THE METABOLISM OF POLYUNSATURATED FATTY ACIDS BY VASCULAR TISSUE

by

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ABSTRACT

Blood vessels convert arachidonic acid to prostacyclin (PGI_2), which is a potent inhibitor of platelet aggregation and thrombus formation. Polyunsaturated fatty acids (PUFA) or their hydroperoxy metabolites can cause a reduction in PGI_2 production. Therefore, in the present study, the metabolism of PUFA and their effects on PGI_2 production have been investigated.

Linoleic acid is converted by aorta to hydroperoxy metabolites, which are either dehydrated to oxo compounds, reduced to monohydroxy products, or converted to epoxyhydroxy-octadecenoic acids. The latter are then hydrolyzed to trihydroxy metabolites by aortic epoxide hydrolases. Aorta also converts eicosatrienoic acid to similar products, which are formed via 12-hydroperoxy-8,10-heptadecadienoic acid.

GC-MS analysis indicated that the major oxygenated PUFA metabolite formed by rat and bovine blood vessels was 6-oxo-prostaglandin-$F_{1\alpha}$. Substantial amounts of free and esterified monohydroxy and trihydroxy metabolites of linoleic acid were detected, especially in rat and rabbit aortae. Reduction of glutathione peroxidase activity by administration of a selenium-deficient diet had no significant effect on the formation of any of the above products.

The presence of esterified monohydroxy and trihydroxy PUFA metabolites in aortic lipids indicates that their esterified hydroperoxy precursors are present. An excess of these products could inhibit PGI_2 synthesis and contribute to the onset of atherosclerosis.

ABRÉGÉ

L'acide arachidonique est transformé en prostacycline (PGI_2) par les vaisseaux sanguins. Celle-ci est un puissant inhibiteur de l'aggrégation plaquettaire et de la formation de thrombus. Les acides gras polyinsaturés (AGPI) ou leurs métabolites hydroperoxydes peuvent réduire la synthèse de la PGI_2. Dans cette étude, nous avons examiné le métabolisme des AGPI et leurs effets sur la synthèse de la PGI_2.

L'acide linoléique est transformé au niveau de la paroi aortique en métabolites hydroperoxydes, qui sont ensuite soit déshydratés en composés oxoïques, soit réduits en composés monohydroxylés voire transformés en composés époxyhydroxylés. Ces derniers sont ensuite hydrolysés en métabolites trihydroxylés par les hydrolases époxidiques de l'aorte. Des produits semblables sont formés par l'aorte à partir de l'acide eicosatriénoïque, par l'intermédiare de l'acide 12-hydroperoxy-8,10-heptadécadiénoïque.

L'analyse par chromatagraphie en phase gazeuse et spectrométrie de masse démontre que le principal métabolite oxygéné des AGPI produit au niveau des vaisseaux sanguins du rat et du boeuf est la 6-oxo-prostaglandine-$F_{1\alpha}$. Par ailleurs des quantités importantes de métabolites monohydroxylés et trihydroxylés libres et estérifiés de l'acide linoléique ont été mises en évidence au niveau de l'aorte du rat et du lapin. Nous avons réduit l'activité enzymatique de la glutathion-peroxydase au moyen d'un régime alimentaire dépourvu de sélénium. Aucun effet significatif sur la formation des produits, mentionnés plus haut n'a été observé.

La mise en évidence de métabolites estérifiés monohydroxylés et trihydroxylés d'AGPI parmi les lipides aortiques y suggère la présence de précurseurs hydroperoxydes estérifiés. Un excès de ces produits pourrait inhiber la synthèse de la PGI_2 et contribuer au déclenchement de l'athérosclérose.

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ABBREVIATIONS

kg	kilogram
g	gram
mg	milligram
µg	microgram
ng	nanogram
l	liter
ml	milliliter
µl	microliter
mol	mole
mmol	millimole
µmol	micromole
nmol	nanomole
pmol	picomole
M	molar
mM	millimolar
µM	micromolar
m	meter
cm	centimeter
mm	millimeter
µm	micrometer
min	minute
s	second
cpm	counts per minute
dpm	disintigrations per minute
Ci	curie
µCi	microcurie
nCi	nanocurie

°C	degrees Celcius
vol	volume
ID_{50}	half maximal inhibitory dose
t_R	retention time
ODS	octadecylsilyl
HPLC	high pressure liquid chromatography
NP	normal-phase
RP	reverse-phase
m/z	mass to charge ratio
GC-MS	gas chromatography-mass spectrometry
TIC	total ion current
V_{max}	maximal velocity
K_m	Michaelis Menton constant
PUFA	polyunsaturated fatty acid
PG	prostaglandin
ep	endoperoxide
Me_3Si	trimethylsilyl
TMS	trimethylsilyl
nBu	n-butylboronate
e	epoxy
h	hydroxy
th	trihydroxy
hp	hydroperoxy
Me	methyl
LT	leukotriene
eV	electron volt
^3H	tritium
^2H	deuterium

18:2	linoleic acid
20:3	di-homo-γ-linolenic acid
20:4	arachidonic acid
PGI_2	prostacyclin
TX	thromboxane
12hp-17:2	12-hydroperoxy-8,10-heptadecadienoic acid
12h-17:2	12-hydroxy-8,10-heptadecadienoic acid
12h-17:3	12-hydroxy-5,8,10-heptadecatrienoic acid
11,12e-10h-17:1	11,12-epoxy-10-hydroxy-8-heptadecenoic acid
10,11,12th-17:1	10,11,12-trihydroxy-8-heptadecenoic acid
8,11,12th-17:1	8,11,12-trihydroxy-9-heptadecenoic acid
9hp-18:2	9-hydroperoxy-10,12-octadecadienoic acid
9h-18:2	9-hydroxy-10,12-octadecadienoic acid
9o-18:2	9-oxo-10,12-octadecadienoic acid
12h-18:2	12-hydroxy-9,13-octadecadienoic acid
13hp-18:2	13-hydroperoxy-9,11-octadecadienoic acid
13h-18:2	13-hydroxy-9,11-octadecadienoic acid
9,10e-11h-18:1	9,10-epoxy-11-hydroxy-12-octadecenoic acid
9,10e-11h-18:0	9,10-epoxy-11-hydroxyoctadecanoic acid
12,13e-11h-18:1	12,13-epoxy-11-hydroxy-10-octadecenoic acid
12,13e-11h-18:0	12,13-epoxy-11-hydroxyoctadecanoic acid
9,10e-13h-18:1	9,10-epoxy-13-hydroxy-11-octadecenoic acid
12,13e-9h-18:1	12,13-epoxy-9-hydroxy-10-octadecenoic acid
9,10,11th-18:1	9,10,11-trihydroxy-12-octadecenoic acid
9,10,13th-18:1	9,10,13-trihydroxy-11-octadecenoic acid
9,10,13th-18:0	9,10,13-trihydroxyoctadecanoic acid
9,12,13th-18:1	9,12,13-trihydroxy-10-octadecenoic acid

9,12,13th-18:0	9,12,13-trihydroxyoctadecanoic acid
19:2	10,13-nonadecadienoic acid
14h-19:2	14-hydroxy-10,12-nonadecadienoic acid
10,11,14th-19:1	10,11,14-trihydroxy-12-nonadecenoic acid
10,13,14th-19:1	10,13,14-trihydroxy-11-nonadecenoic acid
5h-20:4	5-hydroxy-6,8,11,14-eicosatetraenoic acid
8h-20:4	8-hydroxy-5,9,11,14-eicosatetraenoic acid
9h-20:4	9-hydroxy-5,7,11,14-eicosatetraenoic acid
11h-20:4	11-hydroxy-5,8,12,14-eicosatetraenoic acid
12h-20:4	12-hydroxy-5,8,10,14-eicosatetraenoic acid
15h-20:4	15-hydroxy-5,8,11,13-eicosatetraenoic acid

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INTRODUCTION

1.1. History

The group of compounds referred to as "fatty acids" were so designated because they were originally found to be constituents of animal and vegetable fats and fatty oils. Chevreul was the first to isolate fatty acids such as butyric, valeric, and caproic acids as well as impure oleic acid, between 1814 and 1818 (1). But it was in 1930 that two important but seemingly unrelated observations were made. Burr and Burr (2,3) carried out detailed investigations on the effects of fat exclusion from the diet of rats. They found that when rats were reared on a fat-free diet a deficiency disease developed which had not been previously described. The symptoms included: retardation of growth, reproductive disturbances, scaly skin, poor lactation, kidney lesions and excessive water consumption. The animals always died at an early age, unless they were fed a curative dose of fat. Linoleic acid, but not saturated fatty acids, was able to cure the rats suffering from the low fat disease. This led to the emergence of the term "essential fatty acid", referring to those fatty acids which prevent or relieve the symptoms of fat-deficiency. Fatty acids containing methylene-interrupted double bonds belonging to the linoleate and linolenate families came to be known as the "polyunsaturated fatty acids" and this term broadened to include all aliphatic monocarboxylic acids with two or more double bonds in any positional arrangement or geometric configuration.

In the same year as the discovery of the essential fatty acids (1930), Kurzrok and Lieb (4) reported that human uterus responded to fresh human semen by strong contraction or relaxation. Subsequently, in 1933-34, Goldblatt (5), in Britain, and von Euler (6), in Sweden, discovered independently a factor with vasodepressor and smooth muscle-stimulating activity in accessory genital glands and human semen. This factor which had the properties of a fatty acid was termed "prostaglandin" by von Euler. However, it was not until approximately 30 years later that Bergstrom and Samuelsson and their co-workers (7-9) established the link between the early studies of Burr and Burr, and Kurzrok, Lieb, Goldblatt and von Euler. They elucidated the structures of the "classical" prostaglandins (7,8) and found that these substances were produced from the essential fatty acid, arachidonic acid (9,10). It was the diverse and potent biological actions of prostaglandins on almost all organs which then stimulated the research in this field.

A discovery of utmost importance occurred in 1971 when Vane (11) found that the biosynthesis of prostaglandins was inhibited by aspirin-like drugs. Shortly after, Willis and Kuhn (12) described an unstable principle which induced platelet aggregation and was inhibited by aspirin. At approximately the same time Hamberg and colleagues (13) isolated the prostaglandin endoperoxides and found that they were strong inducers of platelet aggregation. Further studies revealed that these endoperoxides could be

transformed by platelets to an unstable substance with proaggregatory and vasoconstrictor properties which they named thromboxane A_2 (TXA_2) (14). The activity of TXA_2 had been previously described under the name of rabbit aorta contracting substance by Piper and Vane (15) in 1969.

In 1975, Vane and co-workers (see 16) attempted to look for TXA_2 synthesis in vascular tissue. Although they did not observe its synthesis they found that its endoperoxide precursor was enzymatically transformed into an unknown product (17). This product, initially referred to as PGX, was labile and relaxed the coeliac and mesenteric arteries of the rabbit (16,17). They found that it was a very potent inhibitor of platelet aggregation and that it was the major metabolite of arachidonic acid in vascular tissue. PGX was shown to be the unstable intermediate in the formation of 6-oxo$PGF_{1\alpha}$, which had previously been identified as a metabolite of 20:4 by rat stomach by Pace-Asciak and Wolfe (18). PGX was shown to be a bicyclic acid-labile allylic ether prostaglandin and was renamed prostacyclin (19,see 20).

1.2. Nomenclature and Structure

1.2.1. Polyunsaturated Fatty Acids (PUFA)

Many PUFA are known by trivial names, such as linoleic acid and arachidonic acid. However, a more exact chemical nomenclature of PUFA has been developed based on the number of carbon atoms and the position and configuration of double bonds (see 21). Numbering of the carbon chain starts at the

carboxyl carbon and proceeds toward the terminal methyl group while double bond location is noted by the carbon closest to the carboxyl group bearing the double bond. Most naturally occurring PUFA have double bonds present in the cis configuration. Therefore, linoleic acid is known as cis,cis-9,12-octadecadienoic acid and arachidonic acid is referred to as all-cis-5,8,11,14-eicosatetraenoic acid. The use of systematic names becomes cumbersome, however, so various abbreviations have been developed to facilitate discussion on PUFA (22). When the double bond positions are unimportant to the discussion the simple abbreviations, 18:2 and 20:4, may be used for linoleic and arachidonic acids, respectively. The first number refers to the number of carbons within the compound while the second refers to the number of double bonds. This notation has the most widespread use within the literature.

A variant of this notation has developed in discussions where the metabolic relationships between fatty acids are important (22). In these cases the terminal methyl carbon is referred to as the ω carbon and the first double bond from the methyl group is denoted by counting from the methyl group as number one. Thus, linoleic acid and arachidonic acid can be designated 18:2ω6 and 20:4ω6, respectively, since their terminal double bonds are between positions 12 and 13, and 14 and 15, respectively.

1.2.2. Eicosanoids

Corey introduced the term eicosanoid to comprise the large number of biologically active compounds derived from carbon-20 unsaturated fatty acids (23). These include prostaglandins, thromboxanes, leukotrienes and lipoxins.

The nomenclature of prostaglandins is based on prostanoic acid, a 20 carbon monocarboxylic acid containing a central cyclopentane ring. Prostaglandins have either one, two or three double bonds in the side chains and have functional groups with oxygen at carbons 9, 11 and 15 of prostanoic acid. The prostaglandins are classified by these functional groups by use of a capital letter while the number of double bonds is denoted by a subscript. The F-type prostaglandins have an additional subscript (α or β) to denote the stereochemistry of the C-9 hydroxy group. Prostacyclin and thromboxanes A_2 and B_2 differ somewhat from the basic prostaglandin structure. Prostacyclin contains a 6(9)-oxy ring and thromboxane A_2 has a fused oxetane-oxane ring system.

The term leukotriene was introduced for compounds which are non-cyclized 20 carbon carboxylic acids, with one or two oxygen substituents and three conjugated double bonds (24). They are formed from an allylic epoxide intermediate (leukotriene A). Leukotrienes with different functional groups are distinguished by capital letters, whereas a subscript denotes the number of double bonds. The peptido-leukotrienes contain a glutathionyl or metabolized glutathionyl substituent at C-6.

The lipoxins, the newest group of eicosanoids, contain four conjugated double bonds and three hydroxyl substituents in their 20 carbon structure (25). Two of these compounds have been described to date: lipoxin A (5,6,15L-trihydroxy-7,9,11,13-eicosatetraenoic acid) and lipoxin B (5D,14,15-trihydroxy-6,8,10,12-eicosatetraenoic acid).

1.3. Polyunsaturated Fatty Acids

1.3.1. General Introduction

There are 2 major groups of PUFA found in mammalian lipids. The ω6 family of PUFA is derived from linoleic acid (18:2ω6) while α-linolenic acid (18:3ω3) is the precursor of the ω3 PUFA. The mammalian organism is not capable of introducing double bonds at either the ω3 or ω6 positions of long chain fatty acids, so these fatty acids must be obtained in the diet (26,27). Linoleic acid, the principle essential fatty acid, can be desaturated and elongated to form the "derived" essential fatty acids, di-homo-ɣ-linolenic acid (20:3ω6) and arachidonic acid (20:4ω6), precursors of the 1 and 2 series of prostaglandins, respectively (see 28). Linoleic acid is found in vegetable oils, such as soybean, cottonseed, corn and safflower oils, where it comprises 50-80 % of the total fatty acid content (see 29). α-Linolenic acid, on the other hand, is approximately eleven times less potent than linoleic acid in reversing the effects of essential fatty acid deficiency in the rat (30). It is indispensible to certain species of fish (31). It can be elongated and desaturated, primarily

in the liver, to 20:5ω3 and 22:6ω3. Linseed oil contains over 50 % α-linolenic acid, whereas soybean oil comprises 6 to 8 % of this fatty acid (29). α-linolenic acid is the major fatty acid of chloroplast lipids in plants.

PUFA are normal constituents of cellular membranes, maintaining their fluidity and structural integrity. PUFA are esterified to phospholipids primarily in the 2-acyl position (32), and they can also be found esterified to triglycerides (33) and cholesteryl esters (34).

Linoleic acid and arachidonic acid are usually the most abundant PUFA in cellular lipids, although high concentrations of certain ω3 PUFA such as 22:6ω3, can be found in phospholipids of brain (35) and retinal photoreceptors (36).

Cellular PUFA content can be greatly influenced by the diet. Greenland Eskimos, for example, whose diet is mainly of marine origin, have a distinct fatty acid composition (37). Eicosapentaenoic acid (20:5ω3), rich in fish oils, almost completely replaces arachidonic acid (20:4ω6) in plasma lipids of this group (37).

1.3.2. Control of Free Cellular PUFA

The level of free arachidonic acid, the most abundant eicosanoid precursor, is rigidly controlled. In the resting state free cellular levels of this fatty acid are extremely low (see 28). A highly specific acyl-CoA synthetase enzyme rapidly esterifies C-20 PUFA (38). C-18 PUFA are esterified much less efficiently by this enzyme (38), and consequently

higher levels of free C-18 fatty acids are found within the cell. Subsequently, an acyl transferase enzyme transfers the PUFA CoA esters into the 2-acyl position of phospholipids (39).

Liberation of 20:4 from phospholipid stores can occur in response to a variety of stimuli. Thrombin (40), bradykinin (41), angiotensin II (42) and adrenocorticotropin (43) stimulate 20:4 release by receptor-mediated mechanisms, whereas the calcium ionophore, A23187 (44), mellitin (45), and mechanical agitation (46) release 20:4 by less specific means.

A complex series of reactions within the cell membrane appears to control the deacylation of 20:4 from phospholipids. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are hydrolyzed by phospholipase A_2, an enzyme that requires calcium for its activation (47). This enzyme seems to be regulated by a number of factors in certain cell types (48). Sequential methylation of PE, located on the cytoplasmic surface of the cell, to PC, concentrated on the outer membrane surface, can occur upon receptor-mediated stimulation. This results in a local increase in membrane fluidity and consequent activation of adenylate cyclase and calcium-dependent adenosine triphosphatase, which initiates the calcium influx responsible for phospholipase A_2 activation and subsequent arachidonate release (48).

Phosphoinositol compounds represent another important

class of 20:4-containing phospholipids. A major component of this phospholipid class is 1-stearoyl-2-arachidonoyl-glycero-3-phosphoinositol (PI), along with phosphatidylinositol 4'-monophosphate (DPI) and phosphatidylinositol 4'5'-bisphosphate (TPI) (49,50). Phospholipase C is capable of hydrolyzing all three of these compounds (51) and may (52) or may not (51) require the presence of calcium for its activation. A number of mechanisms for the release of arachidonate from the phosphoinositides have recently been proposed. Lapetina et al. (53,54) claim that in platelets phospholipase C activation is the initial receptor-linked event. Hydrolysis, primarily of TPI, by this enzyme leads to release of inositol 1,4,5-trisphosphate (IP_3), along with 1,2-diacylglycerol. The latter compound is then rapidly phosphorylated by diglyceride kinase and the resultant phosphatidic acid and IP_3 are involved in mobilizing calcium and activation of protein kinases. This calcium flux in turn activates phospholipase A_2 which results in release of 20:4 from PE, PC and/or phosphatidic acid. Alternatively, according to Majerus and co-workers (50,55), the 1,2-diacylglycerol formed by action of phospholipase C is rapidly hydrolyzed by a diglyceride lipase resulting in liberation of 20:4.

