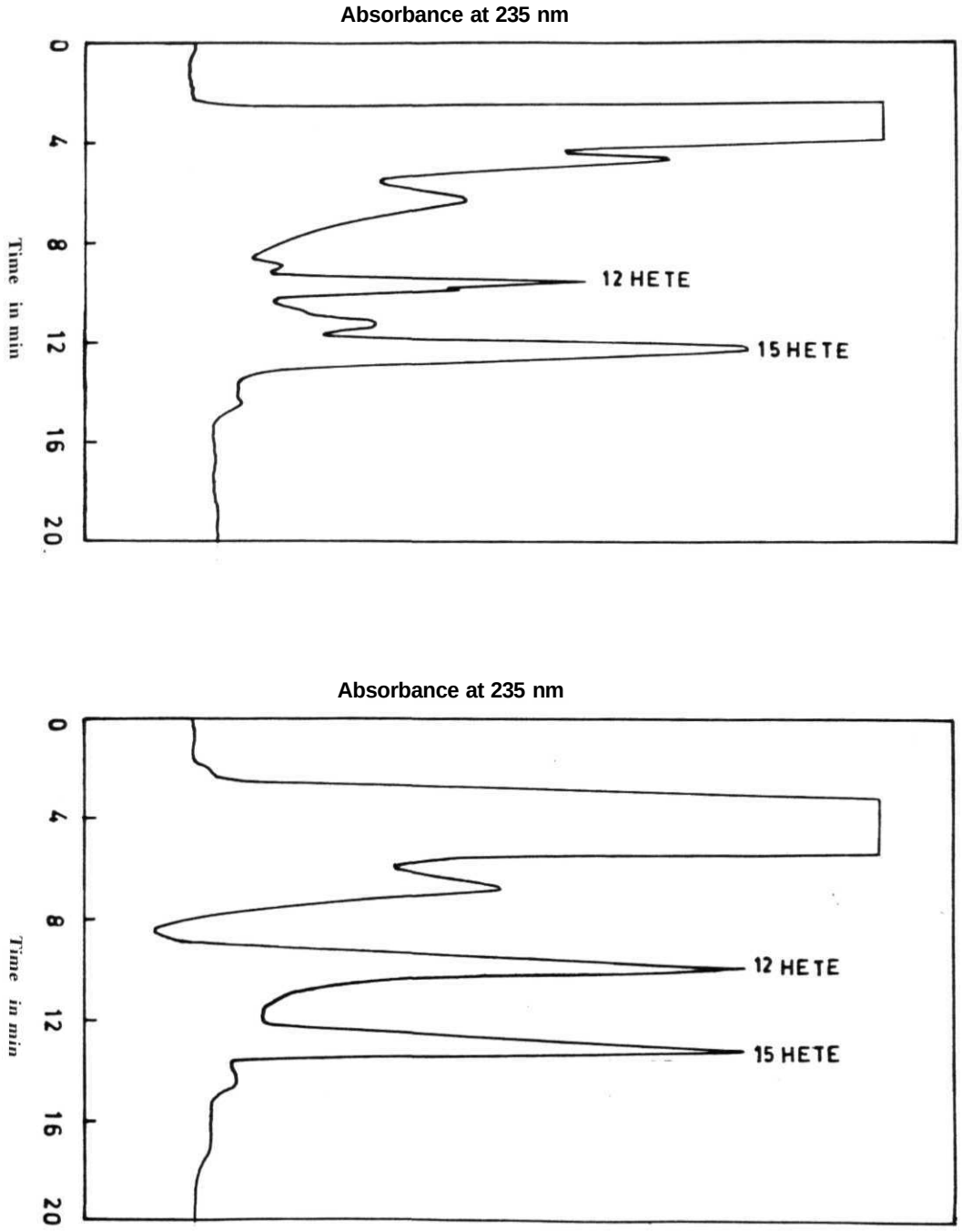

Absorbance at 235 nm
0
4
8
12
16
20
Time in min
12 HETE
15 HETE
Absorbance at 235 nm
0
4
8
12
16
20
Time in min
12 HETE
15 HETE

Fig.42:SP-IIPLC analysis of AA products generated with uterine lipoxygenase at pH 9.0

AA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-IIPLC solvent system. The analysis conditions employed for separation were.

Column	:	CLC-SIL
Solvent	:	Hexane:propane 2-ol:acetic acid (1000:14:1)
Flow rate	:	1mL/min
Detection	:	235nm

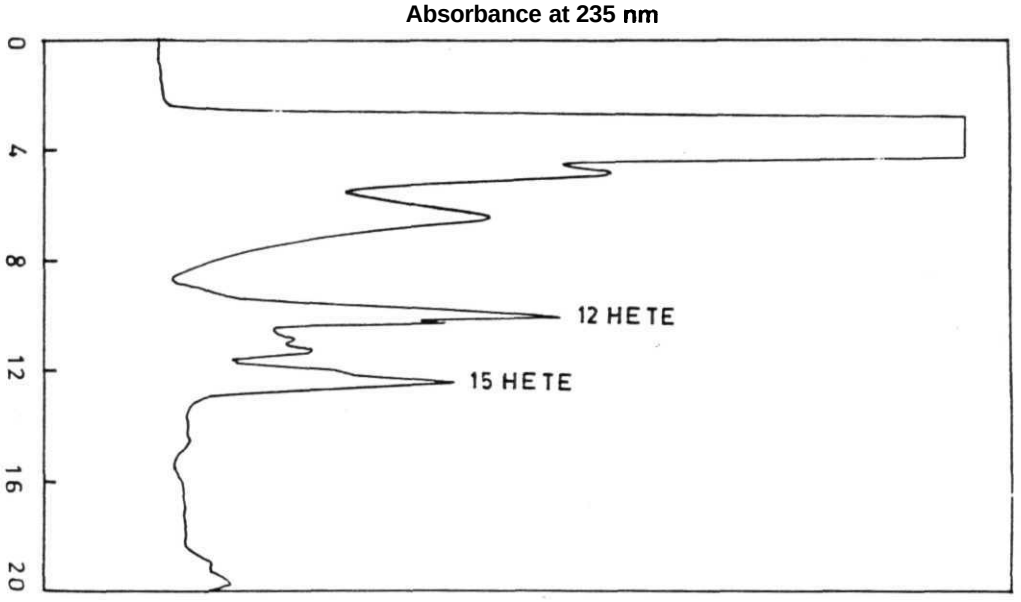
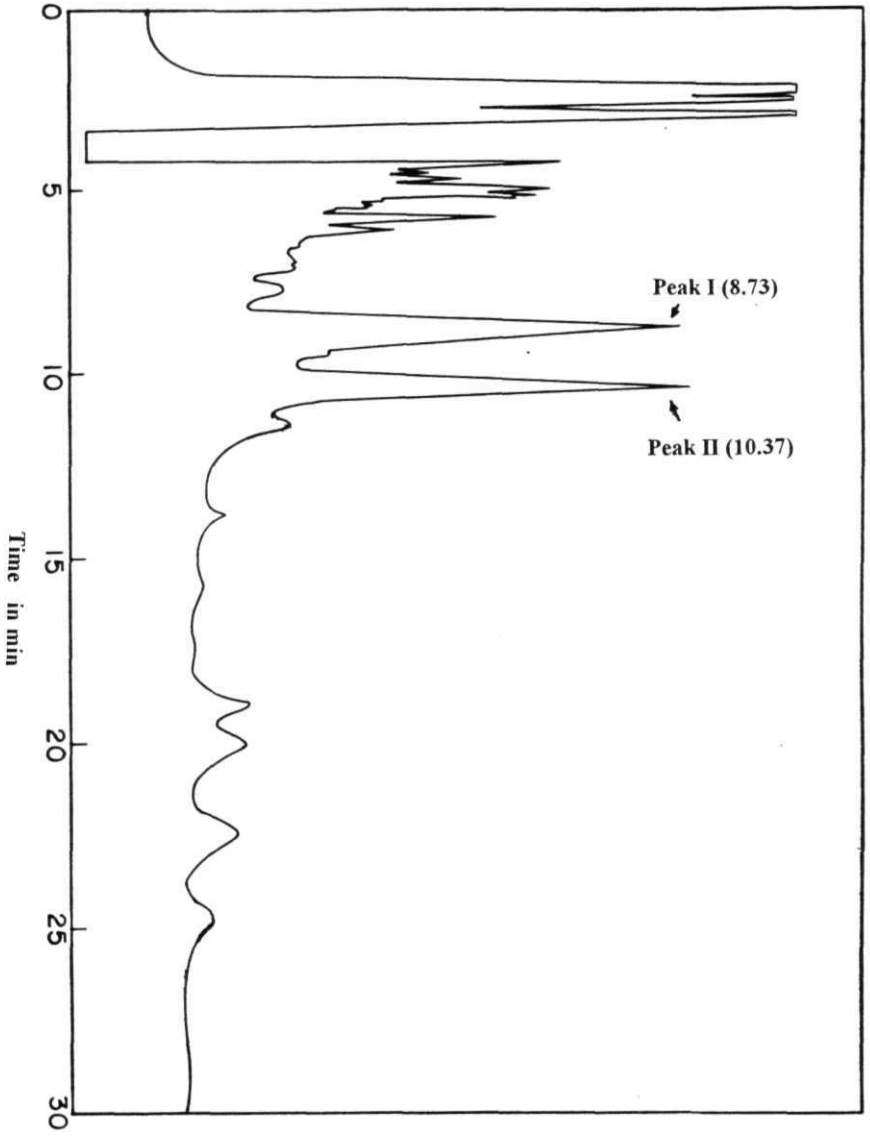

Absorbance at 235 nm
0
4
8
12
16
20
12 HETE
15 HETE

Fig.43:SP-HPLC analysis of GLA products generated with uterine lipoxygenase.

GLA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-HPLC solvent system. The analysis conditions employed for separation were:

Column	:	CLC-SIL
Solvent	:	Hexane:propane 2-ol:acelic acid (1000:14:1)
Flow rate	:	1mL/min
Detection	:	235nm


Absorbance at 235 nm
0
5
10
15
20
25
30
Time in min
Peak I (8.73)
Peak II (10.37)

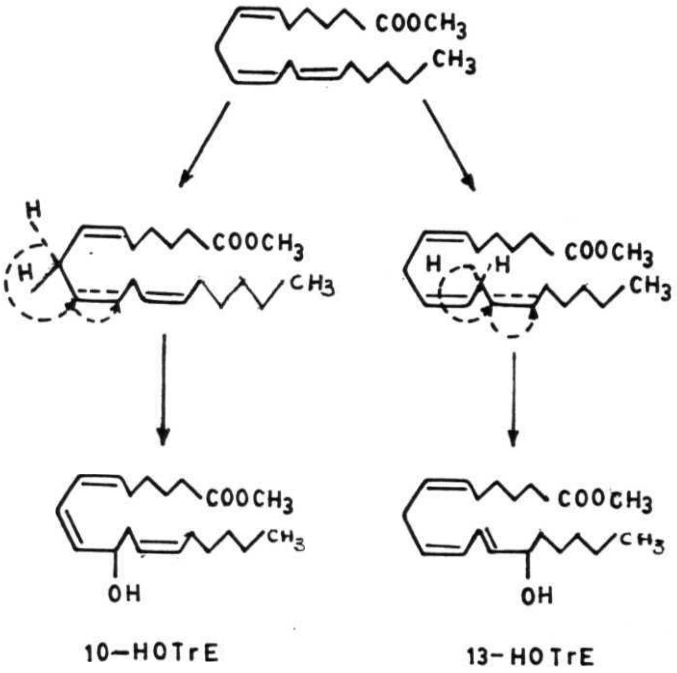


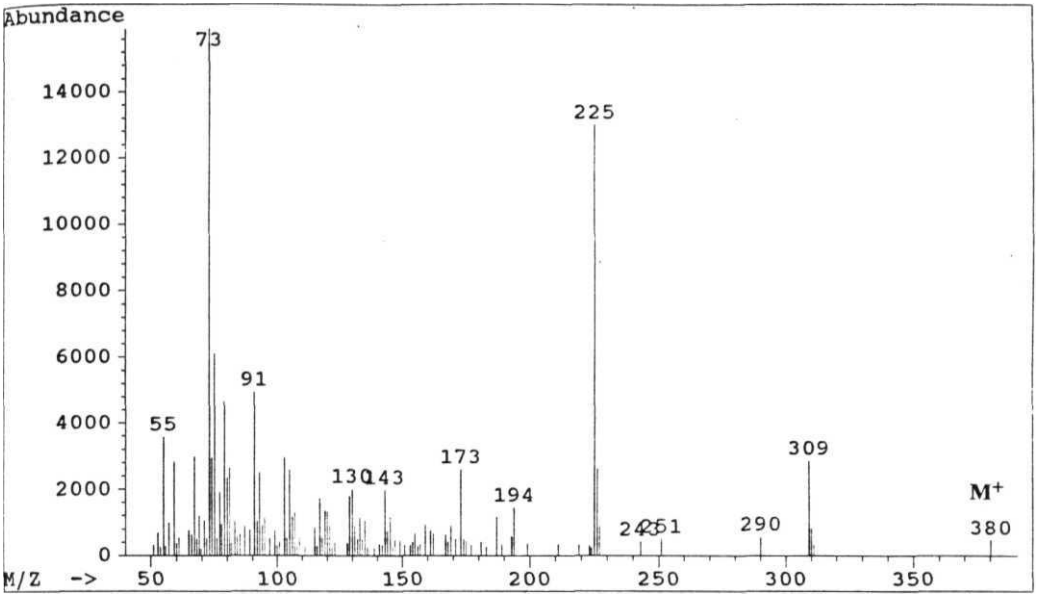
Fig.44: Scheme showing the possible carbon atoms of **GLA** that could be oxidized with uterus **lipoxygenase.**

In order to analyze the peaks on GC/MS, the products were generated in large quantities and after methylation and trimethylsilylation, they were subjected to GC-MS analysis as described in the methodology The fragmentation pattern of peak I i.e retention time of 8.73 min is shown in Fig. 45. The fragmentation pattern with M^+ at 380 and M/Z ions at 309, 290, 251, 243, 225, 173, and 73 confirm the peak as 13-HOTrE. The fragmentation pattern of peak II with retention time of 10.37 min is shown in Fig. 46. The M^+ at 380 and M/Z ions at 365, 269, 173, and 73 confirm this as lOHOTrE.

Thus sheep uterus lipoxygenase exhibits dual regiospecificity both with arachidonic acid and γ-linolenic acid. As a result of dual lipoxygenase activity the enzyme should be able to exhibit LTA4 synthase activity similar to that observed with leukocyte and potato lipoxygenases (Shimizu *et ah,* 1984).

5.1.5.0. 14,15-LTA_4 synthase activity

In view of the dual lipoxygenase activity (12- and 15-) expressed by the purified sheep uterus lipoxygenase, its role in leukotriene bio-synthesis was analyzed by reacting the enzyme with 15-HPETE as the substrate The products were extracted into hexaneether (1:1) and after evaporation analyzed on RP-HPLC. The effluent was monitored at 268 nm for the first 16 min and at 235 nm for the rest of the analysis period, so as to monitor the separation of both diHETEs and H(p)ETEs The products were eluted as two peaks mainly with retention times 6.06 min and 7.82 min when monitored at 268 nm (Fig. 47). These two peaks on spectral analysis showed a typical conjugated triene spectrum with λmax at 268 nm and shoulders at 258 nm and 278 nm (Fig. 47 inset). The peaks were methylated with diazomethane and trimethylsilylated with BSTFA. The methyl esters were then analyzed on Hewlett-Packard GC/MS. The fragmentation pattern gave a molecular ion peak of M^+ 494 and M/Z peaks at 333, 263, 243, 173 and 73 as expected for 8,15-diHETE (Fig. 48, Bild *et al.*, 1977). These two peaks were found to correspond with the hydrolyzed products of synthetic 14,15-LTA_4, thus confirming that the two peaks seen in Fig 47 are the hydrolyzed products of 14,15-LTA_4 i.e 8(S),15(R)-diHETE (Rt 6.06 min) and 8(S),15(S)-diHETE (RT 7.82 min). The unreacted 15-HPETE with the



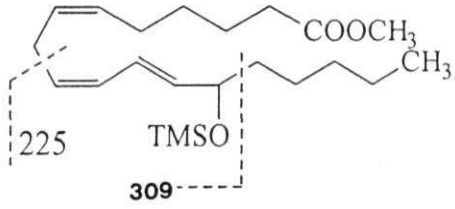
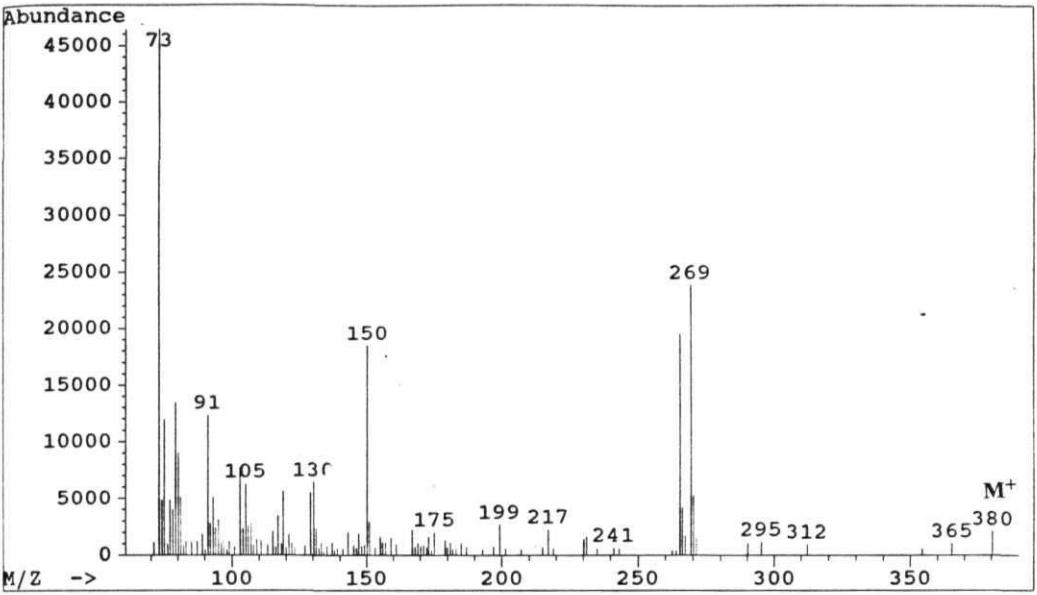


Fig.45:GC/MS analysis of Peak I of Fig. 43(13-HOTrE)