Glucocorticoids appear to play an important role in regulating liberation of 20:4 from phospholipid stores. These steroids are capable of inducing the synthesis of a specific glycoprotein via a receptor-dependent mechanism

(56,57). This protein, known as lipocortin (previously referred to as lipomodulin, macrocortin and renocortin) (58) displays phospholipase A_2 inhibitory action, and therefore inhibits eicosanoid generation by most intact cells (56-58). Lipocortin action is further controlled by a phosphorylation-dephosphorylation mechanism (58). Phosphorylation results in its inactivation, whereas dephosphorylation by alkaline phosphatase restores its activity.

It is obvious that the cellular events involved in the liberation of 20:4 from phospholipid stores are complex and the precise mechanism may vary depending on the type of cell and the initial stimulation event. In any case, the rapidly liberated 20:4 is then free to be oxygenated by various enzymes or may be re-esterified into phospolipids.

1.4. Oxygenation of PUFA

1.4.1. Prostaglandin Endoperoxide Synthase

Prostaglandin endoperoxide synthase (59) (also known as PGH synthase (60)) catalyzes the formation of the endoperoxides which give rise to the prostaglandins and thromboxanes (see 61). This enzyme is often referred to as fatty acid cyclooxygenase (or just cyclooxygenase) (13). However, this term can be somewhat ambiguous because this enzyme contains two activities. The first activity is responsible for the oxidative cyclization of 20:4 to give PGG_2, whereas the second activity catalyzes the conversion of the 15-hydroperoxy group of PGG_2 to the 15-hydroxyl group

of PGH_2 (61) (see Fig. 1). Therefore, the term prostaglandin endoperoxide synthase will be used for the enzyme comprising both activities, while the individual activities will be referred to as cyclooxygenase and prostaglandin hydroperoxidase. PG endoperoxide synthase is nearly ubiquitous, being found in almost all animal tissues (62). This enzyme is located in the microsomal fraction of the cell (63), primarily in the endoplasmic reticulum (64). Heme is an essential component of fully active PG endoperoxide synthase (65).

The detailed mechanistics of this enzyme have proved quite complex. However, Hemler and Lands (66) have proposed a mechanism that attempts to explain all of its properties. Fatty acid hydroperoxides, such as PGG_2, are required to activate the enzyme. The hydroperoxide interacts with the cyclooxygenase-bound ferriheme to produce a peroxy radical. This radical then abstracts the 13-S hydrogen atom from 20:4 giving rise to an activated alkyl radical which subsequently reacts with an oxygen molecule at C-11. Cyclization of the hydrocarbon chain ensues and incorporation of another molecule of oxygen at C-15 occurs. The 15-peroxy radical so formed ends the sequence by abstracting a hydrogen atom from the enzyme-bound hydroperoxide to form the end product, PGG_2. The PG hydroperoxidase activity reduces the C-15 hydroperoxide of PGG_2 with hydrogens abstracted from a hydrogen-donating cofactor via a valency change of the iron moiety of the enzyme-bound heme. During this reduction step

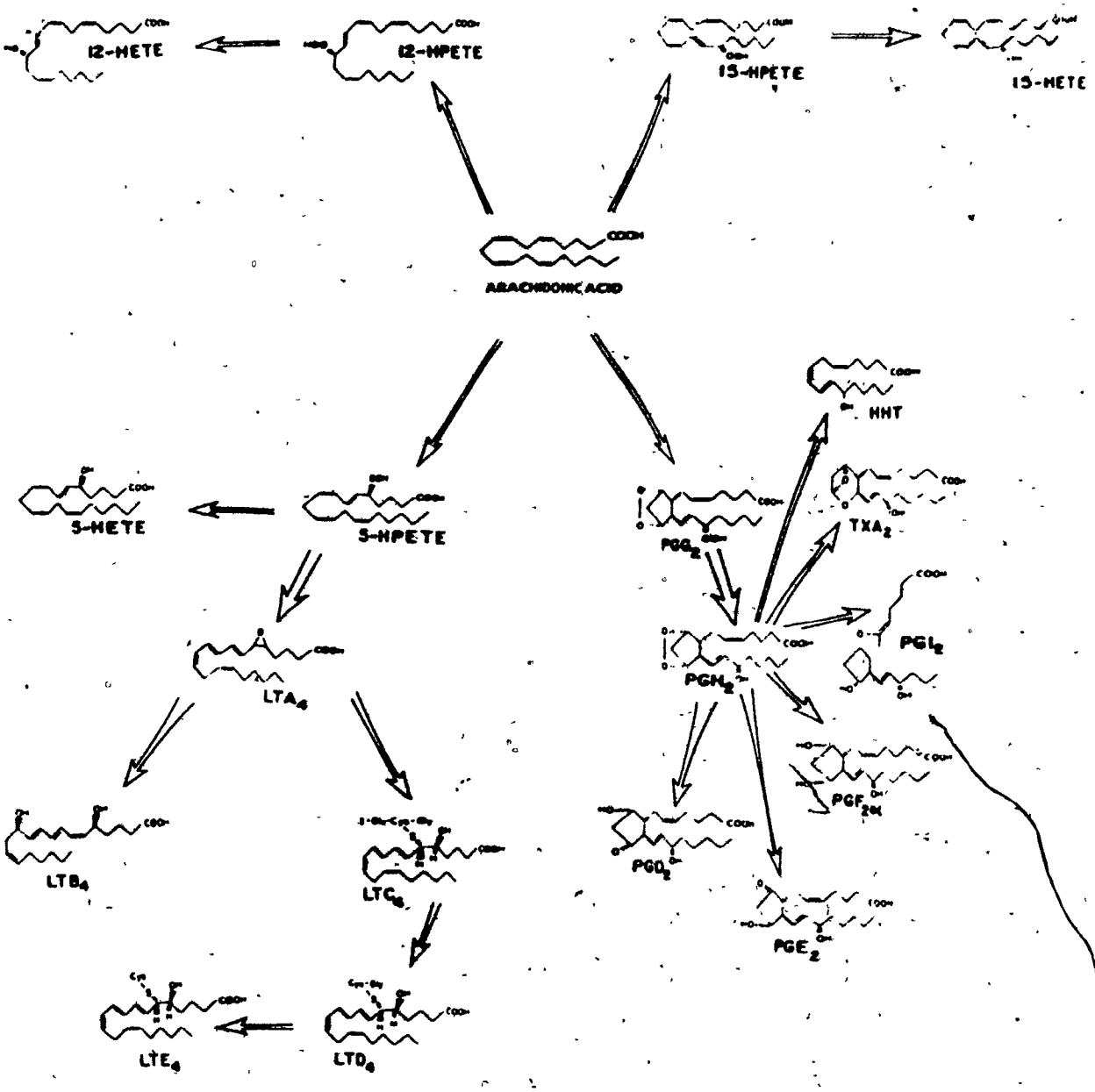

Fig.1. Scheme of transformations of arachidonic acid.

it appears that an active oxygen species is released which can result in destruction of the enzyme.

When the cyclooxygenase reaction is initiated by the presence of small amounts of fatty acid hydroperoxide, the overall consumption of oxygen proceeds at a rapidly accelerating rate, causing the generation of additional activating fatty acid hydroperoxide (67). The oxygenation reaches maximal velocity and then decelerates as a result of self-catalyzed inactivation until finally no further oxygenation occurs. The essential control points of this complex enzyme seem to be the availability of the fatty acid substrate and of the lipid hydroperoxide activator (67). The availability of fatty acid substrate is generally considered to be the rate limiting step in prostaglandin synthesis.

Fatty acid hydroperoxides are required not only for initiation of cyclooxygenase action but also for its continued action (67). Fatty acid hydroperoxides can be produced enzymatically by lipoxygenases or non-enzymatically through a variety of means. The cellular levels of these substances are under intricate control by factors such as vitamin E and glutathione peroxidase. In normal human plasma, Warso and Lands (68) have found a steady state level of 0.5 μM hydroperoxide, by using a new selective assay. This level would be capable of stimulating PG synthesis, for example in blood cells and vascular cells. It is interesting to note that cyclooxygenase, which produces hydroperoxides, also requires hydroperoxide as an activator,

whereas PG hydroperoxidase consumes hydroperoxides. The apparent paradox of these two activities residing in the same enzyme can be explained on the basis of the different affinities for hydroperoxide by the 2 sites on the enzyme. The hydroperoxide requirement for cyclooxygenase (Kp, 20 nM) is much lower than that for PG hydroperoxidase (K_m, 3 μM) (69). Therefore the latter activity is unable to suppress cyclooxygenase activity.

Other factors required for the activation of PG endoperoxide synthase, such as heme and the oxygen co-substrate are not limiting factors in vivo (67). There are many PG hydroperoxidase cofactors, such as tryptophan (59), epinephrine (70), phenol (66,71), guaiacol (72), uric acid (73) and glutathione (74) which have been reported to increase the synthesis of prostaglandins in vitro. The latter two could possibly serve this function in vivo.

PUFA other than 20:4 are also able to serve as substrate for PG endoperoxide synthase. The reaction velocity of the enzyme with di-homo-γ-linolenic acid (20:3) is essentially the same as with 20:4 (75). 20:5 is effectively converted by purified cyclooxygenase from sheep vesicular glands to endoperoxides of the 3 series when sufficiently high levels of hydroperoxide activator are present (75). It is not oxygenated under low peroxide conditions (75). However, recently a prostaglandin product derived from 20:5 was found in human urine suggesting that 20:5 is capable of being oxygenated in vivo (76). Certain dienoic fatty acids like

18:2 (77) and 11,14-eicosadienoic acid (78) also serve as substrates for this enzyme. With these substrates, though, cyclization to endoperoxides can not occur and monohydroxy fatty acids are the end products.

Suppression of prostaglandin endoperoxide synthesis can be achieved by a number of means. Antiinflammatory steroids limit the availability of fatty acid substrate via the synthesis of lipocortin, as mentioned above. Various long chain fatty acids, such as 22:6 (79) and 20:5 (75), under low hydroperoxide conditions, can bind to the substrate site of PG endoperoxide synthase and can effectively block PG endoperoxide synthesis. Acetylenic fatty acids display both competitive and non-competitive inhibitory actions (80). A large group of non-steroidal antiinflammatory agents are effective inhibitors. The two most widely used of these agents, aspirin and indomethacin, bind competitively at the substrate site and additionally cause irreversible inactivation of cyclooxygenase activity (80,81). Antioxidant radical trapping agents also markedly affect enzymatic activity. These agents often exert a biphasic response, stimulating cyclooxygenase activity in high hydroperoxide conditions and inhibiting its activity when the amount of hydroperoxide is low, by lowering the effective concentration of hydroperoxide activator (66).

Thus, the first step in the synthesis of prostaglandins from PUFA is a very complex event resulting from a fatty acid hydroperoxide-initiated free radical chain reaction in which positive feedback aspects of product activation are

countered with negative feedback features of self-catalyzed inactivation.

1.4.2. Prostaglandin Endoperoxide Metabolizing Enzymes

The PGH_2 formed from prostaglandin endoperoxide synthase is at a pivotal stage in the divergent pathways leading to the synthesis of various types of prostaglandins and thromboxanes. Most of the enzymes that utilize PGH_2 as substrate are isomerases which attack the 9,11-endoperoxide bridge of PGH_2 (61). The tissue specificity for prostaglandin and thromboxane biosynthesis depends upon the distribution of these enzymes.

(a) PGE synthase (9-oxo isomerase)

The conversion of PGH_2 to PGE_2 involves isomerization of the 9,11-endoperoxide to 9-oxo and 11α-hydroxyl groups. This enzyme has been localized in microsomes of bovine vesicular glands (82) and coronary artery (83), and rabbit kidney medulla (84). Glutathione is an essential cofactor for the reaction.

(b) PGD synthase (11-oxo isomerase)

PGD synthase is a cytosolic enzyme found in rat lung (85), stomach (85), small intestine (85), skin (85), brain (86), spleen (87) and mast cells (88). The 9,11-endoperoxide group of PGH_2 is isomerized to the 9α-hydroxyl and 11-oxo moieties of PGD_2. The enzyme from spleen and mast cells is stimulated by addition of glutathione, whereas the brain enzyme does not require glutathione. Formation of

PGD_2 from PGH_2 can also be catalyzed by glutathione transferases and albumin (89,90).

(c) Reduction of PGH to PGF

The formation of $PGF_{2\alpha}$ from PGH_2 involves reductive cleavage of the 9,11-endoperoxide. This activity has been reported in microsomes of bovine and guinea pig uterus (91). The agent catalyzing this reaction is not inactivated by boiling so it does not appear to be a protein (91). Non-enzymatic conversion of PGH_2 to $PGF_{2\alpha}$ occurs in the presence of thiol (92) and catechol (93) compounds. Glutathione transferases can also catalyze this reaction (89).

$PGF_{2\alpha}$ can be formed from PGE_2 via a 9-oxo reductase (94) which has been found in blood, heart, liver, kidney, brain and skin.

(d) Prostacyclin synthase

PGI_2 is formed by isomerization of the 9,11-endoperoxide group of PGH_2 into a 6(9)oxy ring and an 11α-hydroxyl group. PGI_2 is unstable, especially at acidic pH, and is readily transformed into 6-oxo$PGF_{1\alpha}$, a stable but biologically inactive product (see 95). Prostacyclin synthase is found in microsomal fractions of artery, vein, heart, lung, kidney, spleen, stomach, and in macrophages (96,97). In smooth muscle cells it displays a bimodal distribution, being found on both the plasma and nuclear membranes (98). Prostacyclin synthase is a ferrihemoprotein (99) that is readily inactivated by alkyl hydroperoxides.

The ferriheme moiety is believed to play an essential role at the active site in the cleavage of the endoperoxide. The factors regulating the synthesis of PGI_2 will be discussed in a later section.

(e) Thromboxane Synthase

The enzyme which produces TXA_2 from PGH_2 is present in microsomal fractions of platelets, lung, spleen and macrophages (100). TXA_2 is very unstable and is rapidly transformed to a stable, biologically inactive degradation product, thromboxane B_2 (101). 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde are formed concomitantly with TXA_2 (102). Thromboxane synthase is inhibited by imidazole and various endoperoxide analogues, and it also shows decreased activity in the presence of high concentrations of salt (101).

1.4.3. Prostaglandin and Thromboxane Catabolizing Enzymes

Since prostaglandins and thromboxanes are extremely potent biological mediators, mechanisms must exist to limit their actions. Prostaglandins and thromboxanes are metabolized by the following pathways (see 61):

1) Oxidation of the 15-hydroxyl group by 15-hydroxy prostaglandin dehydrogenase;
2) reduction of the 13,14-double bond of the 15-oxo product by Δ^{13}-prostaglandin reductase;
3) oxidation of 9-hydroxyl group by 9-hydroxy prostaglandin dehydrogenase;
4) reduction of 9-oxo group by prostaglandin 9-oxo reductase;

5) β-oxidation of the α-side chain giving rise to α-dinor, and α-tetranor compounds and β-oxidation of the ω-chain
6) ω and ω-1 oxidation.

Metabolism of prostaglandins and thromboxanes by the first two pathways is extremely rapid, since these enzymes are present in the lung as well as many other tissues (101). Consequently, prostaglandins and thromboxanes are not considered to be circulating hormones.

1.4.4. Lipoxygenases

Lipoxygenases are enzymes that catalyze the stereospecific insertion of oxygen into polyunsaturated fatty acids containing the cis,cis-1,4-pentadiene grouping. Hydroperoxides with a cis,trans conjugated diene system are the resultant products. Lipoxygenases are widely distributed in the plant kingdom, being found especially in the cereals and legumes. More recently, lipoxygenases have been identified in mammalian tissues.

(a) 12-lipoxygenase

The 12-lipoxygenase system of platelets was the first described mammalian lipoxygenase in 1974 (103). This enzyme was found in the 100,000 x g supernatant of broken platelets from a number of species (104). The oxygenation of 20:4 by this enzyme has been shown to involve, as an initial step, removal of the pro-(S) hydrogen at C-10. This is followed by isomerization of the 11-cis double bond to a 10-trans double bond with concomitant migration of the radical to C-

12. Addition of oxygen to C-12 results in the formation of 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12hp-20:4) (105).

12-lipoxygenase activity has also been localized in a number of other tissues, including lung (106), spleen (106), porcine polymorphonuclear leukocytes (107), peritoneal macrophages (108) and mast cells (109) from a variety of species. In platelets the major route for metabolism of 12hp-20:4 is reduction to 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid. This reductive step involves the action of the enzyme glutathione peroxidase and reducing equivalents from the hexose monophosphate shunt (110). When large amounts of exogenous 20:4 are added to platelets or when a glutathione peroxidase deficiency is induced, 12hp-20:4 is converted to epoxyhydroxy compounds (111,112). The latter are then hydrolyzed to the trihydroxy products, 8,9,12-trihydroxy-5,10,14-eicosatrienoic and 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid (111-113).

(b) <u>5-lipoxygenase</u>

The presence of a 5-lipoxygenase enzyme was demonstrated by the isolation of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5h-20:4) after incubation of rabbit polymorphonuclear leukocytes with 20:4 (114). This activity has also been found in eosinophils (115), alveolar macrophages (116), and mastocytoma (117) and basophilic leukemia (118) cell lines. Insertion of oxygen into the 5 position of 20:4 gives rise to 5(S)-hydroperoxy-20:4. This intermediate can be reduced

by cellular peroxidases to the corresponding monohydroxy product or it may undergo a specific dehydrase reaction, yielding the unstable allylic epoxide, leukotriene A_4 (LTA_4) (119). LTA_4, the precursor of the highly bioactive 5-series leukotrienes can be enzymatically converted via 2 specific pathways, the cell type determining the route of metabolism. One pathway involves a stereospecific hydrolytic reaction by LTA_4 hydrolase to give 5S,12R-dihydroxy-6,8,10,14-eicosatetraenoic acid, better known as LTB_4 (120,121). In the other pathway a specific glutathione-S-transferase enzyme catalyzes the addition of a glutathionyl moiety onto C-6 of LTA_4 to give 5(S)-hydroxy-6(R)-S-glutathionyl-7,9,11,14-eicosatetraenoic acid (LTC_4) (117,122,123). LTC_4 can be further metabolized by a membrane-bound γ-glutamyl transpeptidase (removal of the terminal γ-glutamyl residue) (124) and a dipeptidase (removal of the glycyl residue) (125) to yield LTD_4 and LTE_4, respectively. Addition of γ-glutamic acid (126) and N-acetylation (127) of LTE_4 are two subsequent metabolic pathways which give rise to LTF_4 and N-acetyl LTE_4, respectively.

Alternatively, LTA_4 can undergo non-enzymatic hydrolysis to give two 6-trans isomers of LTB_4 (119).