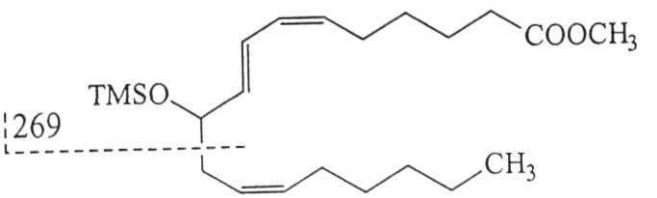
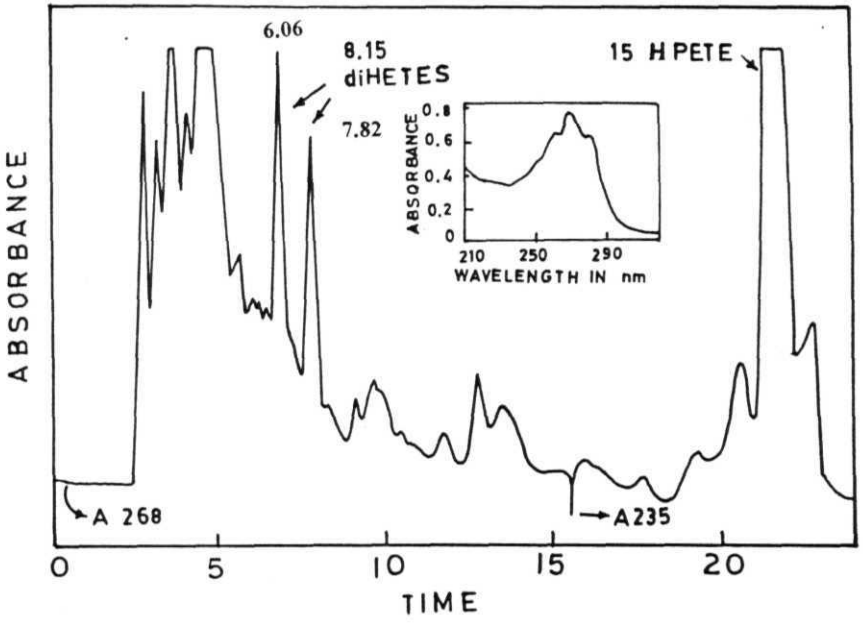
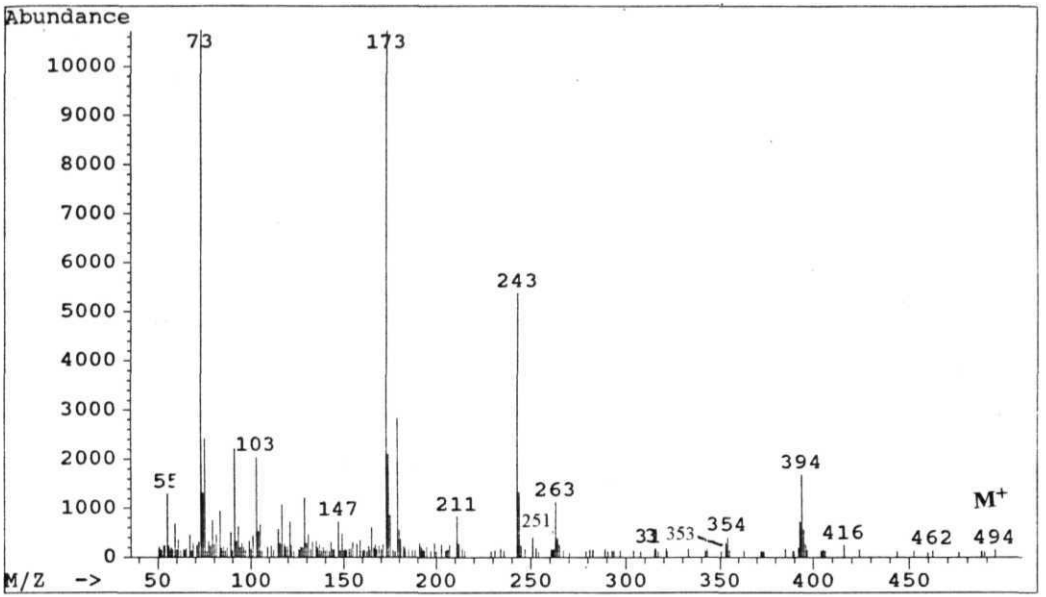


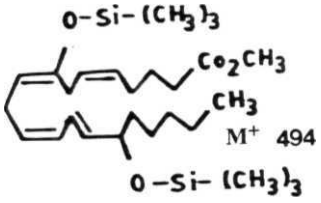
Fig.46:GC/MS analysis of Peak 11 of Fig. 43(10-HOTrE)

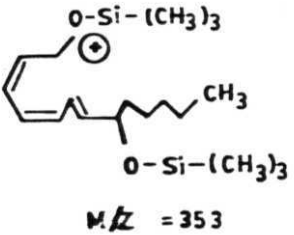
Fig.47:RP-HPLC analysis of 15-HPETE product profile reacted with uterus lipoxygenase: Assay of **14,15-LTA$_4$** synthase activity:
15-HPETE was incubated with uterine LOX in citrate-phophate pH 5.5 buffer. The products were extracted and evaporated under nitrogen. The residue was redissolved in **RP-HPLC** solvent system and analyzed as follows. The individual peaks were collected and checked for spectral analysis on scanning spectrophotometre.

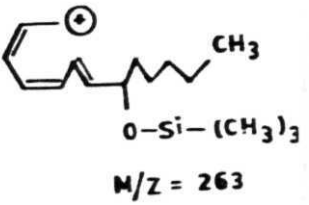
Column	:	CLC-ODS
Solvent	:	Methanol:water:acetic acid (68:32:0.1) **pH** 6.8
Flow rate	:	**1mL/min**
Detection	:	268 and **235 nm**

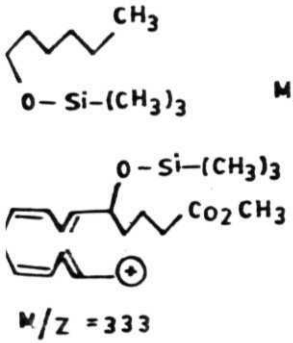

6.06
8.15
diHETES
7.82
15 HPETE
ABSORBANCE
0.8
0.6
0.4
0.2
0
210
250
290
WAVELENGTH IN nm
ABSORBANCE
A 268
A235
0
5
10
15
20
TIME

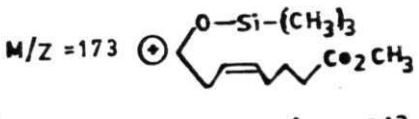












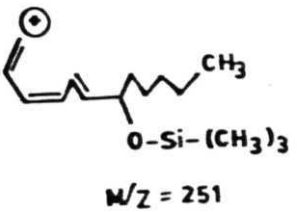
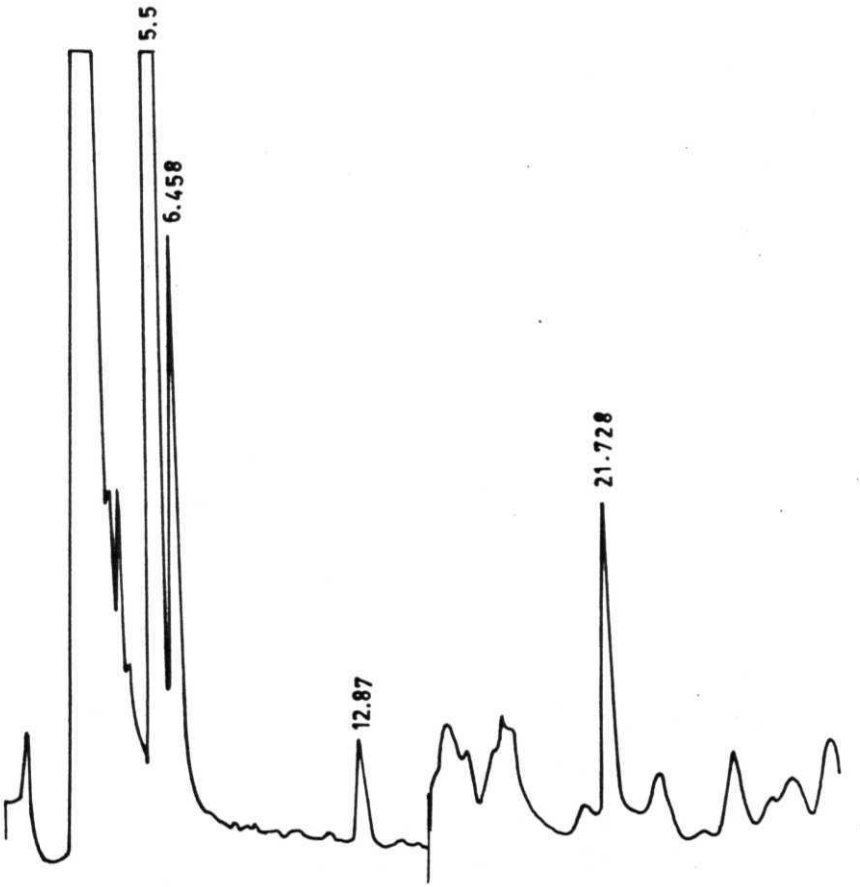


Fig.48:GC/MS analysis of 8,15-diHETEs.

Fig.49: RP-HPLC analysis of endogenous **15-HPETE,** made available with AA and uterine **15-lipoxygenase,** demonstrating endogenous 15-HPETE to be a better substrate for 14,15-LTA4 synthase activity:
AA was incubated with uterine LOX in citrate-phophate pH 5.5 buffer **for 10min** with excess of enzyme. The products were extracted and evaporated under nitrogen. The residue was redissolved in RP-HPLC solvent system and analyzed as follows. The individual peaks were collected and checked fr spectral analysis on scanning spectrophotometre.

Column	:	CLC-ODS
Solvent	:	**Methanol:water:acetic** acid (68:32:0.1) pH 6.8
Flow rate	:	**1mL/min**
Detection	:	268 and 235 **nm**


5.5
6.458
12.87
21.728
A 268
A 235

sheep uterine lipoxygenase eluted as a single peak with retention time of 21.7 5min. This peak was verified for its authenticity by co-chromatography with standard 15-HPETE and on scanning spectrophotometer.

5.2.0.0. Discussion

Lipoxygenases constitute a family of closely related non-heme iron containing dioxygenases that convert polyenoic fatty acids such as arachidonic acid or linolenic acid to their corresponding hydroperoxy derivatives. These enzymes are ubiquitously distributed both in animal and plants, to a higher degree in the latter. In mammalian cells most of the lipoxygenases are grouped into 4 types *viz* 5-lipoxygenases (leukocytes), two 12-lipoxygenases (platelet type and leukocyte type) and the 15-lipoxygenase (reticulocytes), (Yamamoto *et al.*, 1992). However, it should be stressed that the positional specificity of the lipoxygenase is not an absolute property but depends on the substrate structure and the reaction conditions such as temperature, pH and the nature of the solvent (Gardener, 1989). It has been shown, that with 15-HPETE as the substrate soybean lipoxygenase which is incidentally, 15- type exhibits 8- and 5-lipoxygenase activities producing 8,15-and 5,15- diHPETEs (Bild *et al,* 1977). Potato lipoxygenase is known to exhibit multi regiospecificity with arachidonic acid as the substrate producing 5-HPETE as the major product and other HPETEs such as 8-,9-,l 1-, 15- and 12- in minor quantities (Reddanna *et al,* 1990). PMNL lipoxygenase exhibits 5- and 8-lipoxygenase activities by which the enzyme possesses LTA4 synthase activity (Rouzer *et al,* 1985). The reticulocyte 15-lipoxygenase exhibits a minor 12-lipoxygenase activity and hence LTA4 synthase activity (Bryant *et al.*, 1985), whereas the leukocyte 12-lipoxygenase forms 15-HPETE as the minor product along with LTA4 synthase activity (Yokoyoma *et al,* 1986).

In the present study a cytosolic lipoxygenase from sheep uterus was isolated, that exhibits dual regiospecificity with arachidonic acid as the substrate, producing 12-and 15-HPETEs. The two products were formed in equal quantities at the optimum pH unlike other lipoxygenases reported so far (Murray *et al,* 1988). The sheep uterus lipoxygenase

also showed dual regiospecific with y-linolenic acid producing 10- and 13-HOTrEs. Platelet 12-lipoxygenase was also reported to produce two products with y-linolenic acid (Hamberg, 1983) and docosahexaenoic acid (Gardener 1989). This dual regiospecificity is also seen with reticulocyte 15-lipoxygenase on oxygenating with membranes producing 9- and 13-octadecadienoic acids (Kuhn *et al.*,1990).

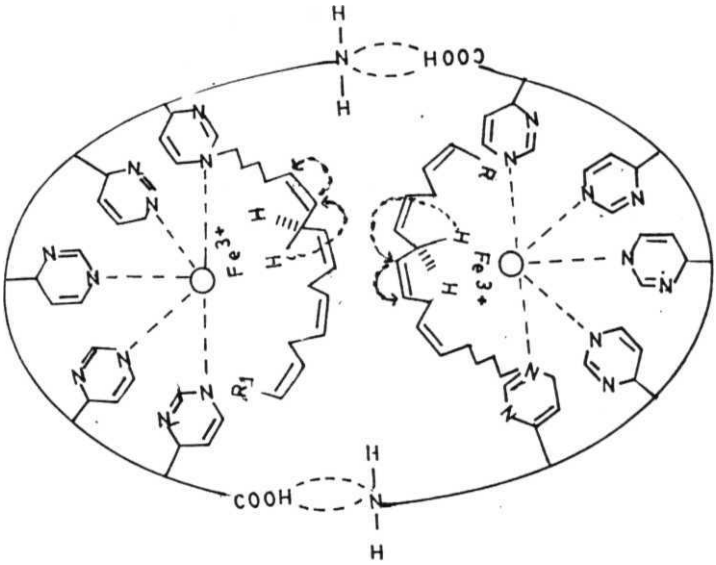
As a result of this dual regiospecificity of the sheep uterine lipoxygenase, the enzyme should exhibit 14,15-LTA_4 synthase activity as reported for other dual lipoxygenases (Yokoyoma *et al.*, 1986, Bryant *et al.*, 1985, Rouzer *et al.*, 1985). In the present study it was demonstrated that the purified lipoxygenase from sheep uterus exhibits 14,15-LTA_4 synthase activity when incubated with 15-HPETE as the substrate. 15-HPETE gets converted to 14,15-LTA_4 which immediately gets hydrolyzed into 8(S),15(R)-diHETE and 8(S), 15(S)-diHETEs (Sok *et al.*, 1982). The hydrolysis of synthetic 14,15-LTA_4 also yields the same two isomers of 8,15-diHETEs confirming that the 8,15-diHETEs seen in the present study are infact the hydrolysis products of 14,15-LTA4. The sequential action of 15- and 12-lipoxygenase activities of the uterine enzyme on arachidonic acid, thus could be responsible for the synthesis of 14,15-LTA_4 as per the mechanism shown in Fig. 9.

Apart from getting converted into 8,15-diHETEs a substantial amount of 15-HPETE was left over in the reaction with uterine lipoxygenase indicating, that not all the 15-HPETE provided externally is converted into 14,15-LTA_4. However, the 15-HPETE generated *in situ* was better utilized for the formation of 14,15-LTA_4 as evidenced by the formation of 8,15-diHETEs when uterine enzyme was incubated with arachidonic acid (Fig 49). From this it appears that the exogenous 15-HPETE is a poor substrate for the formation of 14,15-LTA_4. Similar observation was made in the case of 5,6-LTA4 formation, where only 20% of the exogenous substrate (5-HPETE) was found to be utilized for 5,6-LTA_4 synthesis (Puustien *et al*, 1987 and Rouzer *et al.*, 1986). The 14,15-LTA_4 synthase activity observed in the present study is exactly opposite to that of 5,6-LTA_4 synthase activity of leukocyte and potato lipoxygenase (Bryant *et al.* 1985, Mass *et al.* 1983; and Shimizu *et al.* 1984, Rouzer *et al.* 1985).

Although some lipoxygenases exhibit strict positional specificity multiple or dual lipoxygenase regiospecificity was also observed with several lipoxygenases including the present one (Gardner and Weisleder 1970, Bild *et al,* 1977, Yamamoto *et al,* 1980, Kuhn *et al,* 1985, 1990) But the mechanism of non-regiospecificity or multiregiospecificity of lipoxygenases is still largely unknown. It is known that the reduction of Fe^{3+} to Fe^{2+} allows the oxidation of the 1,4-butadiene systems of the substrate molecule by the abstraction of proton where antarafacial addition of O_2 molecule takes place and rearrange itself to a peroxy group giving 1 hydroperoxy 2,4-*cis, trans* pentadiene systems. It is also known that the reduced iron moiety dissociates itself from the histidine group and the protein gets degraded into smaller mol.wt peptides or fragments (Nelson *et al.,* 1991 and Rouzer *et al,* 1986), there by making it impossible to assume that the enzyme molecule could have another lipoxygenation capacity. The dual regiospecificity of uterus lipoxygenase observed in the present study can only be explained in terms of dimeric nature of the enzyme. As pointed out in the first chapter, only the dimeric enzyme is catalytically active The two enzyme molecules are attached either by hydrophobic nature of the lipoxygenase (Funk *et al,* 1989; Sigal *et al,* 1990) or by electrostatic attractions of the charges present on the enzyme. The carboxylic or terminal group of one protein molecule might be attached to the amino or the terminal group of the other molecule in head to tail arrangement.

The cup like structure formed by the attraction of two molecules could give a neutral charge to the whole molecule as seen in case of the sheep uterine lipoxygenase This dimeric enzyme could oxidize two molecules of substrate at any given time simultaneously, one on either side. Further the orientation of the first substrate molecule might expose C-13 carbon atom for oxidation, possibly restricting the orientation of the 2nd molecule of the substrate to C-10 carbon leading to the production of 15-and 12-HPETEs and thus exhibitingdual regiospecificity. The proposed model to explain dimeric nature and dual regiospecificity is presented in Fig. 50.

Fig.50: Hypothetical working model of uterine lipoxygenase to explain dual **regiospecificity, dimeric** form and neutral nature **of** the uterine LOX. (original model was from Solane et **al.,** 1990)


H
N
H
COOH
Fe3+
H
H
R1
R
H
Fe3+
H
COOH
H
N
H

Chapter-3

6.0.0.0.Introduction

In the preceding chapter data on the structural and functional organization of sheep uterus lipoxygenase was presented In this chapter an attempt is made to understand the significance of such an unique lipoxygenase in the uterine tissue.

It is known that there is a selective retention of arachidonic acid, docosahexaenoic acid, dihomo γ-linolenic acid and eicosapentaenoic acid in the phospholipid moiety of the uterine tissue membranes irrespective of diet and age (Leaver *et al,* 1992, Booney *et al,* 1992). These unsaturated fatty acid substrates could be directly oxygenated by lipoxygenase as in the case of erythroid cells and cause a change in membrane permeability (Schewe *et al,* 1991) which brings about changes in the hemodynamics of the cell, and aids in calcium influx (Cantabrana *et al,* 1991). Reticulocyte lipoxygenase is known to act on phosphatidylcholine to give 15-hydroperoxy product (Jung *et al,* 1985), phosphotidylinositol, 14,15-epoxides and diHETEs directly (Ballon *et al,* 1987). The supplemented γ-linolenic acid by diets is known to be incorporated into the triacyl glycerols in the form of dihomo y-linolenic acid (Gimeno *et al,* 1989) and its 15-lipoxygenation product inhibits the LTB4 formation (Navarette *et al,* 1992, Irvesen *et al,* 1992, and Fischer *et al,* 1992). These products alongwith 12- and 15-HETEs are responsible for suppressing the inflammatory responses in skin (Ziboh, 1992). The depletion of arachidonic acid at the site of implantation shows the importance of its metabolic products in fertilization and implantation of the embryo (Moulton *et al,* 1989).

Lipoxygenase activity was known to be present in uterus from the early 80's (Demers *et al,* 1984) and was found to be regulated by estradiol pathway (Thaler *et al,* 1985). The activity levels and substrate levels at different stages of the estrous cycle would lead to the understanding of the physiological significance of the enzyme in regulating the sexual cycles.

The study on endogenous products at different stages of estrous cycle would provide vital information on the role of lipoxygenase products in uterine physiology. Hence in this chapter an attempt is made to understand the physiological role of abundant

dual lipoxygenase by analyzing fatty acid profile, lipoxygenase activity levels, LTA4 synthase activity and endogenous products at different stages of estrous cycle

6.1.0.0. Results

The unsaturated fatty acids present in the uterine tissue were analyzed by gas chromatography (GC). The endogenous unsaturated fatty acids were extracted, both from the cytosol and microsomal membranes, into methanol : chloroform mixture (3:1 vol/vol). The fatty acids after methylation were separated on GC GC profile of the standard fatty acids is shown in Fig. 51. Relative concentrations of various endogenous fatty acids present in cytosol and microsomes were estimated by using the above technique, the data is presented in Tables III and IV Arachidonic acid was found to be the most predominant fatty acid present in microsomes of uteri at various stages of the cycle However in the cytosol, LA is the predominant PUFA followed by AA. The relative concentrations of arachidonic acid in both the cytosol and microsomes were shown in Fig. 52. The maximum amount of arachidonic acid was present in the microsomes of luteal stage or secretory phase followed by follicular stage and pregnancy stages. However the cytosolic levels of arachidonic acid were found to be increasing from nulliparous to late pregnancy stages.