The 5-lipoxygenase system appears to exist in a relatively inactive state and thus requires activation. For example, when polymorphonuclear leukocytes are incubated with exogenous 20:4 virtually no 5hp-20:4 metabolites are observed (128). However, when the calcium ionophore, A23187, is added simultaneously large amounts of these

metabolites are produced (128,129).

(c) 15-lipoxygenase

The third major lipoxygenase system catalyzes the stereospecific insertion of oxygen at C-15 to yield 15(S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15hp-20:4). This enzyme activity has been found in polymorphonuclear leukocytes (130), reticulocytes (131), lung (132) and sheep vesicular gland (78). The polymorphonuclear leukocyte enzyme is localized within the cytosolic fraction (130). 15hp-20:4 can undergo metabolism by several pathways. Non-enzymatic degradation of 15hp-20:4 to 15-hydroxy-, 15-oxo-, 13-hydroxy-14,15-epoxy-, and 11,14,15-trihydroxy- products has been reported (130). 15hp-20:4 can also be converted to a LTA_4 analogue, having a 14,15-epoxy group instead of a 5,6-epoxy group (14,15-LTA_4; 15-series leukotrienes) (133). The hydrolysis of 14,15-LTA_4 results in the formation of several 8,15- and 14,15-dihydroxy metabolites (133). Perhaps the most interesting of the 15hp-20:4 metabolites are the recently discovered trihydroxy conjugated tetraene compounds (lipoxins A and B), formed by human leukocytes incubated with ionophore A23187 and 15hp-20:4 (25).

(d) Regulation of lipoxygenases

The mechanisms for the regulation of the different lipoxygenase activities are not clearly understood. However, recent evidence suggests that lipoxygenase products formed by one cell type may affect lipoxygenase activity in

other types of cells (134). Maclouf and co-workers (135) reported that the platelet 12-lipoxygenase product, 12hp-20:4, was a potent activator of human blood leukocyte 5-lipoxygenase. 12h-20:4 was inactive. On the other hand, 15h-20:4 inhibited both the platelet 12-lipoxygenase and the leukocyte 5-lipoxygenase (136,137). Its hydroperoxy precursor, 15hp-20:4, was 4 times as potent an inhibitor (134).

Certain drugs can also influence lipoxygenase activity. Vanderhoak and Bailey (138) have shown that ibuprofen, a non-steroidal antiinflammatory drug, stimulates the 15-lipoxygenase pathway of human polymorphonuclear leukocytes while inhibiting the 5-lipoxygenase. They suggest that ibuprofen may interact with a physiological activation process in these cells or alternatively, ibuprofen may mimic the action of an endogenous activator or possibly displace a naturally occurring inhibitor of the 15-lipoxygenase.

1.4.5. Other Pathways of PUFA Oxygenation

PUFA can be oxygenated by enzymes other than cyclooxygenase and lipoxygenases. Liver and kidney cytochrome P-450 enzymes in the presence of NADPH and NADPH-cytochrome P-450 reductase catalyze the oxygenation of PUFA to monohydroxy and epoxy compounds (139,140). Rat liver microsomes oxygenate 20:4 to the 9-, 11-, 12- and 15-monohydroxy products (141), as well as to four epoxy acids: 5,6-epoxy-8,11,14-, 8,9-epoxy-5,11,14-, 11,12-epoxy-5,8,14- and 14,15-epoxy-5,8,11-eicosatrienoic acids (142). The

formation of these epoxy acids by cytochrome P-450 has been referred to as the "epoxygenase" pathway (141). These products can be further metabolized by an epoxide hydrolase enzyme into the corresponding vic-diols (142).

PUFA can be oxygenated by a variety of non-enzymatic means in vivo due to their highly reactive methylene-interrupted double bond structure. When no known catalyst is present the reaction between molecular oxygen and PUFA is referred to as autoxidation. Linoleate autoxidation, for example, leads to addition of oxygen to either end of its 1,4-pentadiene system giving rise to equal amounts of the 9- and 13-hydroperoxy products (143). A mixture of cis,trans- and trans,trans- double bond configurations results depending upon the conditions of autoxidation.

Hemoproteins and transition metal ions are capable of catalyzing the non-enzymatic oxygenation of PUFA (144). In animal tissue, the position of oxygenation and the resulting double bond configuration are highly dependent upon the environment of this non-specific catalysis.

1.5. Physiological Roles of Oxygenated PUFA

Prostaglandins, thromboxanes and leukotrienes have been implicated in numerous cellular homeostatic processes and pathophysiological conditions. The prostaglandins exert pharmacological actions on many tissues. Their physiological roles have been more difficult to delineate. However, prostaglandins, are modulators of certain physiological activities in the healthy state and in

defensive reactions induced by damage and stress. In the kidney, for example, it has been reported that prostaglandins have several roles, including modulation of the renin-angiotensin system, modulation of the effects of kallikrein and inhibition of the response to antidiuretic hormone (145). Prostaglandins may be involved in the stimulation of pituitary hormone release via a stimulatory effect on the release of their releasing factors from the hypothalamus (146). Prostaglandins also have effects on adrenal steroidogenesis (147). In the reproductive system, prostaglandins appear to be required for the action of luteinizing hormone in causing follicular rupture and consequent ovulation (146). They are necessary to promote regression of the corpus luteum in certain species (146), and they are involved in contraction of the myometrium during parturition (148). Prostaglandins are potent inhibitors of gastric acid secretion and also exert a cytoprotective effect in the gastrointestinal system, possibly by increasing mucous production (see 149). These are only a few examples of the possible regulatory roles of prostaglandins in physiological processes. The role of prostaglandins and thromboxanes in vascular homeostasis will be considered in a later section.

Leukotrienes seem to be involved primarily in pathophysiological processes, such as inflammation and immediate hypersensitivity reactions (150). LTB_4 is a potent chemotactic and chemokinetic agent (151,152) and stimulates the aggregation of polymorphonuclear leukocytes

(151). It also augments the adhesion of these cells to the endothelium and elicits their migration from the vascular compartment into dermal tissue (153). The peptido-leukotrienes, LTC_4, LTD_4 and LTE_4, which are the components of slow-reacting substance of anaphylaxis, are involved in the induction of asthma, increasing bronchi mucous production, causing bronchoconstriction and augmenting vasopermeability (154).

The lipoxins have been found to exert certain biological actions. Lipoxin A contracts guinea pig lung strips, generates superoxide anions and stimulates degranulation of leukocytes (28,155). Both lipoxin A and lipoxin B inhibit natural killer cell activity (28,155). The extent to which these compounds appear in vivo and exert physiological effects, however, is not as yet known.

The physiological roles of the various lipoxygenase monohydroperoxy and monohydroxy products are not clearly understood. Although certain biological effects have been attributed to these compounds, further experimental evidence is required to support their physiological roles.

1.6. Metabolism of PUFA by Vascular Tissue

1.6.1. Prostacyclin

Ever since PGI_2 was found to be an important metabolite of vascular tissue by Vane and co-workers in 1976 (17), research has intensified in this area. PGI_2 is the major metabolite of 20:4 in virtually all arteries and veins (95).

Prostacyclin synthase activity, though, appears to be higher in arterial tissues than in veins (156). In microvessels, however, 20:4 metabolism may differ somewhat from the major vessels. Capillary endothelial cells derived from human newborn foreskins produce PGE_2 as their major arachidonate metabolite (157), while rat cerebral microvessels generated predominantly PGI_2 (158). The profile of metabolites released by vascular tissue can be changed markedly by the addition of exogenous 20:4. For example, rat aorta normally produces much more PGI_2 than PGE_2 but when the aorta is perfused with exogenous 20:4 the ratio of these two products is reversed (159). PGI_2 is synthesized in the greatest amounts at the intimal surface and its synthesis progressively decreases towards the adventitia (160).

PGI_2 was originally postulated to be a circulating hormone (161), being continuously released by vascular tissue and lung. This concept seemed to be supported by the fact that PGI_2 was not catabolized by the lung (162) as were other prostaglandins and that there were high plasma PGI_2 levels (measured as 6-oxo$PGF_{1\alpha}$) (95). Subsequent studies, however, have revealed that PGI_2 plasma levels under normal physiological conditions are much too low to exert any appreciable biological effects (163,164).

PGI_2 is unstable and its activity disappears within 10 min at 22 °C at neutral pH (95). In human blood and plasma, though, the half-life of PGI_2 is increased considerably, due to the stabilizing effect of albumin (165).

(a) Roles of prostacyclin in the vascular system

PGI_2 is the most potent known inhibitor of platelet aggregation (17). Applied locally in vivo in low concentrations, PGI_2 inhibits thrombus formation due to ADP in the microcirculation of the hamster cheek pouch (166). When given systemically to the rabbit it prevents electrically-induced thrombus formation in the carotid artery and increases bleeding time (167). PGI_2 causes the disaggregation of platelets in vitro and in extracorporeal circuits of cats where platelet clumps have formed on collagen strips (168). It also dissipates circulating platelet aggregates and prolongs template bleeding time in humans (169). PGI_2 also inhibits thrombus formation in a dog coronary artery model when given locally or systemically (170).

PGI_2 exerts its antiaggregatory action by stimulating platelet adenylate cyclase via a specific platelet membrane receptor (95). The elevation in cAMP levels enhances platelet calcium sequestration, thereby inhibiting platelet activation and thromboxane synthesis.

PGI_2 is also a strong hypotensive agent and a vasodilator of most vascular beds (95). PGI_2 does not act as a circulating vasodepressor hormone under normal physiological conditions (171). However, under certain stressful situations PGI_2 may act alone or in concert with other hormones to adjust local vasomotor tone.

PGI_2, when applied locally to the endothelium of venules and small veins, causes the release of previously firmly

adherent leukocytes (172). Thus, there may be a role for PGI_2 in preventing leukocyte adhesion to vascular endothelium.

(b) Factors affecting prostacyclin synthesis

The mechanisms regulating PGI_2 synthesis are not clearly understood. Brotherton and Hoak (173) have suggested a role for calcium and cAMP in its regulation. They found that preincubation of cultured endothelial cells with an antagonist of cytoplasmic calcium or with an inhibitor of cyclic nucleotide phosphodiesterase (1-methyl-3-isobutylxanthine; MIX) blocked PGI_2 release induced by a variety of stimuli. PGI_2 in the presence of MIX increased cAMP levels 2-fold over levels in the presence of MIX alone. These results led to the hypothesis that intracellular calcium is the mediator leading to the synthesis of PGI_2 from the initial endothelial cell stimulus (e.g. thrombin). The PGI_2 so formed is then capable of inducing an increase of cAMP and leads to a negative feedback signal to regulate its synthesis.

Another control mechanism for PGI_2 production may involve alterations in the enzymatic activities of cyclooxygenase and prostacyclin synthase via hydroperoxy products. Hydroperoxy metabolites of 18:2, 20:3 and 20:4 are potent inhibitors of prostacyclin synthase (174). It has been suggested that increased intracellular hydroperoxide levels could selectively block PGI_2 formation, resulting in the diversion of prostaglandin endoperoxides to other products

such as PGE_2 (175). However, cyclooxygenase can also be inhibited by relatively high concentrations of hydroperoxides (176). In fact, deactivation of cyclooxygenase rather than prostacyclin synthase appears to be the limiting factor of PGI_2 synthesis in cultured vascular endothelium (177), although this deactivation may have occured due to active oxygen species other than hydroperoxides. As was discussed above, low levels of fatty acid hydroperoxides (0.01-0.1 μM) are required to activate cyclooxygenase (69). Hydroperoxides, therefore, could be important factors in controlling the synthesis of PGI_2, the nature of their effects being determined by their precise intracellular concentrations.

Modifying the fatty acid content in the endothelial cell can have a marked effect on PGI_2 production and may be relevent to the effects of diet on PGI_2 production. Linoleic acid enrichment of cultured human umbilical vein (178) and bovine pulmonary artery (179) endothelial cells caused a 60-75 % reduction of PGI_2 production upon calcium ionophore stimulation. Eicosapentaenoic acid, abundant in marine oils, has been reported to exert varying effects on PGI_2 production. For example, humans fed diets high in marine oil were found to excrete substantial amounts of PGI_3 metabolites in their urine, approximately equal to the amounts of PGI_2 metabolites in controls (76). At the same time, there was no reduction in the amounts of PGI_2 metabolites excreted as a result of this treatment. Spector

et al. (180) found that when cultured human umbilical vein endothelial cells were enriched with 20:5 there was a 50-90 % reduction in the capacity of these cells to release PGI_2 when stimulated with thrombin, calcium ionophore or 20:4. Thus, it seems that cellular fatty acid content can substantially influence PGI_2 production although the mechanisms involved have not yet been clearly defined.

High density lipoproteins (HDL) may play a role in stimulating PGI_2 synthesis. By measuring 6-oxo$PGF_{1\alpha}$ one group of investigators (181) found that HDL stimulated PGI_2 synthesis 5-fold over 24 h in cultured porcine aortic endothelial cells. This stimulation could be mediated by provision of the endothelial cells with 20:4 contained within HDL in the form of cholesteryl arachidonate.

Low density lipoproteins (LDL), on the other hand, inhibit PGI_2 synthesis by rat aortic slices (182), superfused bovine coronary arteries (182) and pig aortic microsomes (183). The source of LDL may affect this inhibition, though. It has been suggested that lipid hydroperoxides, present within LDL may be responsible for its inhibitory effect on prostacyclin synthase. However, Beitz et al. (184) oppose this theory and claim that it is the fatty acid pattern and lipid class composition of the LDL which are important for their influence on PGI_2 production.

There appear to be distinct sex differences in the production of PGI_2 by blood vessels. These differences may be mediated by the sex hormones. Pomerantz and co-workers

(185) have shown a higher spontaneous level of PGI_2 production in male rat aorta as compared to the female. Ovariectomy increased PGI_2 production (measured as 6-oxo$PGF_{1\alpha}$) by 6-fold, but castration had no effect. Estradiol treatment of the ovariectomized females suppressed PGI_2 production by 50 % whereas progesterone treatment enhanced it by 2-fold, respectively. Testosterone was without effect in gonadectomized males and females. Seillan et al. (186) found that both estradiol and testosterone stimulated PGI_2 release from cultured piglet aorta endothelial cells obtained from females. In contrast, cells obtained from males were unresponsive to sex hormones. Another study has found higher PGI_2 production in arteries from women than from men (187).

A host of other factors have been reported to stimulate PGI_2 production from vascular tissue. Some of these factors include: thrombin (40), bradykinin (188), angiotensin II (189), kallikrein (190), leukotriene C (191), calcium ionophore, A23187 (40), ADP and ATP (192), noradrenaline (193), interferon (194), immunologic injury (195), decreased extracellular potassium concentration (196), endotoxin (197), phorbol esters (197) and mononuclear cell products (198). Obviously, the factors regulating PGI_2 production in health and under stress are complex. The magnitude of the effects of these agents on PGI_2 synthesis and the duration of their actions are dependent on the specific factor involved and its mechanism of action, as well as the type of

vascular cell (endothelial or smooth muscle) in question.

(c) Prostacyclin and platelet/vascular wall interactions

PGI_2 formation at a site of vascular damage and hemostatic plug formation could be a controlling factor in limiting thrombus size. Thrombin is generated on the platelet surface at the site of vessel damage, via activation of the clotting system. Thrombin can bind to a specific endothelial cell receptor and can stimulate PGI_2 synthesis, possibly by way of phospholipase activation, since mepacrine, an inhibitor of this enzyme, abolishes thrombin-induced PGI_2 synthesis by cultured endothelial cells (40,199). Stimulation of PGI_2 production could limit the hemostatic plug to the site of injury and prevent continuation of thrombus formation down the blood vessel wall.

In addition to vascular 20:4 acting as the substrate for PGI_2 formation, activated platelets could release endoperoxides which could be converted to PGI_2 by vascular cells. The most convincing evidence in support of this was carried out by Marcus and colleagues (200). They incubated [^{3}H]20:4-prelabeled platelets in aggregometer cuvettes with thrombin, A23187 or collagen in the presence of aspirin-treated endothelial cell suspensions. [^{3}H]6-oxo$PGF_{1\alpha}$ was recovered from the supernatants of the combined cell suspensions after stimulation by all three agents. The amounts of platelet [^{3}H]TXB_2 produced were low and platelet aggregation was inhibited at a ratio of 200,000 platelets to

3000-6000 aspirin-treated endothelial cells. At higher platelet levels the proportion of 6-oxo$PGF_{1\alpha}$ to TXB_2 decreased and platelet aggregation occurred. Endogenous 20:4 or exogenous radioactive 20:4 were not converted to PGI_2 after addition of thrombin to aspirin-treated endothelial cells. Therefore, the endothelial cell suspensions could only have used endoperoxides from stimulated platelets, since platelets lack prostacyclin synthase.

Data from Needleman et al. (201,202) suggest that some degree of vascular damage would be necessary for platelet-derived endoperoxides to be used by vascular prostacyclin synthase. They demonstrated that while 20:4 could be readily converted to PGI_2 by perfused rabbit hearts and kidneys, PGH_2 was not readily transformed to PGI_2.

To what extent PGI_2 production is responsible for the inhibition of platelet adhesion (thromboresistance) to the endothelium is debated. Dejana and co-workers (203) studied the effects of inhibition of PGI_2 synthesis by aspirin on platelet adhesion to the endothelial lining of rabbit aorta in vivo and in vitro. They concluded that inhibition of PGI_2 production does not promote platelet adhesion. Using another preparation Curwen et al. (204) found that neither treatment of vascular endothelium with aspirin or indomethacin nor increasing PGI_2 production by addition of 20:4 affected basal platelet adherance. On the other hand, platelet adherence to transformed vascular endothelial cells (obtained after viral infection), which generated only very

small amounts of PGI_2, was markedly increased. Addition of exogenous PGI_2 partially reversed the increased adhesion. Eldor and colleagues (205) conducted experiments using an incubation chamber to measure PGI_2 production at the luminal surface of aortae. They de-endothelialized aorta and found a closely adherent layer of platelets shortly after injury. Several days later only a few platelets adhered to the denuded surface. Results of their experiments revealed that de-endothelialized muscular neo-intima synthesized increasing quantities of PGI_2 with time after injury and that this increase correlated with the formation of a neo-intima and with the acquired thromboresistance of the aorta.

PGI_2 inhibits platelet aggregation at much lower concentrations than those needed to inhibit adhesion (206). Platelets, then, may stick to vascular tissue and interact with it, yet at the same time PGI_2 may prevent or limit thrombus formation. Substantiation of this idea comes from a recent investigation (207). Shortly after balloon de-endothelialization of rabbit aortae there is a closely adherent layer of platelets. A small reduction of adherent platelets could be observed in animals receiving PGI_2 at 50-100 ng/kg/min (a dose sufficient for complete inhibition of platelet aggregation) but concentrations of 650-850 ng/kg/min were necessary to inhibit this adhesion.