DHGLA, EPA, and DHA were found to be present in the cytosol as well as in the microsomes of uterine tissue suggesting their importance in complex uterine physiology, despite being found in low quantities

6.1.1.0.Lipoxygenase activity levels in uterus at different **stages** of **estrous cycle**

The activity levels of lipoxygenase at various stages of the estrous cycle were determined and the data is presented in Fig. 53. The enzyme was found to be highly expressed in luteal stage or the secretory phase of the estrous cycle, where the uterus is under the influence of corpus luteum which secretes progesterone. The activity levels were found to be 3 fold higher than other stages of estrous cycle such as follicular, nulliparous and pregnancy stages. The activity levels at other phases of the cycle remained more or less the same.

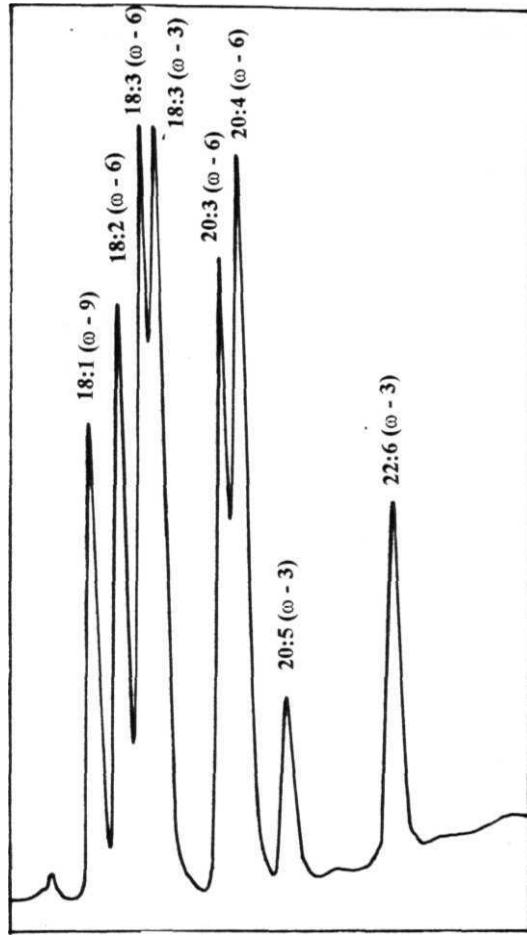


Fig.51:Separation of standard PUFAs on gas chromatograpliy.
PUFAs were methylated using BI_3 **methanol** and analyzed on GC as described in the methodology.

Table III:

Relative concentrations of various PUFAs in uterine cytosol as analyzed on GC.

Stage	LA	AA	DHGLA	EPA	DHA
Nulliparous	11.65	4.7	2.4	1.6	3.3
Follicular	7.5	5.4	0.95	0.87	2.19
Luteal	5.5	7.6	1.72	0.76	1.37
Pregnancy					
Stage I	11.5	8.6	3.1	1.2	2.4
Stage II	12.7	12.9	1.74	0.85	2.7

Table IV:

Relative concentrations of varous PUFAs in uterine microsomal membranes as analyzed on GC.

Stage	LA	AA	DHGLA	EPA	DHA
Nulliparous	9.07	11.99	0.07	0.9	0.9
Follicular	7.37	14.5	0.05	0.9	1.1
Luteal	5.9	19.13	0.01	0.9	1.3
Pregnancy					
Stage I	5.4	8.38	0.03	0.9	0.57
Stage II	5.1	8.9	0.04	0.9	0.8

(The data is obtained from a typical representative uterine sample at each stage of the cycle).

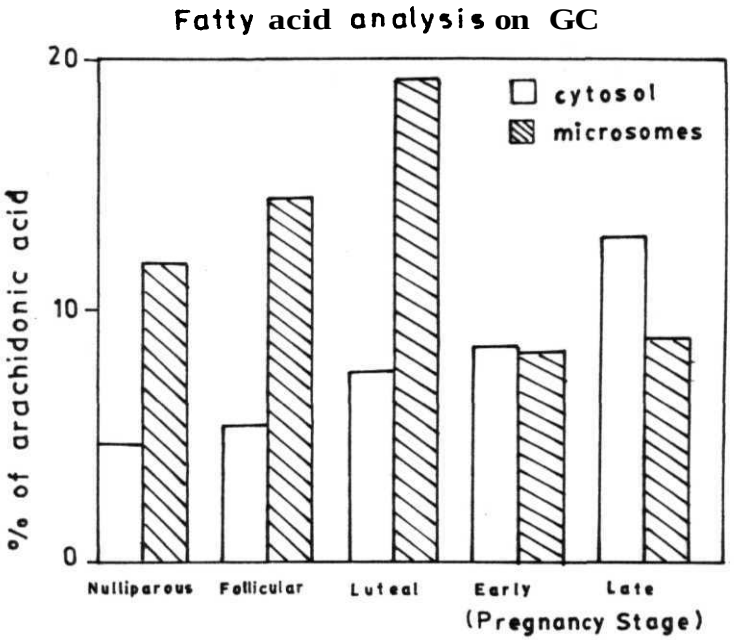


Fig.52:Figure depicting relative concentrations of AA in uterus cytosol and microsomes.

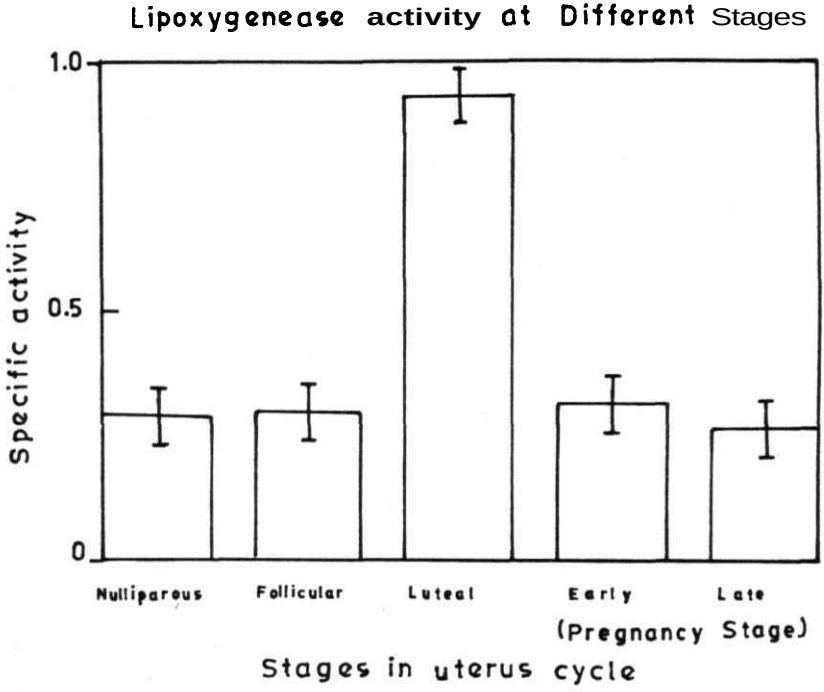


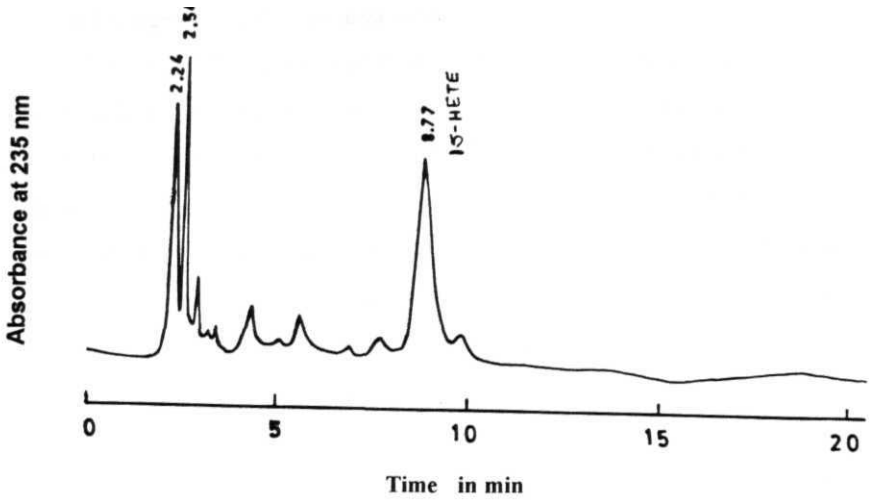
Fig.53: Activity levels **of lipoxygenase** in difTerent phases of estrous cycle in sheep.

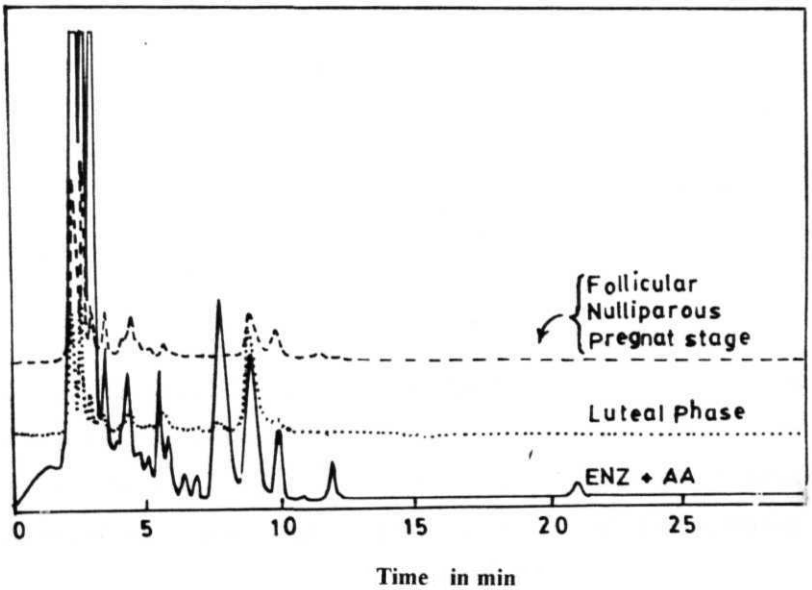
Fig.54:SP-HPLC analysis of endogenous mono'ıydroxides at • luteal phase.