Various factors produced within the platelet affect PGI_2 synthesis. Platelet-derived growth factor (PDGF), in low concentrations greatly enhances PGI_2 release from bovine

endothelial cells (208). It has been postulated that PDGF released in vivo may increase PGI_2 production as part of a negative feedback mechanism controlling platelet aggregation. On the other hand, β-thromboglobulin, another protein released from platelet α-granules, inhibits PGI_2 production from cultured endothelial cells (209). Serotonin, a dense granule platelet constituent, increases PGI_2 synthesis in cultured bovine aortic smooth muscle and PDGF acts synergistically in this response (210). Yet no stimulation is observed with aortic endothelial cells.

It appears, therefore, that platelet-vessel wall interactions are intimately linked and carefully regulated. PGI_2, although apparently not responsible for all the thromboresistant properties of vascular endothelium, plays a crucial role in the control of platelet aggregability, especially in situations in which platelet reactivity might be enhanced due to local tissue damage.

(d) Prostacyclin - Thromboxane balance

A direct physiological antagonism exists between PGI_2 and TXA_2. PGI_2 is a potent platelet antiaggregatory agent and vasodilator while TXA_2 is pro-aggregatory and causes vasoconstriction (211). Both substances are derived from the same substrate, have the same intermediates, are unstable and their biological activity depends on their rate of synthesis, since they are not stored. The balance of these two substances in vivo has been purported to play an important role in the control of vascular homeostasis (211).

There is some evidence in the literature to suggest that an improper balance of the PGI_2/TXA_2 ratio may be linked to the vascular complications in a number of diseases, including atherosclerosis, which will be discussed in the next section.

Platelets from patients with arterial thrombosis, deep vein thrombosis or recurrent venous thrombosis produce more prostaglandin endoperoxides and TXA_2 than normal and have a shortened survival time (212). Diminished PGI_2 production may explain the microvascular complications present in diabetes. PGI_2 production by blood vessels from patients with diabetes is depressed (213) and circulating levels of 6-oxo$PGF_{1\alpha}$ are reduced in diabetic patients with proliferative retinopathy (214). The reliability of this latter result is questionable, though, since circulating levels of PGI_2 have been found to be very low as was discussed above. Platelets from diabetic subjects show diminished sensitivity to PGI_2 and platelets from rats made diabetic release more TXA_2 than normal (95). However, these results have not been confirmed in other studies (215,216). One study reveals that in diabetics with microangiopathy the balance between PGI_2 and TXA_2 is shifted towards PGI_2 (215) and in another, data was presented that do not support an association between reduced PGI_2 and diabetic retinopathy (216).

The balance between PGI_2 and TXA_2 has been reported to be altered in Bartter's syndrome (217), thrombocytopenic purpura (218), pre-eclampsia (219), hypercholesterolemia

(220) as well as angina (221).

Much interest in the pharmacological control of the PGI_2/TXA_2 balance has arisen recently. Attempts have been made to find an aspirin dose which would effectively inhibit platelet TXA_2 synthesis without reducing vascular PGI_2 synthesis. These attempts were based on two in vitro findings: (i) platelet cyclooxygenase appeared to be more sensitive than the vascular enzyme to aspirin inhibition (222), and (ii) the platelet enzyme is inhibited for the lifespan of the platelet, whereas enzyme turnover permits replacement of cyclooxygenase in the vascular endothelium (223). A detailed investigation was carried out by Fitzgerald and colleagues (224). Although they found greater inhibition of TXA_2 synthesis than PGI_2 synthesis with chronic low doses of aspirin (20 and 40 mg/day, for 7 days), inhibition of platelet function was not complete at this dosage. Doses of aspirin in excess of 80 mg/day resulted in substantial inhibition of endogenous PGI_2 synthesis. It was concluded that it would be unlikely to find a dose of aspirin which would maximally inhibit thromboxane generation without reducing endogenous PGI_2 synthesis. More recent studies (235) suggest that simulated slow release of low-dose aspirin (either 1 mg every 30 min x 20 or 5 mg every 2 h x 4) results in a more rapid inhibition of platelet thromboxane formation than acute low-dose (20 mg), while at the same time preserving the capacity of systemic endothelium to synthesize PGI_2.

With the advent of thromboxane synthase inhibitors, selective inhibition of TXA_2 formation has become possible. Dazoxiben, one of these inhibitors, when administered to healthy volunteers in single doses of 25-200 mg, induced a dose-dependent reduction of serum TXB_2 levels and inhibition of ex vivo platelet aggregation induced by 20:4 (226). Endogenous PGI_2 synthesis was increased, possibly by the donation of platelet endoperoxides to the vascular endothelium (226). One potential drawback to the use of thromboxane synthase inhibitors is the fact that platelet-derived prostaglandin endoperoxides can still be formed and they may exert pro-aggregatory activity if not converted to PGI_2 by the endothelium. In any case, pharmacological manipulation of the PGI_2/TXA_2 balance appears to be possible and may have possible therapeutic potential in diseases with vascular complications.

(e) Prostacyclin, lipid peroxides and atherosclerosis

High concentrations of lipid peroxides have been demonstrated in advanced atherosclerotic lesions (227). It has been proposed that the accumulation of lipid peroxides in vascular tissue leads to inhibition of PGI_2 synthesis, which is followed by increased platelet adhesion to endothelium and release of platelet factors (228). Platelet-derived growth factor, one of these factors, induces smooth muscle cell proliferation, an essential step in the process of atherogenesis (229). The finding that PGI_2 synthesis by human (230) and rabbit (175)

atherosclerotic arteries in vitro is lower than in control arteries is consistent with this hypothesis.

On the other hand, it has recently been reported that the amounts of urinary metabolites of PGI_2 are actually higher in patients with atherosclerosis than in normal controls (231). This implies that the total production of PGI_2 by the body is increased, rather than decreased in this disease. The in vitro experiments referred to above would reflect stimulated PGI_2 synthesis, whereas the levels of PGI_2 metabolites in urine would reflect basal PGI_2 synthesis which is normally very low. These results could be reconciled, however. PGI_2 generation might be enhanced in the presence of platelet activation in atherosclerosis. This enhanced PGI_2 formation may derive from healthy vascular tissue that is attempting to minimize the effects of platelet activation, and not from atherosclerotic diseased tissue.

Szczeklik and co-workers (232) have reported striking and prolonged benefits following intra-arterial infusion of PGI_2 in 5 patients with advanced atherosclerotic lower limb peripheral vascular disease. Rest pain disappeared, muscle blood flow was significantly increased for at least 6 weeks after PGI_2 infusion, and ulcers healed. A further detailed study was carried out by this group on 55 patients with advanced peripheral arterial disease of the lower extremities, 38 of the patients being diagnosed for atherosclerosis (233). In 42 % of the patients treated there was a persistent, long lasting improvement. In 40 %

of patients the improvement lasted no longer than 2 months while in the remaining 18 % of patients there were virtually no changes.

1.6.2. Prostaglandins and the Ductus Arteriosus

During fetal life 90 % of right ventricular output passes through the ductus arteriosus to the descending aorta (234). By shunting the blood flow away from the high resistance fetal pulmonary circulation, total workload on the heart is greatly reduced. Fetal patency of the ductus is actively maintained by prostaglandins, apparently of the E type (234). PGE_1 and PGE_2 exert an extremely potent relaxant effect on the ductus arteriosus in vitro, whereas PGI_2 is approximately 3 orders of magnitude less potent (235).

PGI_2 has been reported to be the major metabolite in homogenates and isolated preparations of fetal ductus arteriosus (236,237). PGE_2 and $PGF_{2\alpha}$ were also detected. Fetal lamb ductus was reported to produce about 30 % as much PGE_2 as 6-oxo$PGF_{1\alpha}$ (237).

Olley and Coceani, on the basis of their studies favour locally produced PGE_2 as the prime mediator of ductus patency. In line with this concept is the evidence that prostaglandin endoperoxide synthase inhibitors (e.g. indomethacin) constrict the fetal lamb ductus in vitro (234). Others, however, favour the hypothesis that humoral sources of vasodilator PGE_2 are important for ductus arteriosus patency, since they were not able to detect enzymatic synthesis of PGE_2 from PGH_2 by isolated fetal lamb

ductus arteriosus (238). In any case, PGE's are essential for ductus patency and they are the compounds of choice in the treatment of newborns with congenital heart lesions requiring patency of the ductus to maintain the pulmonary or systemic circulation (234).

The mechanism for closure of the ductus arteriosus at birth is still not clearly understood. However, it is believed that this contraction is triggered by the rise in arterial oxygen tension (pO_2) that occurs with the first breath (234). One hypothesis for closure of the ductus is that prostaglandins, specifically PGE_2, become less effective postnatally (239). Coceani, Olley and colleagues have shown that the relaxant effect of PGE_2 on the ductus is diminished with high pO_2 (240). A decline in the response of the ductus to prostaglandins during the last third of gestation has been reported (241), and this could represent a priming factor for postnatal closure. Concentrations of prostaglandins in the blood are relatively high in the fetus and fall rapidly after birth (242), therefore, their relaxant effect on the ductus is removed. Closure of the ductus, then, may be regarded as a withdrawal of the relaxant actions of prostaglandins combined with the constrictor effect of high pO_2. Others suggest that vasoconstrictor products are important for ductus arteriosus closure (243). TXA_2 produced in the lungs and conveyed to target sites in the ductus by the bloodstream has been suggested as the mediator of oxygen-induced ductus

constriction (243). Contrary to this hypothesis is the finding that TXA_2 did not contract the ductus in vitro (244).

1.6.3. Other Oxygenated PUFA Metabolites Produced by Vascular Tissue

(a) Synthesis of thromboxanes by vascular tissue

TXA_2 has been reported to be produced as a minor product of 20:4 metabolism by vascular tissue. In 1976 Tuvemo and colleagues (245) demonstrated its synthesis by human umbilical artery, and suggested that it might be involved in its closure at birth. Salzman et al. (246) observed synthesis of TXA_2 by intrapulmonary artery strips, which was not attributable to adhering lung tissue or platelets. TXA_2 synthesized by the vessel appeared to impart some basal tone to it.

Ingerman-Wojanski et al. (247) have shown that thromboxane (measured as TXB_2 by radioimmunoassay) is synthesized by cultured bovine aortic endothelial cells but not by vascular smooth muscle cells. The ratio of 6-oxo$PGF_{1\alpha}$ to TXB_2 produced by endothelial cells was reported to be between 5 and 10 to 1 with both exogenous (20:4 and PGH_2) and endogenous (stimulated by ionophore A23187) substrates. Human arterial and venous segments, obtained from patients undergoing operations, converted [1-^{14}C]20:4 to 6-oxo$PGF_{1\alpha}$ and TXB_2 in a ratio of 4:1 (248). In other experiments thromboxane production by intimal cells was negligible, whereas its production by media smooth muscle cells was the main source of this substance (radioimmunoassay measurement) (248). This finding seems to contradict the

above results with cultured bovine vascular cells.

Wolfe and colleagues (249) found that synthesis of TXB_2 from endogenous 20:4 of rat aortic and mesentary artery rings was very low, at the limit of sensitivity of their GC-MS assay (approximately 2 ng/sample). "6-oxo$PGF_{1\alpha}$ was present in amounts 1000 times that of TXB_2." They were not able to detect TXB_2 from perfused rat mesenteric artery preparations. Several other investigators have failed to demonstrate thromboxane synthesis by other blood vessels (243,250).

Generally, the production of thromboxane, both in vivo and in vitro, by vascular tissue is regarded as a minor pathway. Studies employing GC-MS as an analytical tool reveal very small amounts of thromboxane production. Experiments with radioimmunoassay as the measure of thromboxane production have shown a significant thromboxane to PGI_2 ratio (247,248) which possibly is a result of a lack of specificity of the assay. As well, cultured vascular cell production of thromboxane may not accurately reflect its production by intact tissue.

(b) <u>Synthesis of leukotrienes by vascular tissue</u>

Slow-reacting substance of anaphylaxis (SRS-A) has been characterized and shown to consist of LTC_4, LTD_4 and LTE_4. SRS-A was detected after immunological stimulation of guinea pig aorta (251). Piper and co-workers (252) reported the generation of a leukotriene-like substance from porcine coronary and pulmonary arteries when incubated with the

divalent cation ionophore, A23187, in the presence of a cyclooxygenase inhibitor. Further characterization of this substance revealed that it had all the properties of LTE_4 (253). The adventitia was the richest source of LTE_4, followed by the intima. This substance was produced in smaller amounts in the absence of the cyclooxygenase inhibitor, however. Other blood vessels released smaller amounts of LTE_4 whereas no leukotriene-like material was detected from aorta. In shorter incubations, preliminary experiments also revealed the presence of leukotrienes C4 and D4, the precursors of LTE_4 (253).

In addition to LTE_4 a substance which had properties indistinguishable from those of LTB_4 was generated from the pulmonary artery (253).

1.7. Studies in our Laboratory

Prior to the present investigation, 20:4 metabolism by particulate fractions of fetal calf aorta and ductus arteriosus was studied in detail in our laboratory (236,254). Particulate fractions from fetal calf aorta and ductus arteriosus converted 20:4 to 6-oxo$PGF_{1\alpha}$, 6,15-dioxo$PGF_{1\alpha}$, 12-hydroxy-5,8,10-heptadecatrienoic acid, 11h-20:4, and 15h-20:4 (254). The formation of all of these products was inhibited by indomethacin indicating that they were all formed by prostaglandin endoperoxide synthase. No thromboxane synthesis was observed. 12h-20:4, a lipoxygenase product, was also detected in the presence of indomethacin. Its synthesis was presumably enhanced due to

the increased availability of substrate. Factors stimulating prostaglandin hydroperoxidase activity (epinephrine and tryptophan) stimulated the formation of the monohydroxy fatty acids and 6-oxo$PGF_{1\alpha}$. Methemoglobin, on the other hand, selectively stimulated the formation of 6,15-dioxo$PGF_{1\alpha}$. It appeared that this product was formed by dehydration of 15-hydroperoxyPGI_2 or possibly 15-hydroperoxy-6-oxo$PGF_{1\alpha}$. At high concentrations of 20:4, 6,15-dioxo$PGF_{1\alpha}$ formation was substantially increased with respect to 6-oxo$PGF_{1\alpha}$ formation, since the capacity of the particulate fraction to reduce 15hp-PGI_2 (or 15hp-6oxo$PGF_{1\alpha}$) was exceeded and the excess 15-hydroperoxy compound was converted to the corresponding 15-oxo derivative. These 15-hydroperoxy intermediates in the metabolism of 20:4 may be important factors in regulating PGI_2 synthesis in aorta, since prostacyclin synthase is strongly inhibited by such compounds (174). Slices of fetal calf aorta incubated in physiological medium released all the metabolites mentioned above from endogenous stores of 20:4 (254).

Ductus arteriosus particulate fractions metabolized 20:4 in essentially the same manner as fetal calf aorta.

E prostaglandins seem to be important for maintaining ductus arteriosus patency during fetal life (234). Besides 20:4, 20:3 is another possible source of E prostaglandins in fetal blood vessels. Although 20:3 is present in cellular lipids in considerably smaller amounts than 20:4, it cannot be converted to PGI_2 due to the absence of the 5,6-double

bond. Instead, the major product of PGH_1 metabolism by partially purified prostacyclin synthase from rabbit aorta was reported to be 12-hydroxy-8,10-heptadecadienoic acid (12h-17:2) (255). It is possible that in fetal blood vessels, the metabolism of prostaglandin endoperoxides derived from 20:3 could be diverted from prostacyclin synthase to PGE isomerase (if this enzyme is present in this tissue).

Particulate fractions and homogenates from adult and fetal bovine aorta were found to convert 20:3 to the following metabolites: 11-hydroxy-8,12,14-eicosatrienoic acid, 15-hydroxy-8,11,13-eicosatrienoic acid, 12h-17:2 (major product), 12-oxo-8,10-heptadecadienoic acid (12o-17:2), 10,11,12-trihydroxy-8-heptadecenoic acid , 8,11,12-trihydroxy-9-heptadecenoic acid (2 isomers), PGE_1 and $PGF_{1\alpha}$ (256). With the exceptions of 12o-17:2 and PGE_1, all of the above products were shown by gas chromatography-mass spectrometry to be formed from endogenous substrate after incubation of slices of fetal calf aorta in physiological medium. Prostaglandin endoperoxide synthase appeared to be required for the synthesis of all of the above products. Small amounts of 12-hydroxy-8,10,14-eicosatrienoic acid and 11,12,15-trihydroxy-8,13-eicosadienoic acid were also detected (257). The 17-carbon products were all derived from 12hp-17:2.

Methemoglobin increased the formation of all products except PGE_1 and $PGF_{1\alpha}$ by about 50-100 % above the control. Adrenaline stimulated the formation of the monohydroxy fatty

acids by 5 to 6-fold and that of prostaglandins by about 10-fold but had no effect on the synthesis of trihydroxyheptadecenoic acids.

1.B. Basis for the Present Study

At the onset of the present study substantial information was known about 20:4 metabolism by vascular tissue. The study of 20:3 metabolism had been initiated, but the mechanism for the formation of the trihydroxyheptadecenoic acids was not known. We proposed that the trihydroxy products are formed via epoxyhydroxy intermediates. Therefore, an investigation to further characterize this reaction was initiated.

18:2 is an abundant cellular PUFA, often found in quantities equal to, or exceeding, 20:4. It had been reported to inhibit PGI_2 production in cultures of vascular endothelial cells (178,179). We postulated that 18:2 could be oxygenated by vascular tissue to give rise to hydroperoxy intermediates. These intermediates, if not metabolized rapidly, could then cause an inhibition of prostacyclin synthase. Alternatively, 18:2 could compete with 20:4 for the substrate binding site of prostaglandin endoperoxide synthase and thus block its conversion into PGI_2. Therefore, 18:2 metabolism by vascular tissue was studied, as well as the mechanism of its effect on 20:4 vascular metabolism.

Following the characterization of the metabolism of these PUFA we decided to establish an assay for measuring the

major oxygenated PUFA products synthesized from endogenous substrate by vascular tissue. Previous work from our laboratory has demonstrated that a variety of fatty acid hydroperoxides are formed during the metabolism of PUFA by aorta (254,256,257). As discussed above, these products could have inhibitory effects on PGI_2 synthesis. By measuring the monohydroxy and trihydroxy products derived from fatty acid hydroperoxides, a reliable assessment of their amounts within vascular tissue may be obtained. In the past, lipid peroxides have usually been measured by determining the amount of thiobarbituric acid-reactive material present in the sample. However, this is not an entirely satisfactory procedure (258). Therefore measurement of the monohydroxy and trihydroxy products represents an alternative and much more specific approach.

An impairment in the metabolism of hydroperoxy fatty acids to hydroxy fatty acids by peroxidases in vascular tissue could be reflected by a decreased PGI_2 synthesis and an increase in other hydroperoxy metabolites, such as trihydroxy products. One cellular peroxidase enzyme, glutathione peroxidase, requires selenium as an essential component (259). By feeding rats and rabbits a selenium-deficient diet, we attempted to induce a deficiency in this enzyme. Measurement of the oxygenated PUFA metabolites formed from endogenous substrates by aorta was then carried out to determine whether an alteration in the profile of these metabolites would result.

MATERIALS AND METHODS

2.1. Materials

2.1.1. Sources of Animals and Blood Vessels

Bovine blood vessels were obtained from a local slaughterhouse. They were placed on ice immediately after dissection and then transported to the laboratory.

Male New Zealand White rabbits were purchased from Ferme Cunicole C. Leonard, Inc., Ste. Scholastique, Quebec. Male Sprague-Dawley rats were purchased from Charles River, St. Constant, Quebec.

2.1.2. Diets

For the selenium deficiency study weanling rats and rabbits were fed ad libitum specially prepared diets from Teklad, Madison, WI., as shown in Table 1. All animals had free access to water.

2.1.3. Chemicals

PGE_1, $PGF_{1\alpha}$, [3,3,4,4-^{2}H]PGE_2 and 6-oxo[3,3,4,4-^{2}H]$PGF_{1\alpha}$ were kindly provided by Dr. J.E. Pike of the Upjohn Co., Kalamazoo, MI. Indomethacin was obtained from Dr. C. Gleason of Merck, Sharpe and Dohme, Montreal, Quebec.

Unlabeled fatty acids were purchased from NuChek Prep. Inc., Elysian, MN., whereas [1-^{14}C]18:2 (54.7 Ci/mol), [1-^{14}C]20:3 (55.1 Ci/mol) and [1-^{14}C]20:4 (55.4Ci/mol) were obtained from New England Nuclear Canada, Lachine, Quebec. These compounds were purified by normal-phase high pressure liquid chromatography (NP-HPLC) immediately prior to use (254).

TABLE 1

Composition of Rat and Rabbit Selenium-Deficient and Control Diets

RAT (Teklad #170698)

	g/kg
Torula Yeast	300.0
Sucrose	598.2967
Lard, tocopherol-stripped	50.0
Mineral Mix, Hubbell-Mendel-Wakeman (#170790)	50.0
Thiamin HCl	0.0004
Riboflavin	0.0025
Pyridoxine HCl	0.002
Calcium Pantothenate	0.02
Niacin	0.1
Biotin	0.001
Folic Acid	0.002
Vitamin B_{12} (0.1% trituration in mannitol)	0.1
Choline Chloride	1.0
Dry Vitamin A Palmitate (500,000 U/g)	0.028
Dry Vitamin D_3 (500,000 U/g)	0.0064
Dry Vitamin E Acetate (500 U/g)	0.44
Menadione	0.001

* The selenium control diet (Teklad TD83466) has a sodium selenite-sucrose mix (0.0445 % Na_2SeO_3) added at 2.5 g/kg diet and the sucrose concentration is adjusted to 595.7967 g/kg to balance the formula. This diet is designed to give 0.5 ppm selenium.

RABBIT (Teklad #TD84156)

	g/kg
Torula Yeast	320.0
DL-Methionine	3.0
Sucrose	441.7
Corn Oil	50.0
Fiber (cellulose)	130.0
Mineral Mix, Williams-Briggs (#170910)	35.0
Calcium Carbonate	7.7
Vitamin Mix, AIN-76A (#40077)	10.0
Choline Bitartrate	2.6

* The selenium control diet (Teklad #TD84309) has sodium selenite ($Na_2SeO_3.5H_2O$) added at 0.0017 g/kg and the sucrose concentration is decreased to 441.6983 g/kg. The control diet contains approximately 0.52 ppm selenium, whereas the selenium deficient diet is estimated to contain 0.02 ppm selenium.

[9β-^{3}H]$PGF_{1\alpha}$ was synthesized by reduction of PGE_1 with sodium boro[^{3}H]hydride (260).

Soybean lipoxygenase (Type 1, 152,200 Units/mg), methemoglobin, adrenaline bitartrate, guaiacol, nordihydroguaiaretic acid and glutathione reductase (Type III, 160 Units/mg) were all purchased from Sigma Chemicals, St. Louis, MO.

Pierce Chemical Co., Rockford, IL., supplied N-methyl-N-trimethlysilyltrifluoroacetamide, Tri-Sil Z reagent (trimethylsilylimidazole in pyridine) and methoxylamine hydrochloride.

The following chemicals were purchased from the indicated companies: platinum(IV)dioxide (Alfa, Danvers, MA.), n-butylboronic acid and 2,2-dimethoxypropane (Supelco, Inc., Bellefonte, PA.), Diazald (N-methyl-N-nitroso-p-toluenesulfonamide) (Aldrich Chemical Co., Milwaukee, WI.) and purified pyridine (J.T Baker Chemical Co., Phillipsburg, NJ).

All other chemicals were analytical reagent grade quality and were obtained through local suppliers.

2.1.4. Solvents

Solvents used for HPLC were HPLC grade and were purchased from Fisher Scientific Co., Fairlawn, NJ. Methyl formate, practical grade (Eastman Kodak, Rochester,NY.) was distilled prior to use. Solvents used for other purposes were reagent-grade.

Formula 947 and Formula 963 aqueous liquid scintillation

counting cocktails were obtained from New England Nuclear, Boston, MA. Ready-Solv was obtained from Beckman Instruments Inc., Fullerton, CA.

Water was always deionised and glass-distilled prior to use.

2.2. Methods

2.2.1. HPLC

Initial studies were carried out using a Milton Roy duplex minipump with a Valco injector and a Berthold radioactivity monitor (Munich, Germany). Subsequent work was performed with a Waters HPLC system (Milford, MA), consisting of two M-45 pumps, a model 680 gradient controller and a Lambda-Max Model 481 spectrophotometer. Columns of 5 µm Partisil (either 350 x 4.2 mm or 350 x 4.6 mm) and phenylsulfonate bound to silicic acid (RSilCAT, 5 µm particle size) obtained from Alltech Associates, Deerfield, IL., were used for NP-HPLC and argentation HPLC, respectively. The cation exchange column was treated with silver nitrate before use (261). RP-HPLC was performed on a column (250 x 4.2 mm) of ODS-silica (5 µm Ultrasphere) obtained from the Beckman Instrument Co., Palo Alto, CA. Unless otherwise stated the flow rates for NP-HPLC and RP-HPLC were 2.0 ml/min and 1.5 ml/min, respectively.

2.2.2. GC-MS

GC-MS was carried out either at the Biomedical Mass Spectrometry Unit of McGill University on a LKB-9000

instrument (ionizing voltage, 70 eV) with a column (0.003 x 2 m) of OV-101 (1%) on Chromosorb W-HP (100-120 mesh) at a helium flow rate of 35 ml/min, or at the Clinical Research Institute of Montreal on a Hewlett-Packard 5985B instrument (ionizing voltage, 70 eV) with a column (0.002 x 2 m) of SP2250 (3%) on Supelcoport (100-120 mesh) at a helium flow rate of 30 ml/min.

2.2.3. Derivatization Procedures

Samples were converted to their methyl esters by treatment with 0.5-1.0 ml ethereal diazomethane for either 5 min at room temperature or 30 min at 0 °C. The samples were then dried under a stream of nitrogen and taken up in the appropriate solvent. TMS derivatives were prepared by treatment with either 15 µl of N-methyl-N-trimethylsilyl-trifluoroacetamide (30 min, room temperature) or 15 µl of Tri-Sil Z reagent (5 min at 60 °C).

Hydrogenations were performed in methanol (0.4 ml) containing 1.3 mg platinum dioxide. After bubbling hydrogen through the methanol - platinum dioxide mixture for 30 s, the sample was added in 100 µl of methanol. Hydrogen was bubbled for an additional 90 s, and the sample was passed through a column (1 x 0.6 cm) of silicic acid (Biosil HA, BioRad Laboratories, Richmond, CA) and eluted with a further 1.5 ml methanol.

O-methyloxime derivatives were formed by addition of 1 mg methoxylamine hydrochloride in 100 µl pyridine to the dried sample. After heating for 1 hour at 55 °C, 2 ml of diethyl

ether were added to precipitate excess reagent. The precipitate was removed by centrifugation and the ether supernatant was evaporated under a stream of nitrogen.

n-Butylboronate derivatives were prepared by treatment with a mixture of n-butylboronic acid and 2,2-dimethoxypropane for 2 min at 60 °C (262).

2.2.4. Extraction Procedure

Samples were brought to 15 % aqueous ethanol by addition of either water or ethanol and then were acidified to pH ca. 3 by addition of 1 N HCl. They were loaded onto pre-washed ODS silica Sep Paks (Waters Associates, Milford, MA) using 20 ml glass syringes. Subsequent washes with 15 % aqueous ethanol (20 ml), water (20 ml) and petroleum ether (10 ml) were performed (Fig. 2). Reaction products were eluted from the Sep Pak with 10 ml of methyl formate.

New Sep Paks were washed prior to use with 20 ml ethanol, followed by 20 ml of water. Used Sep Paks were eluted with 80 % aqueous ethanol (20 ml) prior to the other two washes. All Sep Paks were discarded after being used five times.

Unless otherwise stated all extractions were performed by the above method.

2.2.5. Preparation of Standards (see Table 2)

(a) Internal Standards for GC-MS

14-Hydroxy-10,12-nonadecadienoic acid (14h-19:2) and trihydroxynonadecenoic acid (th-19:1) were prepared by incubation of 10,13-nonadecadienoic acid with soybean lipoxygenase in the presence of guaiacol (cf 263).

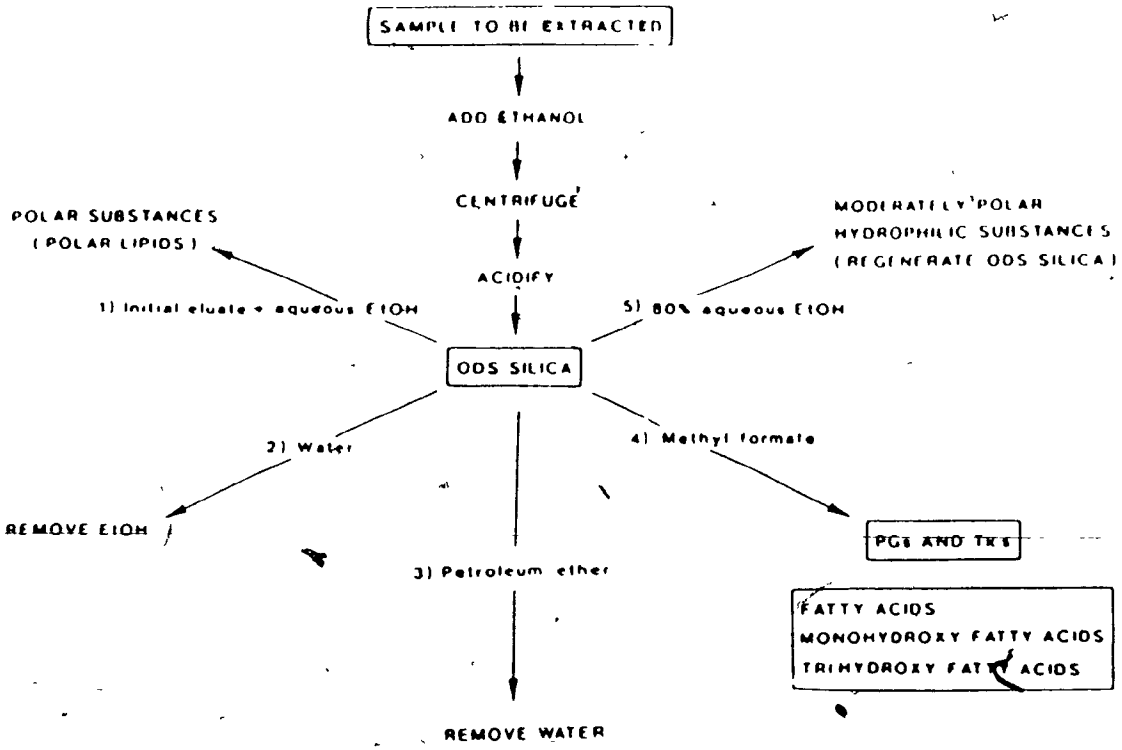

Fig.2. Scheme for the extraction of oxygenated PUFA using octadecylsilyl (ODS) silica.

Nonadecadienoic acid (3 mg) was dissolved in 0.019 M ammonium hydroxide (0.15 ml), and added to a mixture of soybean lipoxygenase (90,000 Units) in 0.1 M sodium borate buffer, pH 9.0 (30 ml) containing guaiacol (20 mM) at 0 °C. The mixture was stirred at 0 °C and the addition of enzyme and substrate was repeated after 15 and 30 min. The reaction was terminated by the addition of ethanol 45 min after the initial addition of substrate, and the products were extracted using Sep-Pak cartridges containing ODS silica as described above. Hydroperoxides were reduced with sodium borohydride (1 mg) in methanol (0.3 ml), for 20 min at 0°C then 20 min at room temperature. 12 % aqueous ethanol (9.7 ml) was added, the mixture acidified, and the products were extracted with ODS silica. 14h-19:2 (t_R, 10 min) and th-19:1 (t_R, 46.5 min) were purified by NP-HPLC using a mobile phase consisting of hexane:n-propanol:acetic acid (98.75:1.25:0.1) for the first 30 min, followed by a linear gradient over 25 min to hexane:n-propanol:acetic acid (70:30:0.1).

The identities of the purified products were confirmed by GC-MS. The mass spectrum (Fig.3) of the methyl ester, TMS ether derivative of 14h-19:2 (C-value, 20.7) had major ions at m/z 396 (M), 381 (M-15), 325 (base peak, cleavage between C-14 and C-15), 225 and 129.

The mass spectrum (Fig.4A) of the TMS ether derivative of the methyl ester of th-19:1 (C-value, 23.4) had major ions at m/z 559 (M-15), 474 (M-100, loss of hexanal), 401

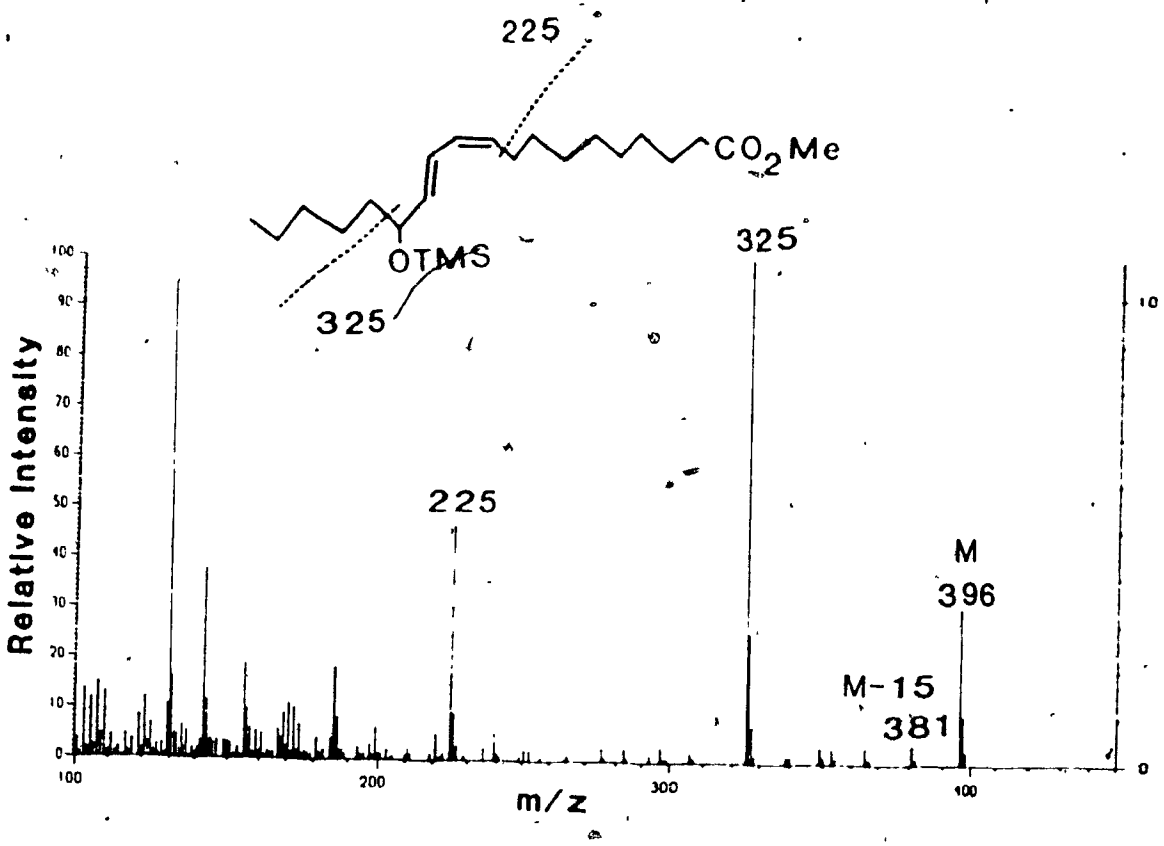

Fig.3. Mass spectrum of the trimethylsilyl ether derivative of the methyl ester of 14-hydroxy-10,12-nonadecadienoic acid.

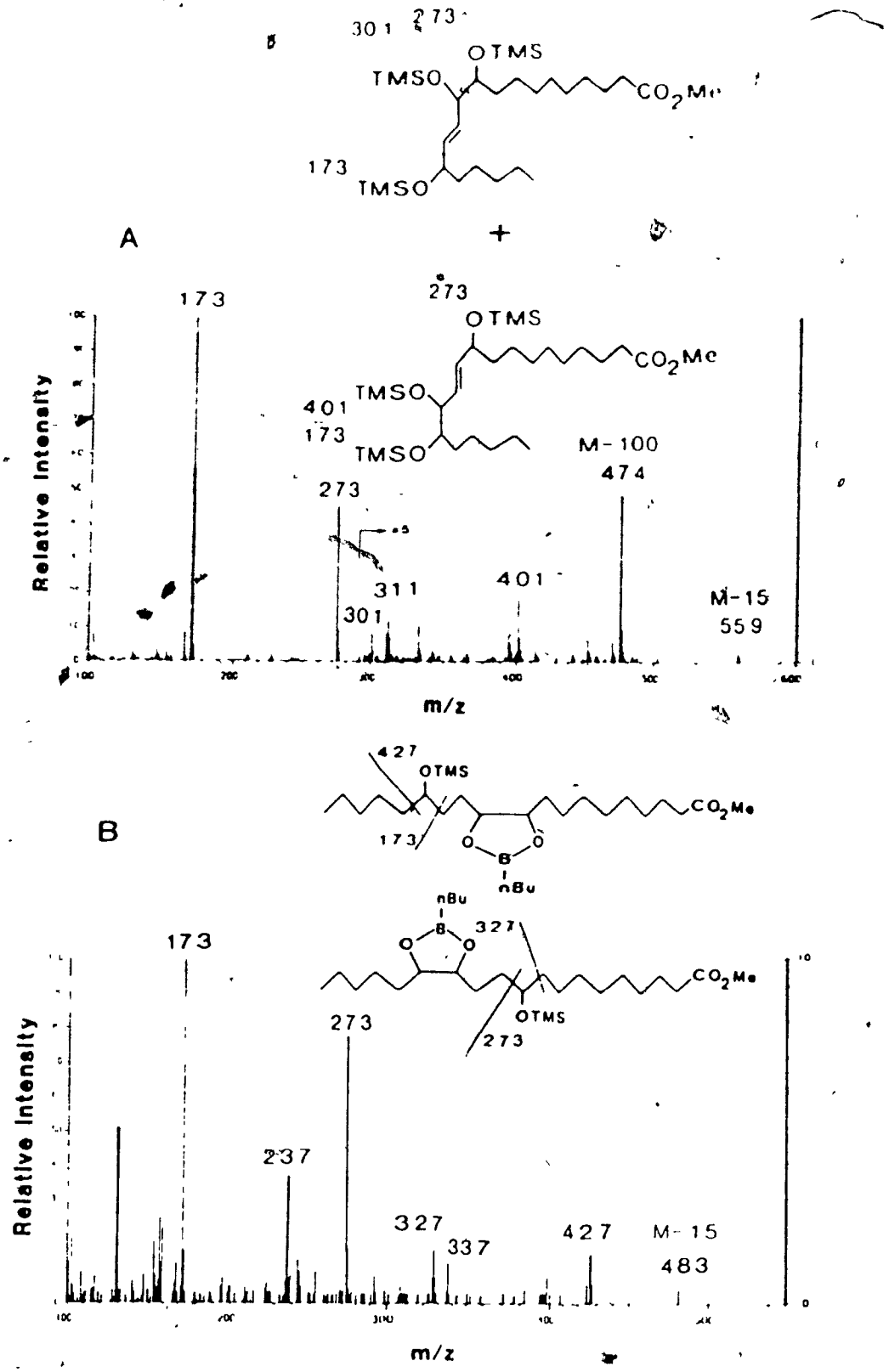

Fig.4. Mass spectra of 10,11,14-trihydroxy-12-nonadecenoic acid and 10,13,14-trihydroxy-11-nonadecenoic acid after (A) conversion to their trimethylsilyl ether, methyl ester derivatives, and (B) hydrogenation and conversion to their trimethylsilyl ether, n-butylboronate, methyl esters.