The endogenous metabolites were extrac-ted into hexane :ether after acidfying the homogenate and precipitating the proteins. The organic extract was evaporated to dryness and the residue was dissolved in SP-HPLC solvent system and analyzed in the following conditions:

Column	:	CLC-S1L
Solvent	:	Hexane:propane 2-ol:acetic acid (1000:14:1)
Flow rate	:	1mL/min
Detection	:	235nm

Fig.55: Overlaying chromatogram of SP-HPLC analysis of endogenous monohydroxides at different stages of estrous cycle.


Absorbance at 235 nm
2.24
2.51
8.77
15-HETE
0
5
10
15
20
Time in min


Follicular
Nulliparous
pregnat stage
Luteal Phase
ENZ + AA
0
5
10
15
20
25
Time in min

6.1.2.0. Endogenous arachidonic acid metabolites

The oxygenated metabolites of the PUFAs present in the uterine tissue were extracted from the 10,000 X g supernatants of the sheep uterus, and analyzed on SP-HPLC for the identification of monohydroxides, and on RP-HPLC for the identification of dihyroxides. The data on endogenous hydroxy metabolites of the uterus at luteal phase of the reproductive cycle is presented in Fig. 54. As shown in the chromatogram, a prominent peak with RT of 8.77 min was obtained with typical conjugated diene spectrum. The peak was identified as 15-HETE by co-chromatography and GC/MS analysis which gave a fragmentation pattern similar to the standard 15-HETE. A comparison of the relative concentrations of the endogenous hydroxy metabolites was also taken up from other phases of the estrous cycle and the chromatograms are presented in Fig. 55. As shown in the figure 15-HETE was found to be present in comparatively higher concentrations in luteal phase than follicular, nulliparous and pregnancy stages An important observation is the conspicuous absence of 12-HETE among the endogenous products, though the same was formed from uterus lipoxygenase *in vitro*. The RP-HPLC analysis of the endogenous products only showed the presence of 8,15-diHETE, that eluted out with RT 5.5 min, and 15-HETE that eluted out with RT 21 min, as observed in luteal phase (Fig 56). The peak with RT 5.5 min gave a typical conjugated triene spectrum with absorption maximum of 268.5 nm.

6.1.3.0. Endogenous leukotrienes

The peptido leukotrienes, known for their uterotonic effects have been extracted from sheep uterus homogenate, and analyzed on RP-HPLC as described in the methodology. The products when separated on RP-HPLC gave a peak with retention time of 6.31 min (Fig. 57) with absorption maximum of 280 nm and shoulders at 270 and 290 nm (Fig. 58) The absorption spectrum is typical of any peptido-leukotriene. In order to find the identity of the endogenous peptido-leukotriene, the peak with RT 6.31 min was co-chromatographed with different 5,6- and 14,15-peptido-leukotrienes. As shown in the Fig. 59, the 6.31 min peak co-eluted with standard 14,15-LTC_4. These results confirm the

Fig.56: RP-HPLC analysis of endogenous AA metabolites.

The endogenous metabolites were extracted into hexane :ether after acidifying the homogenate and precipitating the proteins. The organic extract was evaporated to dryness and the residue was dissolved in RP-HPLC solvent system and analyzed:

Column	:	**CLC-ODS**
Solvent	:	**Methanol:water:acetic** acid **(68:32:0.1) pH** 6.8
Flow rate	:	**1mL/min**
Detection	:	268nm and **235nm**

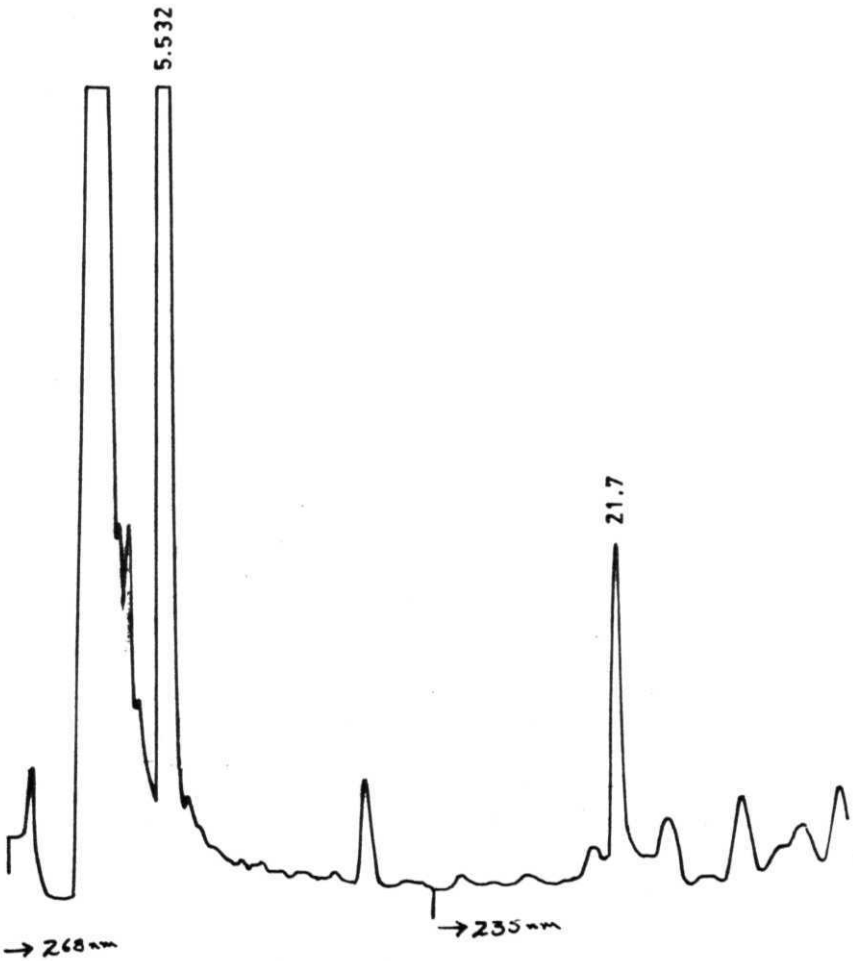
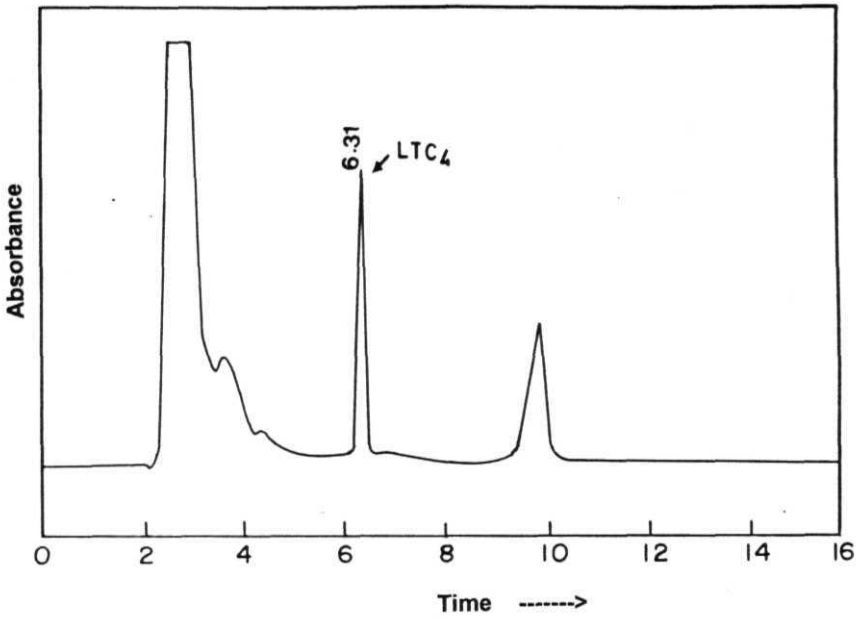

5.532
21.7
→ 268 nm
→ 235 nm

Fig.57: RP-HPLC analysis of endogenous peptido **leukotrienes.**

Endogenous leukotrienes were extracted according to the methodology described in the text; The following conditions were employed for the analysis **of** the endogenous leukotrienes, the individual peaks were collected and checked for spectral analysis on scanning **spectrophotometer.**

Column	:	CLC-ODS
Solvent	:	**Methanol:water:acetic** acid (70:30:0.1) **pH** 6.0
Flow rate	:	**1mL/min**
Detection	:	280nm


Absorbance
6.31
LTC4
0
2
4
6
8
10
12
14
16
Time ------->

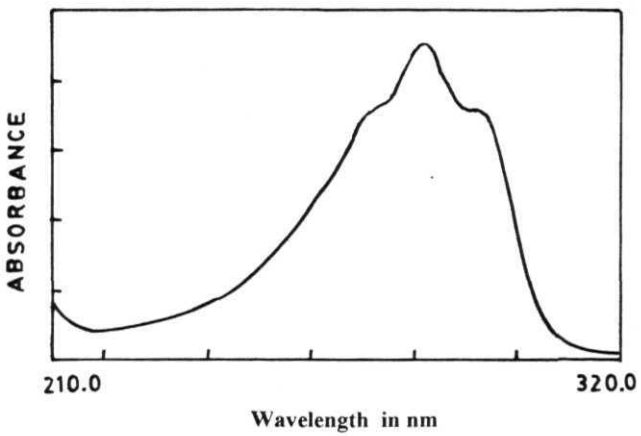


Fig.58: Spectral analysis of peak with RT 6.31min of Fig. 57.

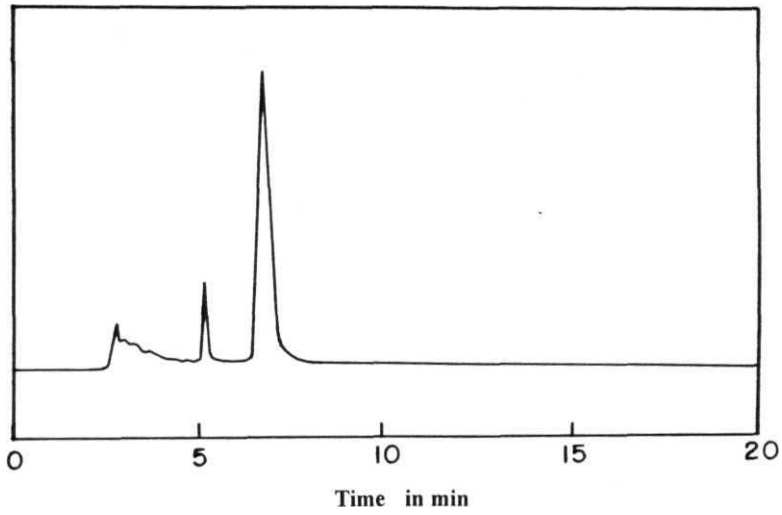
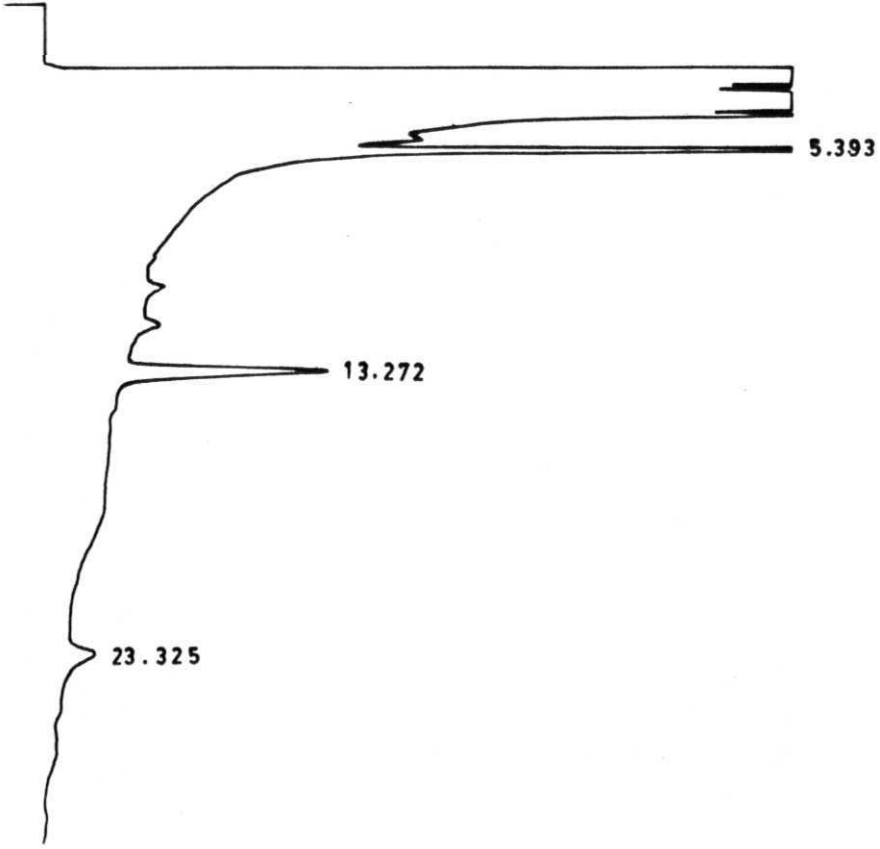


Fig.59:Co-chromatography of peak with RT 6.31min and standard 14,15-LTC_4.

Fig. 60: Assay of **LTC_4synthase** activity of sheep uterus particulate proteins using **14,15-I TA_4** Me as the substrate.

14,15-LTA_4 Me was reacted with uterus microsomal membrane proteins in presence of glutathione and the products were extracted into cold methanol and analyzed on **RP-HPLC.**

Column	:	CLC-ODS
Solvent	:	**Methanol:water:acetic** acid (70:30:0.1) pH 6.0
Flow rate	:	**1mL/min**
Detection	:	280nm


5.393
13.272
23.325

presence of 14,15-LTC_4 as an endogenous component of the bio-active lipids found in sheep uterus. 5,6-LTC_4 under similar RP-HPLC conditions eluted much later than that of 14,15-LTC_4.

6.1.4.0. LTC4 synthase activity

The presence of endogenous 14,15-LTC_4 and the formation of 14,15-LTA_4 by dual regiospecificity of sheep uterus lipoxygenase suggests the presence of LTC4 synthase activity in the sheep uterus. In order to test the activity of LTC4 synthase, 14,15-LTA4 Me was incubated for 10 min with sheep uterus microsomal membrane proteins (105,000 X g pellet) in the presence of glutathione. The reaction products were extracted and then separated on RP-HPLC as per methods described in the methodology. As shown in the chromatogram (Fig. 60) only the peak with RT 5.94 min gave a typical peptido leukotriene spectrum (Fig 58) The peak with RT 5.94 min was found to co-elute with 14,15-LTC_4 Me standard. On other hand 5,6-LTA_4 free acid and its methyl ester were found to be poor substrates for the LTC4 synthase present in the sheep uterus (data not shown).

6.2.0.0. Discussion

In the first chapter, an unique lipoxygenase from sheep uterus was isolated and purified to apparent homogeneity. Also the structural organization of the purified lipoxygenase was described. In the second chapter the functional organization of the lipoxygenase was presented. The purified uterus enzyme with dual (15- and 12-) lipoxygenase activity was shown to synthesize new series of leukotrienes (14,15-LTs). The lipoxygenase with dual regiospecificity was shown to catalyze the conversion of arachidonic acid to HPETEs and further to leukotrienes by sequential peroxidation and dehydrase action producing an epoxide These products of the lipoxygenase pathway were implicated in many physiological functions, such as defense mechanism, inflammation, myometrial contractions and luteolysis.

As expected, arachidonic acid was found to be the predominant polyunsaturated fatty acid present in all stages of uterine tissue microsomes with traces of other fatty acids such as EPA, DHA, and DHGLA. Thus among the endogenous products identified the arachidonic acid metabolites were predominant.

The high levels of lipoxygenase activity seen in luteal phase of the estrus cycle is in correlation with high amounts of arachidonic acid present in both the cytosol and microsomal membranes. The high Km values for arachidonic acid obtained in the present study (chapter I) could be due to the presence of high levels of arachidonic acid in the tissue. It is also known that phospholipase A_2 activity, which is responsible for the liberation of arachidonic acid from phospholipids, is higher in the luteal phase than the proliferative phase (Bonney *et al,* 1992). The liberated arachidonic acid, when acted upon by the uterus lipoxygenase, should get converted into 12- and 15-HETEs. However only 15-HETE was identified among the endogenous products extracted from sheep uterus. In all in vitro studies both the 12- and 15- HETEs were generated when uterus lipoxygenase was incubated with arachidonic acid (chapter II). From this it appears that 12-HETE formed in vivo is utilized or diverted to other pathways. The acidic conditions prevailing in the uterus must be favoring the formation of 15-HETE as observed in the present study (Chapter II)

These lipoxygenase products are known for their powerful vascular effects and control of blood flow in the feto-placental unit and implantation bed (Parasi *et al,* 1986). 12-HETE is known to stimulate the generation of LTB_4, a known inflammatory mediator (Bray *et al,* 1981). Also it is reported to play an important role in implantation of the fertilized egg with its role highly regulated in the fetal and utero-placental hemodynamics and immunological adaptations of pregnancy (Mitchell *et al,* 1987; Radmark *et al,* 1980) LTB_4 is also responsible for the chemokinesis, chemoattraction and aggregation of leukocytes in secretory phase of the estrus (Bray *et al,* 1981), and eoisnophils in estrogen primed uterus (Benitez *et al,* 1989). The endogenous 8,15- diHETEs seen in the RP-HPLC analysis (Fig. 56) could be perfoming similar actions.

The lipoxygenase products are known for their myometrial contractions in uterus (Carraher *et al,* 1983). Luteolysis was found to be delayed when lipoxygenase pathway was inhibited by NDGA, a specific lipoxygenase inhibitor, suggesting the importance of this pathway in the regulation of the estrous cycle (Hamilton *et ed.,* 1990, Mitchell *et al.,* 1987).

The infiltration of blood (leukocytes) flow caused by the lipoxygenase products in luteal phase is known to increase the oxygen tension in the surrounding milieu, which in turn leads to increased expression of LTA4 synthase activity (Ohawada *et al,* 1990), resulting in the formation of increased production of 14,15-LTA_4. This epoxide being unstable gets hydrolyzed to 2 isomers of 8,15-diHETEs, or gets converted to LTB4, or upon addition of glutathione transforms into LTC4 (Radmark *et al .,* 1980; Bach *et al,* 1984; Jakschik *et al,* 1983; 82).

The biologically active leukotrienes (5,6-LTs) could be acted upon by the 15-lipoxygenase of the uterus producing 15-hydroperoxy leukotrienes, that are least potent in their biological actions (Sirosis.P., 1979; Vonakis *et al,* 1992; Orning *et al.,* 1983).

So the dual lipoxygenase of sheep uterus lipoxygenase could perform both the roles of aggregatory and anti-aggregatory of inflammation i.e, have the double role of an auto inhibitory effect on the metabolism of arachidonic acid pathway in sheep uterus.

The endogenous 14,15-LTC_4 identified in the present study supports the operation of 14,15-LTA_4 pathway in uterus (Fig. 57). The formation of peptido leukotrienes and activation of its receptors in the smooth muscles of uterus are known to trigger its contraction (Chegini *et al,* 1988a; 1988b, Cheng *et al,* 1985) in estrogen primed uterus and in preimplantation periods (Ledwozyw *et al,* 1989; Malathy *et al,* 1986), suggesting that these products act in concert with normal uterine hormones in establishing the complex physiology of uterus and in general female reproductive system It is also known that, there is an interaction between leukotrienes and prostaglandins in the induction of uterine decidual response (Tawfik *et al,* 1987a; 1987b; Gupta *et al,* 1990) .

The endogenous 14,15-LTC_4, observed in the present study indicates the conversion of 14,15-LTA_4 to 14,15-LTC_4 catalyzed by an 14,15-LTC_4 synthase. In fact the sheep uterine microsomes alone showed the conversion of 14,15-LTA_4 to 14,15-LTC4 Me, supporting the presence of LTC4 synthase in the tissue. Further studies, however, are required to isolate and characterize the nature of 14,15-LTC_4 synthase in the uterus.