(loss of 173, cleavage between C13 and C14), 311 (401-90), 273 ($TMSO^+=CH-(CH_2)_9-CO_2CH_3$), 173 (base peak, $TMSO^+=CH-(CH_2)_4-CH_3$). This indicates that this substance is a mixture of 10,13,14th-19:1 and 10,11,14th-19:1. This was confirmed by the mass spectrum of the butyl boronate derivative of the methyl ester of the hydrogenated derivative of th-19:1 (Fig.4B), which had major ions at m/z 427 (M-71, loss of C-15 to C-19), 337 (427-90), 327 (loss of $(CH_2)_8-CO_2CH_3$)), 273, 237 (327-90), and 173. The ratio of the ions at m/z 327, 237, and 273 to those at m/z 427, 337, and 173 suggests that the ratio of 10,13,14th-19:1 to 10,11,14th-19:1 was approximately 1.25:1.

Both 14h-19:2 and th-19:1 were quantitated by gas chromatography using stearic acid and $PGF_{1\alpha}$, respectively, as internal standards.

(b) <u>Preparation of Monohydroxy 20:4 Compounds</u>

Monohydroxy 20:4 compounds were prepared as described by Boeynaems et al (264). 20:4 (5 mg, 2.6 x 10^4 cpm) and cupric chloride (10 µmol) were dissolved in methanol (2 ml) and the pH was brought to 7 by addition of 0.2 M Tris buffer, pH 8.5 (approx. 0.5 ml). Hydrogen peroxide (0.36 mmol, 40 µl of a 30% solution) was added and the reaction mixture was stirred for 30 min at room temperature. The concentration of MeOH was adjusted to 15 % by addition of water, and the mixture was extracted with ODS silica Sep-Paks. The hydroperoxides were then reduced with sodium borohydride and re-extracted as described above. The reaction products were purified by

TABLE 2

Preparation of Oxygenated PUFA Standards for GC-MS

Standard	Initial Substrate	Means of Synthesis
12h-17:2	20:3	fetal calf aorta particulate fraction
12h-17:3	20:4	porcine platelets
th-17:1	20:3	fetal calf aorta particulate fraction
9h-18:2	18:2	tomato lipoxygenase (crude homogenate)
13h-18:2	18:2	soybean lipoxygenase
th-18:1	18:2	fetal calf aorta particulate fraction and slices
14h-19:2	19:2	soybean lipoxygenase + guaiacol
th-19:1	19:2	soybean lipoxygenase + guaiacol
5h-20:4	20:4	porcine polymorphonuclear leukocytes
8h-20:4	20:4	$CuCl_2$ + H_2O_2
9h-20:4	20:4	$CuCl_2$ + H_2O_2
11h-20:4	20:4	$CuCl_2$ + H_2O_2
12h-20:4	20:4	porcine polymorphonuclear leukocytes
15h-20:4	20:4	soybean lipoxygenase

NP-HPLC employing a mobile phase consisting of hexane:n-propanol:acetic acid (98.75:1.25:0.1). 11h-20:4, 9h-20:4 and 8h-20:4 were subsequently quantitated by GC using stearic acid as the internal standard.

(c) 13h-18:2 and 15h-20:4

13h-18:2 and 15h-20:4 were prepared by incubation of 18:2 (5 mg) or 20:4 (5 mg), respectively, with soybean lipoxygenase (72,500 Units) in 3 ml of 0.1 M sodium borate buffer, pH 9.0, at 0 °C (265). Addition of enzyme was repeated after 15 min and incubation was continued for a further 15 min. Ethanol was then added to give a final concentration of 15 % and reaction products were extracted with ODS silica Sep Paks. After reduction of the hydroperoxides the products were purified by NP-HPLC as described above for monohydroxy 20:4 isomers.

(d) 12h-17:3

Porcine blood (2 l) was mixed with 6 % Dextran T-500 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.9 % NaCl (0.2 vol). After 45 min, the upper layer containing leukocytes and platelets was removed and centrifuged at 200 x g for 10 min. The pellet (leukocytes, see below for preparation of 12h-20:4) and supernatant (platelets) were separated and the supernatant was centrifuged at 750 x g for 25 min. The pellet was resuspended in 0.14 M NaCl-0.01 M Tris-HCl-1.5 mM EDTA (pH 7.4) and re-centrifuged at 750 x g for 20 min. The wash was repeated and platelets were taken up in 20 ml of calcium-free phosphate buffered saline, pH

7.2. 20:4 (2 mg, 2 x 10^4 cpm) was added and the mixture was shaken for 10 min at 37 °C. Two volumes of ethanol were added and the reaction products were extracted as described above. 12h-17:3 (t_R, 28 min) was purified by RP-HPLC using a mobile phase consisting of methanol:water:acetic acid (72:28:0.05).

(e) 12h-20:4

The pellet containing porcine polymorphonuclear leukocytes (see above) was resuspended in 0.15 M NaCl and the mixture was layered over Ficoll-Paque (Pharmacia) and centrifuged at 400 x g for 30 min. The pellet was resuspended in 0.135 M ammonium chloride in 0.015 M Tris-HCl, pH 7.2, and incubated for 10 min at 37 °C. The mixture was centrifuged and the pellet washed twice by centrifugation in 0.15 M NaCl at 200 x g for 10 min and resuspended in phosphate buffered saline. 12h-20:4 was synthesized by incubation of 20:4 with porcine polymorphonuclear leukocytes in the presence of the divalent cation ionophore, A23187 (10 μM), for 10 min at 37 °C and was purified by RP-HPLC.

(f) 9h-18:2

A firm, red tomato was peeled and, after the seeds were removed, the flesh was diced and washed in water at 0 °C. The slices (10 g) were homogenized in 35 ml of 0.1 M sodium acetate buffer, pH 5.5. 18:2 (5 mg), was dissolved in 0.019 M ammonium hydroxide (0.25 ml) and added to the homogenate

and the mixture was shaken for 20 min at 25 °C (266). Oxygen was bubbled through the mixture for 15 s every two minutes. Ethanol was added to stop the reaction and the products were then extracted using ODS silica. Hydroperoxides were reduced with sodium borohydride and 9h-18:2 was purified by NP-HPLC using a mobile phase of hexane:n-propanol:acetic acid (98.75:1.25:0.1). 9h-18:2 had a t_R of 23 min.

(g) th-18:1

th-18:1 was prepared either by incubation of 18:2 with a fetal calf aorta particulate fraction in 0.05 M Tris buffer, pH 7.5, or by incubation of fetal calf aorta slices in Krebs-Ringer Tris buffer, pH 7.4, for 60 min at 37 °C. Aorta slices were homogenized in chloroform/methanol (2:1). The homogenate was filtered through a sintered glass funnel and the filtrate was evaporated in a rotary evaporator. The residue was dissolved in 0.28 N potassium hydroxide in 95 % EtOH and hydrolysis of lipids was carried out for 45 min at 55 °C. After hydrolysis, the sample was extracted with ODS silica. The products obtained from the two procedures were combined and the th-18:1 isomers (t_R between 34 and 37 min) were purified by NP-HPLC using a linear gradient between 3 % and 100 % solvent B (toluene:ethyl acetate:acetonitrile: methanol:acetic acid (30:40:30:2:0.5)) in solvent A (hexane:toluene:acetic acid (50:50:0.5)) over 50 min. The th-18:1 isomers were combined and, after solvent evaporation, were further purified by RP-HPLC employing a

mobile phase of acetonitrile:water:acetic acid (35:65:0.05).

(h) 12h-17:2 and th-17:1

The standards, 12h-[^{14}C]17:2 and th-[^{14}C]17:1, used as markers for the monohydroxy and trihydroxy regions, respectively, on NP-HPLC, were prepared by incubation of [1-^{14}C]20:3 (35 x 10^6 cpm) with a bovine aorta particulate fraction (4 ml), containing 10 µM methemoglobin, for 60 min at 37 °C (256). After extraction, 12h-17:2 (t_R, 22 min) and th-17:1 (t_R, 62-65 min) were purified by NP-HPLC employing a mobile phase consisting of 97 % A and 3 % B over 30 min followed by a linear gradient over 50 min between 3 % and 100 % solvent B in solvent A. 12h-17:2 (t_R, 28 min) was re-chromatographed by NP-HPLC using hexane:2-propanol:acetic acid (98.8:1.2:0.1) as the mobile phase, whereas th-17:1 (t_R, 11.5 min) was re-chromatographed by RP-HPLC using an acetonitrile:water:acetic acid (30:70:0.05) system.

2.2.6. Measurement of PUFA Metabolites Formed from Exogenous Substrate by Homogenates and Particulate Fractions

(a) Preparation of homogenates and particulate fractions

Aorta and ductus arteriosus were cleaned and the adventitia removed. The remaining tissue was minced in 0.05 M Tris-HCl, pH 7.5, (4 ml/g tissue) and homogenized in an ice-water bath with a Vir-Tis homogenizer (6 x 10 s with 50 s in between to allow for cooling). The homogenate was either incubated with radioactive substrates or centrifuged at 400 x g for 10 min at 4 °C. The supernatant was filtered through one layer of cheesecloth and the pellet was

resuspended to the original volume of the homogenate by addition of Tris buffer, and rehomogenized with a Vir-Tis homogenizer (3 x 10 s). This homogenate was centrifuged for 10 min at 400 x g and, after filtration, the two filtered supernatants were combined and centrifuged at 100,000 x g for 60 min at 4 °C in a Beckman model L-8 ultracentrifuge. The pellet was resuspended in Tris buffer (0.25 ml/g tissue) which, in some cases, contained either methemoglobin (10 μM) or adrenaline (1 mM).

(b) Incubation procedures for subcellular fractions and homogenates

Homogenates or 100,000 x g supernatant or particulate fractions from aorta or ductus arteriosus were incubated with mixtures of unlabeled and ^{14}C-labeled 18:2, 20:3, 20:4, 13hp-18:2, 9,10e-11h-18:1, or isomers of 11,12e-10h-17:1 for various times at 37 °C. Incubations were terminated with 2 volumes of ethanol and the products were extracted with ODS silica. For experiments in which the products were to be quantitated, [9β-^{3}H]$PGF_{1\alpha}$ (approx. 1 μg, 5 x 10^5 dpm) was added before extraction as an internal standard to monitor recovery.

(c) Purification and quantitation of products

The products in the methyl formate fraction, either untreated or in some cases methylated by treatment of diazomethane, were analyzed by NP-HPLC. Products were quantitated by measuring the radioactivity in fractions (each collected for a period of 1 min) by liquid

scintillation counting after the addition of 3 ml of Ready-Solv or Formula 949. All values were corrected for recovery on the basis of the percent recovery of [9β-^{3}H]$PGF_{1\alpha}$.

In some experiments the total amounts of ^{14}C-labeled oxygenated metabolites formed from the corresponding ^{14}C-labeled substrates were measured by separating the substrates from their more polar oxygenated metabolites by chromatography on open columns of silicic acid (0.5 g). The dried methyl formate extract was dissolved in hexane/toluene/methanol/acetic acid (95:5:0.002:0.1). Unconverted substrate was eluted with 22 ml hexane/diethyl ether/acetic acid (98.5:1.5:0.1). The oxygenated metabolites were then eluted as a single fraction with 8 ml methyl formate/methanol (90:10). After evaporation of the solvent under nitrogen the radioactivity was measured by liquid scintillation counting.

2.2.7. Measurement of Products Formed from Endogenous Substrate by Slices of Aorta

(a) Preparation of tissues and incubation conditions

Rat and rabbit aortae were rapidly removed, cleared of adhering fatty tissue, and placed in a modified Krebs-Ringer medium (127 mM NaCl, 5 mM KCl, 1.27 mM $MgSO_4$, 1.27 mM KH_2PO_4, 2.7 mM $CaCl_2$, and 5.5 mM glucose), containing 15 mM Tris-HCl, pH 7.4, at 0 °C. Bovine blood vessels were cleared of adhering fatty tissue, and in some cases sectioned into inner (intima and inner media) and outer (outer media and adventitia) layers, before being placed in Krebs-Ringer Tris medium at 0 °C. Slices of aorta or ductus

arteriosus (0.3 to 1.0 g) were incubated for 20 min at 37 °C in Krebs-Ringer Tris media. The medium was then removed, and the amounts of various oxygenated PUFA metabolites in the medium and the tissue were determined as described below and illustrated in Fig. 5.

(b) Extraction of oxygenated PUFA metabolites and hydrolysis of lipids

The media obtained after incubation of slices of blood vessels was divided into two parts in a ratio of 3:1. A mixture of the internal standards, [3,3,4,4-^{2}H]PGE_2 (200 ng) and 6-oxo[3,3,4,4-^{2}H]$PGF_{1\alpha}$ (200 ng), was added to the smaller part (fraction A), whereas a mixture of 14h-19:2 (200 ng) and th-19:1 (200 ng) was added to the larger part (fraction B). Both mixtures were extracted using ODS silica as described above.

The tissue slices from which the medium had been removed were immediately frozen in liquid nitrogen and were kept on dry ice. Within 3 h of the incubations, the frozen tissue was pulverized using a tissue pulverizer (Thermovac Industries) which was cooled in an acetone-dry ice bath. The resulting powder was homogenized at -20 °C in a ground glass homogenizer in chloroform:methanol (2:1) containing 0.05% butylated hydroxytoluene, 14h-19:2 (400 ng), and th-19:1 (400 ng), which had been bubbled with argon. The homogenate was rapidly filtered through a sintered glass funnel, and the filtrate was concentrated nearly to dryness in a rotary evaporator. Chloroform:methanol (2:1) (6 ml)

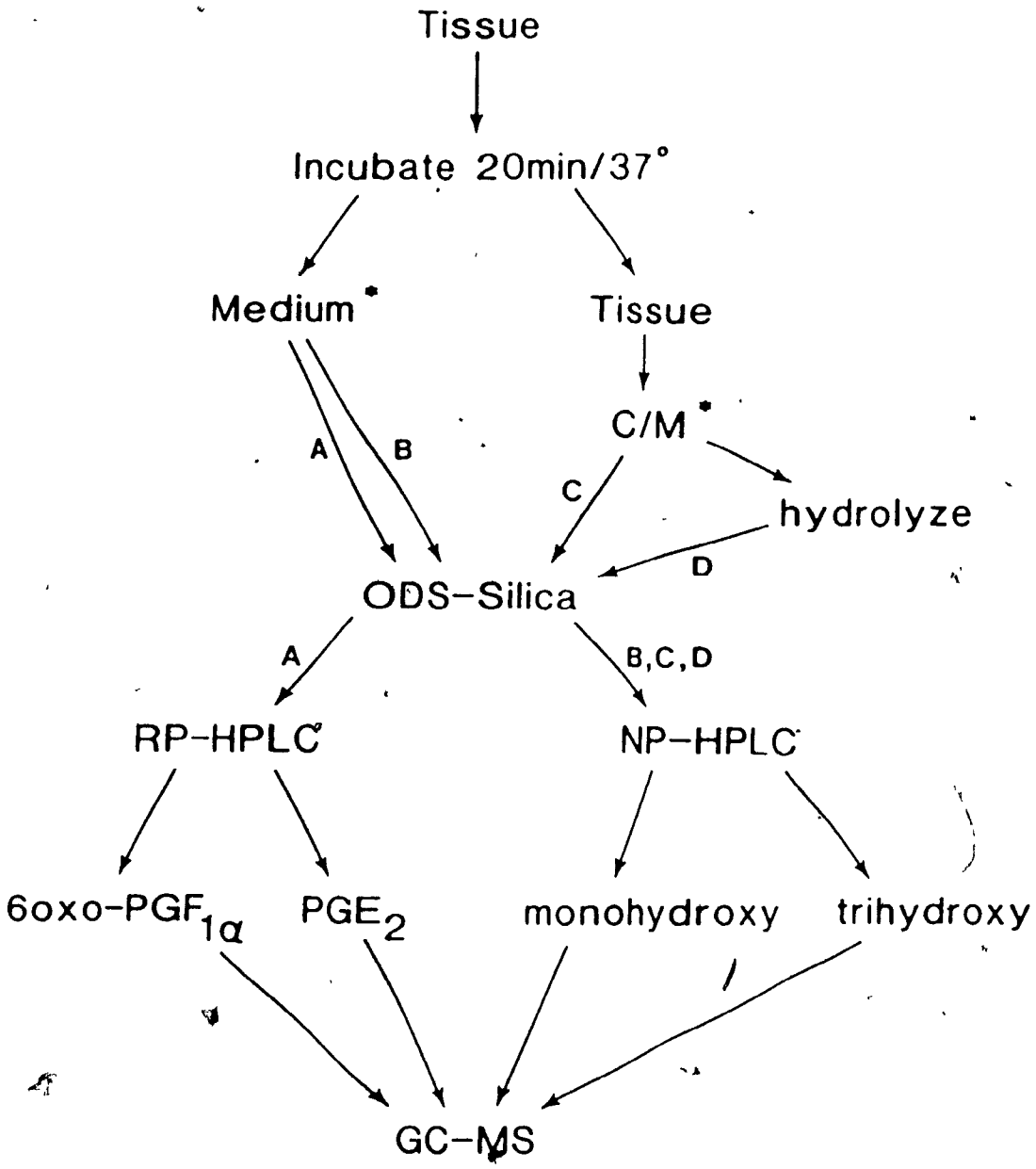

Fig.5. Scheme for the purification and analysis by GC-MS of oxygenation products of 18:2 and 20:4. * The internal standards were added at these points.