The cysteinyl leukotrienes thus formed are known to effect smooth muscle contractions as seen in case of guinea pig ileum (Lewis *et al,* 1984). Elevated levels of these peptido-leukotrienes (LTC4) were found to be responsible for the contraction of bronchial muscles (Sally *et al.,* 1990). LTD4 was shown to be the major mediator of asthma. Thus LTC4 and its metabolic products are responsible for the contractile properties of the smooth muscles. In view of generally known smooth muscle contractile properties of peptido-leukotriene, and their formation in the uterus observed in the present dtudy, it can be suggested that these may be playing a vital role in uterine contractions, either in the transport of gametes or in the parturition process (Vane and Williams, 1973; Borell *et al.,* 1986). It was also shown that the oxytocin induced contraction, were found to be inhibited when specifically LTC4 synthase inhibitor, ethacrynic acid, is employed (Leung, 1986, Vallina *et al.,* 1991). These reports support the possibility of involvement of peptido-leukotrienes in the normal parturition process. However it is widely known that $PGF_{2\alpha}$ is the major uterine contractile factor involved in the parturition process. The present results and the literature evidence challenge the dogma that the prostaglandins are universal mediators of labor.

Further the lipoxygenase activity in the uterus as observed in the present study, is much higher than PGH synthase (data not shown), the enzyme involved in the formation of $PGF_{2\alpha}$ in the uterus, which may be involved in uterine contractions and probably in labor. Further studies, however, are required to analyze the levels of peptido-leukotrienes and $PGF_{2\alpha}$ during labor

Estrogen is known to induce uterine growth, and epidermal growth factor is an inducing agent of growth in uterus and female reproductive tract (Nelson *et ed.,* 1991) Inhibition of cyclooxygenase and lipoxygenase pathway in combination was found to inhibit the estrogen and epidermal growth factor induced uterine growth, suggesting the involvement of eicosanoids in estradiol action. The potential candidates involved in uterine growth could be the hydroxymetabolites of arachidonic acid, such as 15-HETE. It is known that the hydroxymetabolites of arachidonic acid are involved in the mitogenic responses in cultured cells (Kiran kumar *et al.,* 1992). 15-HETE, was shown to enhance the proliferation rate of BHK-21 cell lines *in vitro*. The presence of endogenous 15-HETE in the uterus at luteal phase suggests the possible involvement of arachidonic acid hydroxymetabolites in the mediation of uterine growth. Further indepth studies, however, are required to probe into the possible involvement of arachidonic acid products in the mediation of estrogen and progesterone induced uterine growth.

Summary

Many disorders of human health are linked to an unbalanced overproduction of hormone like materials called eicosanoids. Eicosanoid is the term given to a group of oxygenated derivatives of eicosapolyenoic fatty acids, especially arachidonic acid Arachidonic acid, the most abundant polyunsaturated fatty acid in cell membrane phospholipids, once released, will be oxygenated by two important pathways : the cyclooxygenase pathway producing prostaglandins, thromboxanes and prostacyclin, and the lipoxygenase pathway leading to the production of hydroperoxides, leukotrienes and lipoxins. These are extremely potent biologically active compounds with bewildering variety of actions. There is not a single system or process that is not affected, one way or the other, by the eicosanoids, including reproduction. There is extensive literature and many reviews on the role of prostanoids in reproduction. However there is very little information on lipoxygenase pathway in reproduction. In the present study an attempt is made to analyze the lipoxygenase pathway in sheep uterus

Purification of sheep uterus lipoxygenase

An abundant lipoxygenase activity, as high as 10.7 units/g wet tissue was observed in the cytosolic fraction of sheep uterus. This is one of the most abundant sources for lipoxygenases ever reported in animal tissues. The protein was purified by conventional chromatographic as well as HPLC techniques. Since the lipoxygenase activity was in the flowthrough of both cation and anion exchange columns, the enzyme was passed through both the columns connected in series. On HPLC using PA-DEAE ion exchange column also, the protein eluted in the flowthrough fractions*

The enzyme was purified to apparent homogeneity with 14.6 fold purification and an overall yield of 42.66%. The highly purified enzyme showed specific activity as high as 14.0 units/mg protein.

Structural characterization

The purified protein on SDS polyacrylamide gel electrophoresis showed a single band with molecular weight of 66 kDa The same molecular weight was observed when the protein was run on HPLC using gel filtration column (Diol-300). However, on non-denaturing gels, the highly purified lipoxygenase resolved into two bands 132 kDa and 66 kDa. Both the bands cross reacted with polyclonal antibodies raised against the highly purified lipoxygenase on Western blotting, indicating that the high molecular weight band could be the dimeric form of the lipoxygenase. In order to find out the catalytically active form of the enzyme, the proteins resolved on non-denaturing gel was incubated with archidonic acid (substrate) solution and the hydroperoxides formed were later reacted with O-dianisidine color reagent. These studies revealed that only the dimeric form to be catalytically active.

The uterus lipoxygenase was found to be a neutral protein with a Pi value of 7.0 as indicated by non-binding to both anionic as well as cationic exchangers and electrofocussing. The enzyme expresessed maximum activity at pH 5.5. The substrate dependent kinetics with arachidonic acid as the substrate revealed a high Km i.e 180 μM. The affinity towards the γ-linolenic acid (GLA) was much higher when compared to that of arachidonic acid as evidenced by low Km for GLA (98 uM). Further the enzyme was found to be unique in that no cofactors like Ca^{2+}, Mg^{2+}, ATP are required for expression of maximum activity.

Functional characterization

In order to identify the type, the purified uterus lipoxygenase was incubated with arachidonic acid as the substrate and the products were extracted into organic layer and then separated on straight phase HPLC. The products separated were identified based on their UV/Vis spectral characterization and GC/MS analysis. From these studies it was observed that both 12-HETE and 15-HETE were formed in almost equal concentration when highly purified enzyme was incubated with arachidonic acid as the substrate,

indicating dual lipoxygenase nature of the enzyme. The relative concentration of 12- and 15- HETEs, however, varied with the pH of the incubation medium. At pH below the optimum, 15-HETE was formed in higher concentration. At alkaline pH, the reaction favoured the formation of 12-HETE when compared to 15-HETE. Similar to arachidonic acid, the enzyme showed dual lipoxygenase activity with GLA producing 13-HOTrE and 10-HOTrE in equal concentration at the optimum pH. The enzyme thus, is unique in exhibiting dual regiospecificity. The dual regiospecificity of lipoxygenase can be explained based on the dimeric nature of the enzyme. Since only the dimer is catalytically active, two substrate molecules should be binding to the two catalytic centres, one facilitating the formation of 12-HETE and the other catalytic centre producing 15-HETE

As a result of dual regiospecificity, the enzyme should exhibit LTA4 synthase activity similar to other known dual lipoxygenases reported Leukocytes which exhibit 5- and 8- lipoxygenase activities, exhibit 5,6-LTA_4 synthase activity also (Shimizu *et al.*, 1985). In order to test the formation of leukotrienes, uterus lipoxygenase was incubated with 15-HPETE and the products formed were separated on reverse phase HPLC and identified basing on the UV/VIS spectra and GC/MS analysis. Two major products (peak I and II) were formed, with typical conjugated triene spectra and absorption maximum of 268 nm, with 15-HPETE as the substrate. Both the products (peak I and II) were identified as 8,15-diHETEs based on GC/MS analysis The elution pattern was similar to the hydrolyzed products of 14,15-LTA_4 Further peak I and peak II co-eluted with 14,15-LTA4 hydrolyzed 8(S), 15(R)- and 8(S), 15(S)-diHETEs, confirming the formation of 14,15-LTA_4 when 15-HPETE was incubated with uterine lipoxygenase. These studies have thus demonstrated that the uterine dual lipoxygenase exhibits 14,15-LTA_4 synthase activity. This is the first report on the formation of 14,15-series of leukotrienes in mammalian reproductive tissues.

Physiological role of lipoxygenases in uterus

In order to understand the physiological role of lipoxygenases in uterus, PUFAs were analyzed in different phases of estrous cycle on GC. Arachidonic acid was found to be the predominant unsaturated fatty acid present both in the cytosol and microsomal membrane fractions.

Lipoxygenase activity was the highest in luteal phase as compared to the other phases of estrous cycle. Also the endogenous products extracted from uterus, at different phases of estrous cycle, were higher in the luteal phase. In luteal phase, uterus is under the influence of estrogen and progesterone with active mitogenic activity. From this it appears that lipoxygenases may be involved in the mediation of steroid hormone actions.

In order to identify the lipoxygenase products involved in uterine functions, the endogenous products were extracted, separated on HPLC and identified. Among the hydroxy metabolites only 15-HETE was identified with a conspicuous absence of 12-HETE, as against expectations. The results suggest the possible diverstion/ utilization of 12-HETE specfically under *in vivo* conditions. 12- and 15-HETEs may be acting as chemotactic agents for leukocytes leading to their infilteration into the uterus. In addition to hydroxy metabolites, dihydroxymetabolites and leukotrienes were observed among the endogenous metabolites of arachidonic acid in the uterus. The dihydroxymetabolites were identified as 8,15-diHETEs. Since 8,15-diHETEs are known to play an important role in the mediation of immunological cross reactions, the diHETEs formed in the uterus in the present study must be involved in leukocyte infilteration and other hypersensitivity reactions.

Among the leukotrienes, a product with 280 nm absorption maximum was isolated It was identified as 14,15-LTC_4 based on the co-chromatography with standard leukotrienes. The formation of 14,15-LTC_4 was further confirmed by demonstration of LTC4 synthase activity in uterine microsomes. Since peptido-leukotrienes are known for

smooth muscle contractile properties, 14,15-LTC_4 formed in the uterus must be playing vital role in uterine contractions which are helpful either in normal parturition or in the transport of spermatozoa towards the site of fertilization and fertilized eggs to the site of implantation.

From these studies it can be concluded that a lipoxygenase, with dual regio specificity producing 12- and 15-HETEs with arachidonic acid as the substrate is present abundantly in the sheep uterus. As a result of dual regiospecificity the enzyme also showed 14,15-LTA_4 synthase activity Since arachidonic acid is the major PUFA in the uterus, the arachidonic acid metabolites formed via the lipoxygenase pathway must be playing vital role in uterine functions such as growth and contractions

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