C/M, chloroform/methanol (2:1).

was then added, and the mixture was divided into two equal parts. One part (fraction C) was extracted immediately with ODS silica to give an estimate of the amounts of free oxygenated PUFA metabolites contained within the tissue. The solvent was evaporated from the other part under a stream of argon, and the residue was dissolved in 0.28 N potassium hydroxide in 95% ethanol which had been degassed and bubbled with argon. Hydrolysis of lipids was carried out in this solution for 45 min at 55 °C under argon. After hydrolysis, the samples were extracted with ODS silica. The material in the extracts was dissolved in toluene containing butylated hydroxytoluene (0.05%), and stored under argon at -20 °C. This fraction (fraction D) was used to determine the total amounts of free plus esterified oxygenated PUFA metabolites present in the tissue.

(c) Purification of oxygenated PUFA metabolites by HPLC

6-oxo$PGF_{1\alpha}$ (t_R, 13 min) and PGE_2 (t_R, 23.5 min) and their deuterated analogs were purified from fraction A by RP-HPLC using a linear gradient between water:acetonitrile:acetic acid (75:25:0.05) and water:acetonitrile:acetic acid (55:45:0.05) over 25 min. [9β-^{3}H]$PGF_{2\alpha}$ (10^5 cpm; t_R, 22 min) was added as a marker.

The materials in fractions B, C, and D were methylated, and separated by NP-HPLC into 2 fractions containing (i) monohydroxy PUFA (t_R's between 11 and 17 min) and (ii) trihydroxy products (t_R's between 43 and 44 min). The mobile phase consisted of hexane:isopropanol:acetic acid

(99.5:0.5:0.1) for the first 25 min, followed by a linear gradient to hexane:isopropanol:acetic acid (65:35:0.1) over 25 min. In order to help determine which fractions to combine, 12-hydroxy-8,10-[1-^{14}C]heptadecadienoic acid (2500 cpm; t_R, 15 min) and 8,11,12-trihydroxy-9-[1-^{14}C]heptadecenoic acid (4000 cpm; t_R, 44 min) were added as markers for the monohydroxy and trihydroxy fractions, respectively.

(d) Quantitation of products by GC-MS

Prostaglandins were quantitated as their methyl ester, TMS ether, O-methyloxime derivatives using an SP2250 column at 250 °C. Ions were monitored at m/z 598 and 602 for 6-oxoPGF$_{1\alpha}$ and its tetradeuterated analog (t_R, 1.8 min), respectively. Ions at m/z 508 and 512 were monitored for PGE_2 and d_4-PGE_2 (t_R, 1.8 min), respectively.

Trihydroxy-18:1 (t_R, 2.3 min) was quantitated as the TMS derivative of its methyl ester by comparing the intensity of the ion at m/z 259 to that for the internal standard, th-19:1 (t_R, 3 min), at m/z 273. Ions at m/z 173 (both compounds) and 460 (th-18:1) were also monitored to provide further evidence for the structures of the substances being measured. The column temperature was 225 °C for 1 min, and then increased at a rate of 10 °C/min.

Monohydroxy derivatives of 17:3, 18:2, 19:2, and 20:4 were hydrogenated and converted to their methyl ester, TMS ether derivatives and analyzed by GC-MS. The column temperature was 203 °C for one minute, and was then increased at a rate of 2 °C/min. Three sets of ions were

monitored for these products as shown in Table 3. The amounts of each of the above products was determined by determining the ratio of the peak area for their specific ions, to that at m/z 329 for the internal standard, 14h-19:2.

The amounts of released monohydroxy and trihydroxy products were calculated from the sum of the products in fractions B (released into medium) and C (unesterified products present in the tissue after the incubation). The amounts of esterified products were determined by subtracting the amounts of products in fraction C from those in fraction D (total content of products in the tissue, after hydrolysis).

2.2.8. Measurement of Fatty Acid Content of Aorta

Sections of aortae (50-100 mg) were homogenized using a ground glass homogenizer in 20 volumes of chloroform:methanol (2:1) containing 100 µg arachidic acid as an internal standard. The homogenate was filtered and the solvent removed from the filtrate using a rotary evaporator. The residue was subjected to basic hydrolysis as described above. Non-saponifiable materials were then extracted with hexane (2 x 5 ml), and the sample was then acidified and the fatty acids extracted with hexane (2 x 5 ml). After removal of the hexane under a stream of nitrogen, the material in the residue was methylated with diazomethane and analyzed by gas chromatography on a 10% Silar 10C column (2 x 0.015 m). The temperature was

TABLE 3

Quantitation of Monohydroxy PUFA Metabolites by GC-MS

The monohydroxy fatty acid fraction from aorta, containing the internal standard, 14h-19:2, was purified by NP-HPLC. The products in this fraction were hydrogenated and converted to their methyl ester, TMS ether derivatives prior to analysis by GC-MS as described in the Methods section.

Ions monitored			
Set 1 (1-3 min)	Set 2 (3-3.9 min)	Set 3 (3.9-5 min)	Product (t_R)
173			12h-17:0 + 13h-18:0
259			9h-18:0 (2.7 min)
301			12h-17:0 (2.2 min)
315			13h-18:0 (2.85 min)
	173		14h-19:0 (3.6 min)
	259		-
	301		-
	329		14h-19:0
		173	15h-20:0 (4.7 min)
		287	11h-20:0 (4.4 min)
		301	12h-20:0 (4.5 min)
		343	15h-20:0 (4.7 min)

increased at a rate of 2 °C/min, from 150 °C to 210 °C.

2.2.9. Glutathione Peroxidase Assay

Glutathione peroxidase activity was assayed by coupling the reaction to the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of glutathione reductase and measurement of the decrease in optical density at 340 nm (267). The coupled reaction is illustrated in Fig. 6.

Rat or rabbit aorta (0.05-0.1 g) was chopped into small pieces and homogenized in 2 ml ice cold 0.05 M Tris buffer, pH 7.55, containing 0.1 mM EDTA. The homogenate was centrifuged for 1 h at 100,000 x g and the supernatant was removed. Aliquots (100-400 µl) of the 100,000 x g supernatant were assayed for activity in 3 ml of buffer containing 1 mM glutathione, 0.2 mM NADPH, 1 U glutathione reductase and 0.25 mM hydrogen peroxide. All components of the assay, except for the hydrogen peroxide, were preincubated for 2 min at room temperature prior to the addition of substrate. The conversion of NADPH to NADP+ was followed by continuously recording the absorbance of the system at 340 nm between 1 and 4 min after initiation of the reaction. Since glutathione can be oxidized non-enzymatically by air, the rate of oxidation of NADPH in the absence of the 100,000 x g supernatant was measured and subtracted from the total rate in the presence of the 100,000 x g supernatant.

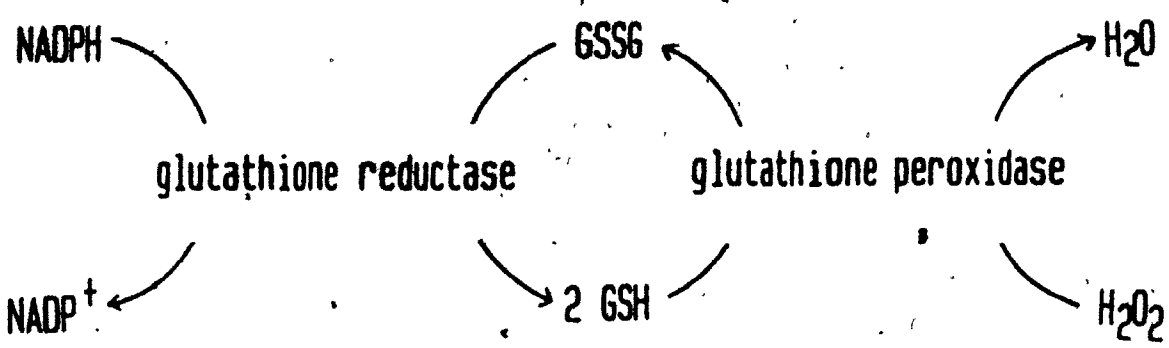

Fig.6. Assay of glutathione (GSH) peroxidase activity by coupling the reaction to the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of glutathione reductase. GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide substrate.

2.2.10. Cell Culture

(a) Endothelial cells

Bovine aorta endothelial cells were cultured according to the method of Macarak et al. (268). Calf thoracic aortas (20-25 cm) were immediately placed in ice cold Dulbecco's phosphate-buffered saline (PBS) supplemented with glucose (2 g/l), penicillin (100 U/ml), streptomycin (100 µg/ml) and mycostatin (100 U/ml). The aortas were washed with PBS and the adhering fat was dissected away to free the intercostal arteries. These small vessels were tied and one end of the aorta was clamped and the collagenase distributed throughout the lumen by inverting the clamped vessel several times. The vessel was then allowed to incubate for 40 min at room temperature. Following the incubation period the collagenase solution was discarded and the aorta was washed four times with 10 ml of culture medium (Medium 199 supplemented with 20 % fetal bovine serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and mycostatin (50 U/ml)). The medium from the washes was pooled and evenly distributed in tissue culture dishes (100 x 20 mm style, approx. 10 ml/dish). Cells were cultured at 37 °C in a humidified atmosphere of 5 % carbon dioxide in air. Culture medium was changed after 24 h and subsequently every 48 h. All experiments were carried out on primary cultures that had reached confluence (5-9 days). Cells exhibited a characteristic monolayer, "cobblestone" appearance by light microscopy.

(b) Smooth muscle cells

Smooth muscle cells were grown from intimal segments (approx. 1 mm^2) obtained from calf aorta (269). Five to ten segments were placed in dry tissue culture dishes (100 x 20 mm style) and were allowed to adhere to the bottom of the dishes. After 10 min, culture medium (Dulbecco's Modified Eagle's medium supplemented with 10 % fetal bovine serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and mycostatin (50 U/ml)) was carefully added to the dishes so as not to dislodge the tissue segments. The dishes were then placed in an incubator at 37 °C with a humidified atmosphere of 5 % carbon dioxide in air and were left undisturbed for 3 days. Culture medium was replaced every 72 h. The cells which grew from the explants were nearly confluent after approximately 3 weeks. At this time the cells were subcultured by trypsinization (trypsin/EDTA (1X) without calcium and magnesium; Gibco, Grand Island, NY) for 5 min at 37 °C. The cells released due to treatment with trypsin were split in a 1/3 ratio and subcultured. Subcultured cells grew rapidly, reaching confluence within one week and then growing into multiple overlapping layers.

2.2.11. Incubation of 18:2, 13h-18:2, and th-18:1 with Endothelial and Smooth Muscle Cells

Confluent endothelial (8 days in culture) and smooth muscle (4 passages) cells were incubated with either [1-^{14}C]18:2, 13h-[1-^{14}C]18:2, or th-[1-^{14}C]18:1 (7.3 x 10^5 cpm) for 4 h at 37 °C. The medium was then removed and the cells were washed once with medium (5 ml) prior to extraction of

lipids. After removal of the medium, methanol (2 ml) was added to the culture dishes, and the cells were removed by scraping with a rubber policeman. Chloroform (4 ml) was added, and after mixing with a Pasteur pipette, the mixture was filtered. The solvent was removed under a stream of nitrogen, and the residue dissolved in chloroform (3 ml) and passed through a column of silicic acid (0.3 g). The column was eluted with a further 7 ml chloroform to give a fraction containing neutral lipids. Polar lipids were than eluted with methanol (4 ml), followed by methanol:water (1:1, 2 ml). Neutral lipids were analyzed by RP-HPLC using water:tetrahydrofuran (24:76) at a flow rate of 0.7 ml/min as the mobile phase. Polar lipids were analyzed by NP-HPLC with acetonitrile:methanol:sulfuric acid (100:6:0.05) at a flow rate of 1 ml/min as the mobile phase.

RESULTS

3.1. 20:3 Metabolism

Di-homo-γ-linolenic acid (20:3), precursor of the monoene prostaglandins, is present in considerably smaller amounts in lipids of animal tissues than arachidonic acid (20:4). In vascular tissue 20:3 cannot be converted to PGI_2 due to the absence of the 5,6-double bond. 20:3 had been shown in our laboratory to be converted predominantly to products derived from 12-hydroperoxy-8,10-heptadecadienoic acid (256). 10,11,12-trihydroxy-8-heptadecenoic acid and 2 isomers of 8,11,12-trihydroxy-9-heptadecenoic acid were identified by GC-MS, but the precise mechanism for their formation had not been worked out. We proposed that these products are formed via epoxyhydroxy intermediates.

3.1.1. Identification of 11,12-epoxy-10-hydroxy-8-heptadecenoic acid

Incubation of 20:3 with a particulate fraction from fetal calf aorta for 5 min resulted in the formation of a considerable number of products (Fig.7). In addition to the major product (t_R, 20 min), which was previously identified as 12h-17:2 (256), there were a number of more polar products (a to e). Compound c had previously been identified as 10,11,12th-17:1, whereas compounds d and e were stereoisomers of 8,11,12th-17:1 (256). Compounds a (t_R, 39 min) and b (t_R, 42 min), which had not previously been identified, were only present in large amounts when incubations were carried out for short times. Analysis of

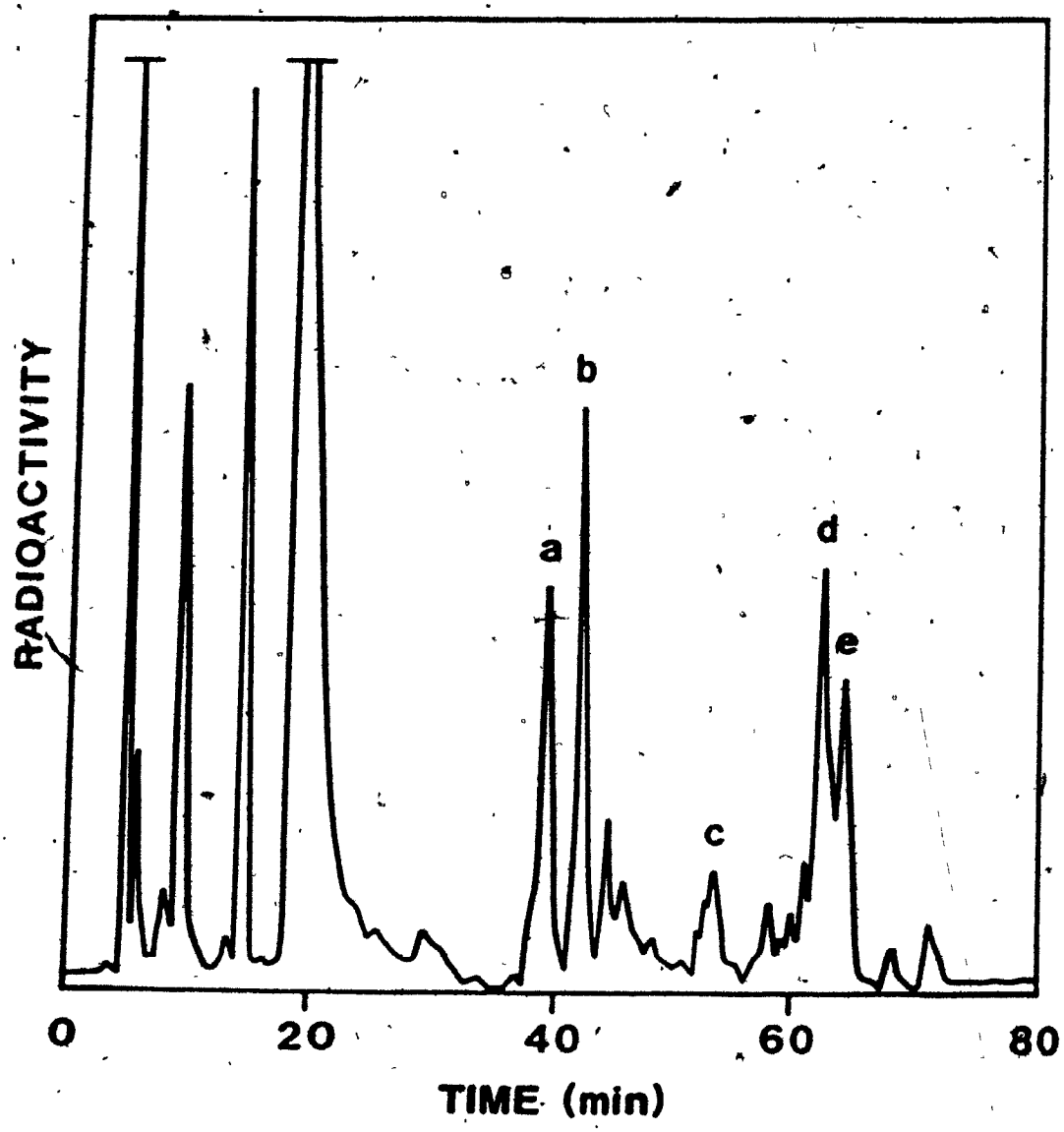

Fig.7. High pressure liquid radiochromatogram of the products formed after incubation for 5 min at 37 °C of a particulate fraction (2.5 ml) from fetal calf aorta with [1-^{14}C]20:3 (2 μCi, 36 nmol) in the presence of 10 μM methemoglobin. The incubation was terminated with ethanol (5 ml) and the products were extracted using ODS silica. The residue from the extract was analyzed by NP-HPLC using a mobile phase consisting of 3% solvent B_m (toluene:ethyl acetate:acetonitrile:methanol (30:40:30:2)) in solvent A_m (hexane:toluene (50:50)) for 30 min, followed by a linear gradient over 50 min between 3% and 100% solvent B_m in solvent A_m.

these compounds by GC-MS indicated that they had identical C-values (19.8) and mass spectra, suggesting that they were stereoisomers. The mass spectrum of compound b (Fig.8A) has major fragment ions at m/z 369 (M-15), 313 (M-71), 285, 271 (cleavage between epoxy and TMSO substituents) and 255. This is quite similar to the mass spectrum of the TMS ether derivative of the methyl ester of 12,13-epoxy-11-hydroxy-9-octadecenoic acid (270) except that most of the above ions occur at m/z values 14 units higher in the mass spectrum of the octadecenoic acid derivative. The mass spectrum of the TMS derivative of the methyl ester of compound b after hydrogenation (Fig.8B) has major fragment ions at m/z 371 (M-15), 287, 273, (cleavage between epoxy and TMSO substituents), 257 and 169. This is analogous to the mass spectrum of 12,13-epoxy-11-hydroxyoctadecanoic acid reported in the literature (263). These results therefore indicate that compounds a and b are stereoisomers of 11,12e-10h-17:1.

3.1.2 Time Course for the Conversion of 20:3 to Epoxyhydroxy and Trihydroxy Products

Particulate fractions from fetal calf aorta were incubated for various times with [1-^{14}C]20:3 in the absence of any added cofactors, and the products were measured by HPLC (Fig.9). Although the 2 stereoisomers of 11,12e-10h-17:1 (a and b) were formed rapidly during the initial period of the incubation, the amounts of these products reached maxima at about 10 min and then declined. On the other hand, only small amounts of 10,11,12th-17:1 (c) were

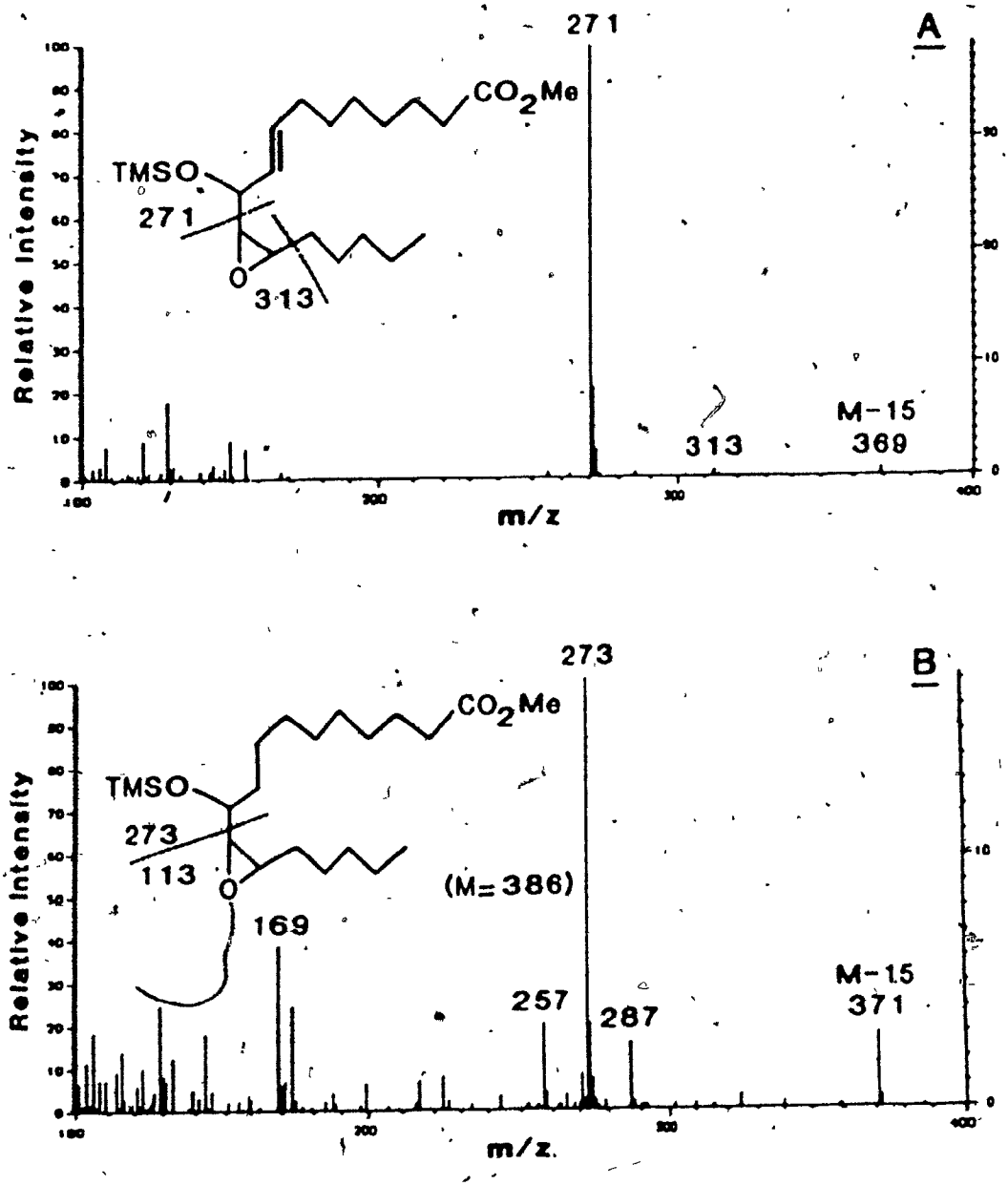

Fig.8. Mass spectra of the trimethylsilyl ether derivatives of the methyl esters of 11,12-epoxy-/10-hydroxy-8-heptadecenoic acid (compound b) before (A) and after (B) hydrogenation.

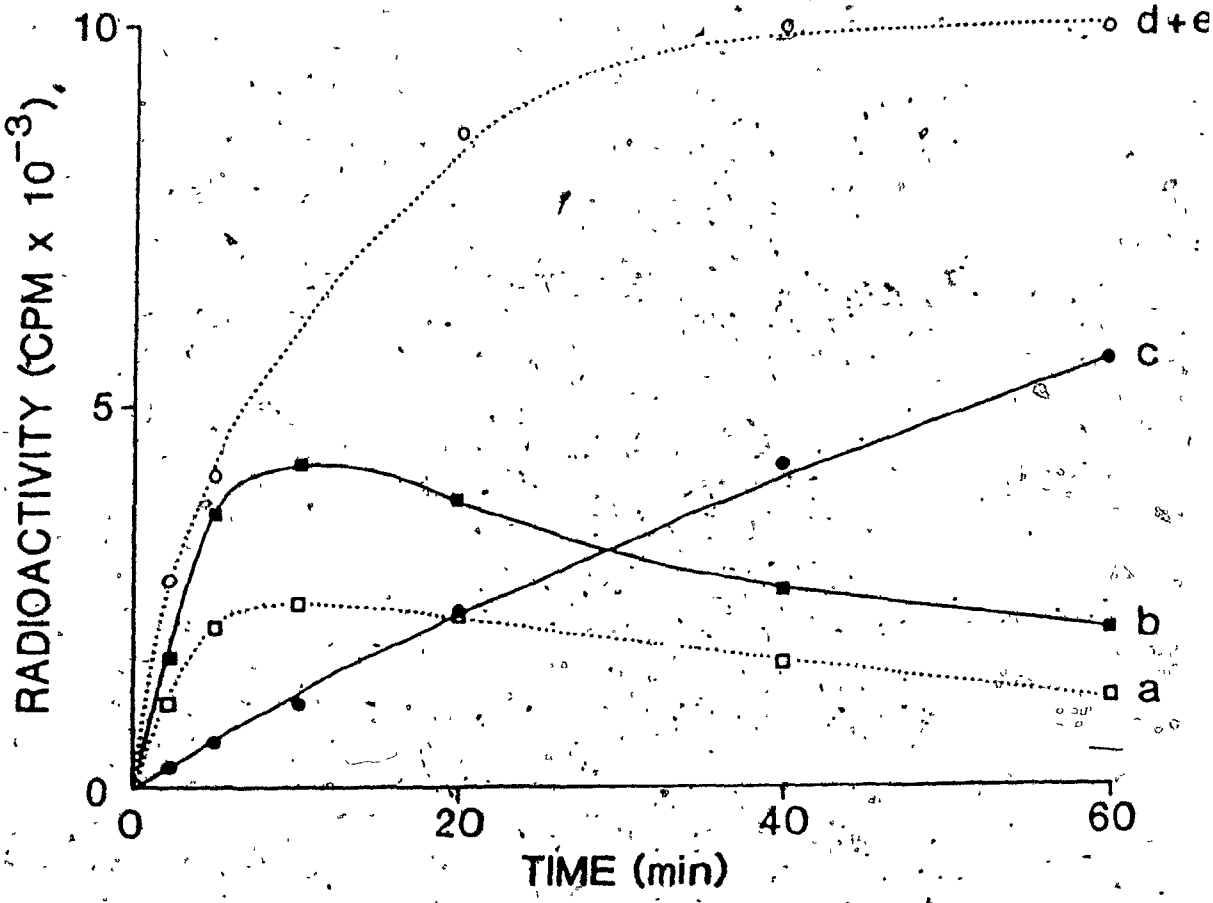

Fig.9. Time course for the conversion of 20:3 to compound a (□; 11,12e-10h-17:1, first isomer), compound b (■; 11,12e-10h-17:1, second isomer), compound c (●; 10,11,12th-17:1) and compounds d + e (○; 2 isomers of 8,11,12th-17:1). Particulate fractions (0.5 ml) from fetal calf aorta were incubated in the absence of added cofactors at 37 °C with [1-^{14}C]20:3 (390,000 cpm, 9.8 nmol). At the desired time intervals, incubations were terminated with 1.0 ml ethanol. [9β-^{3}H]$PGF_{1\alpha}$ (5 x 10^5 dpm, 1 μg) was added to each mixture to serve as an internal standard and the products were extracted using ODS silica. The residue from each extract was then analyzed by normal-phase HPLC. The values are means of duplicates and have been corrected for recovery.

detected initially, but the formation of this product was nearly linear up to 60 min. This is in contrast with the 2 stereoisomers of 8,11,12th-17:1 (d and e), since the amounts of these products did not increase after 40 min. These results suggest that the epoxyhydroxy compounds a and b are intermediates in the formation of product c.

3.1.3. Metabolism of 11,12e-10h-17:1

The 2 stereoisomers of 11,12e-10h-17:1 were synthesized by incubation of [1-^{14}C]20:3 with a particulate fraction from fetal calf aorta. Incubation of compounds a or b with either a particulate or 100,000 x g supernatant fraction from fetal calf aorta resulted in the formation of a product with a t_R in each case identical to that of 10,11,12th-17:1 (c). The isomers of c derived from a and b had the same t_R, even when cochromatographed using isocratic conditions. In order to confirm the identitities of these products, a (13 µg, 50,000 cpm) and b (11 µg, 42,000 cpm) were incubated separately with a 100,000 x g supernatant fraction (2.5 ml) from fetal calf aorta for 70 min at 37 °C. The products were extracted, purified by HPLC and analyzed by GC-MS after conversion to the TMS ether derivatives of their methyl esters. Both products had mass spectra identical to that which was previously reported for the corresponding derivative of 10,11,12th-17:1 (256).

The time course for the conversion of 11,12e-10h-17:1 (isomer a) to 10,11,12th-17:1 (c) is shown in Fig.10. Nearly 80 % of compound a was metabolized by each of these

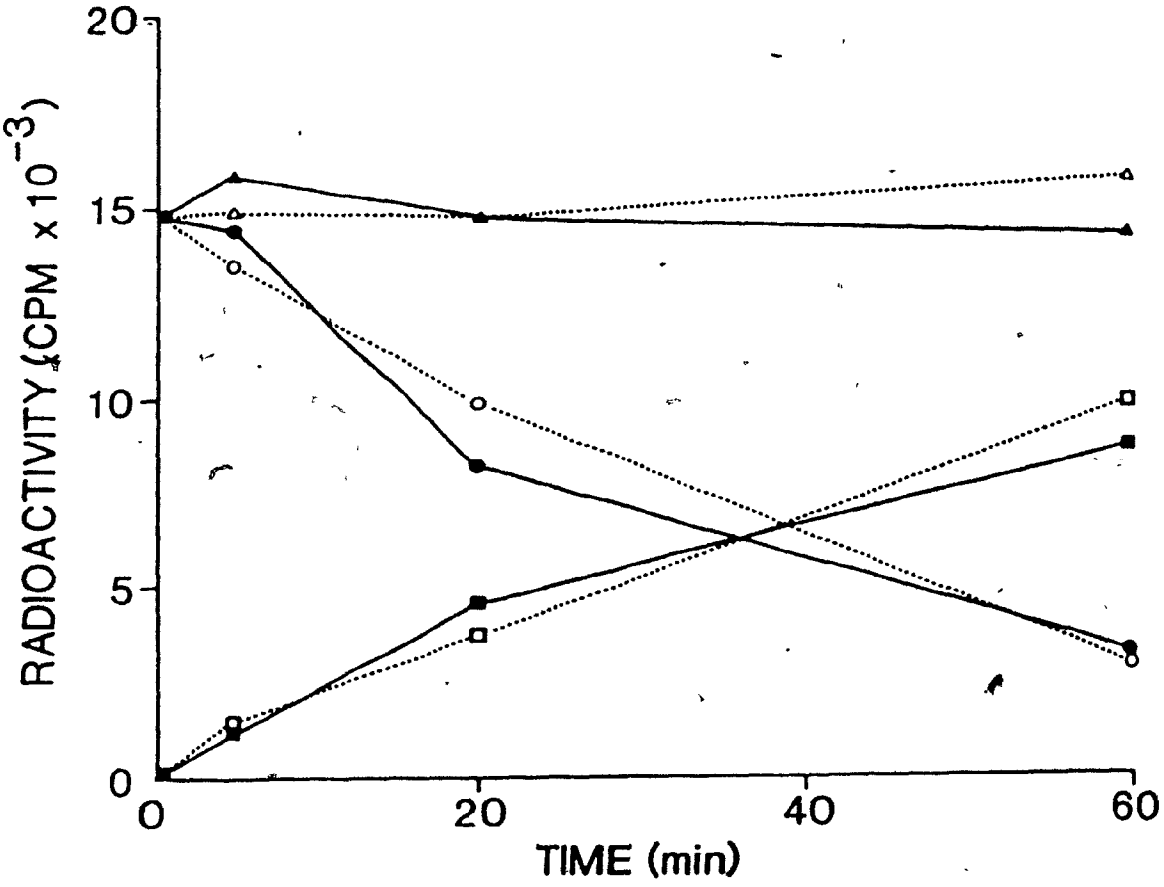

Fig. 10. Conversion of compound a (11,12e-10h-17:1; O,●,Δ and ▲) to compound c (10,11,12th-17:1; □ and ■) in the presence of particulate (___ ; ●,■) and cytosolic (...; O,□) fractions from fetal calf aorta. No conversion of compound a was observed in the presence of boiled particulate (▲) or cytosolic (Δ) fractions. [1-^{14}C]-labeled compound a (15,000 cpm, 0.15 nmol), prepared as described in the text, was incubated with the above fractions for various times at 37 °C in the absence of added cofactors. The products were extracted and analyzed as described in the legend to Fig.9. The protein concentrations of the particulate and cytosolic fractions were 4.1 and 1.7 mg/ml, respectively. All values are means of duplicates and have been corrected for recovery.

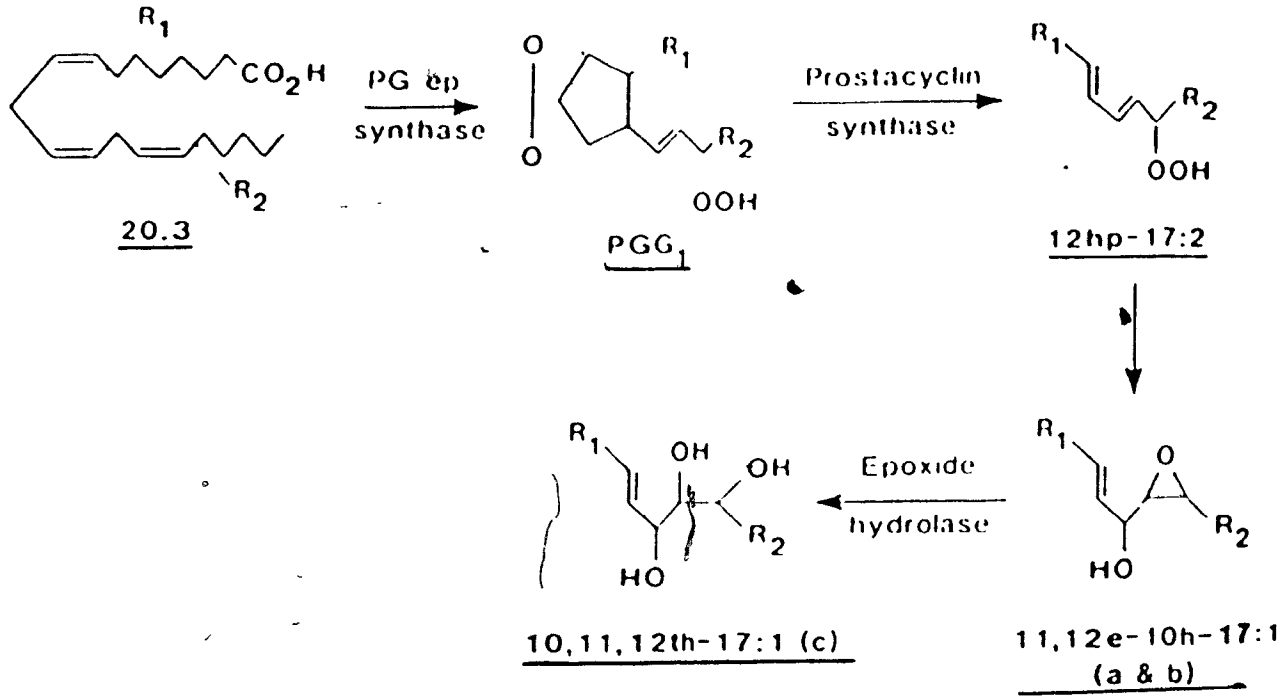

Fig.11. Biosynthesis and metabolism of 11,12-epoxy-10-hydroxy-8-heptadecenoic acid (compounds a and b) by aorta.

fractions after 60 min. There were corresponding increases in the amounts of 10,11,12th-17:1 formed during the incubations. No metabolism of compound a was observed after incubation with boiled particulate or supernatant fractions. Similar results were obtained with compound b. These results indicate that products a and b were enzymatically converted to product c, presumably by an epoxide hydrolase. The mechanism for the formation of epoxyhydroxy and trihydroxy products from 20:3 is shown in Fig.11.

3.2. Metabolism of Linoleic Acid

18:2, found abundantly in many cellular lipids, has been reported to inhibit PGI_2 production in cultures of vascular endothelial cells (178,179). We postulated that 18:2 could be oxygenated by vascular tissue to give rise to hydroperoxy intermediates. If not metabolized rapidly these intermediates could cause an inhibition of prostacyclin synthase, since this enzyme is inhibited by hydroperoxides. Alternatively, 18:2 could compete with 20:4 for the substrate binding site of prostaglandin endoperoxide synthase and thus block its conversion to PGI_2. Therefore, we undertook an investigation of the metabolism of 18:2 by vascular tissue, as well as the mechanism of its effect on 20:4 vascular metabolism.

3.2.1. Metabolism of 18:2 by Particulate Fractions from Fetal Calf Aorta

Particulate fractions (7.5 mg protein/ml) from fetal calf aorta were incubated with [1-^{14}C]18:2 (2.0 μCi, 57 μM) for

Fig.12. High-pressure liquid radiochromatograms of 18:2 metabolites formed by aorta. Particulate fractions from fetal calf (A) or adult (B) aorta (1.0 ml, 7.5 mg protein) were incubated for 40 min at 37 °C with [1-^{14}C]18:2 (2.0 µCi, 57 µM). A homogenate (4.0 ml, 6.7 mg protein/ml) of fetal calf aorta (C) was also incubated with [1-^{14}C]18:2 under similar conditions. Incubations were terminated with ethanol (2 vol.) and the products were extracted using ODS silica. The residues from the extracts were analyzed by NP-HPLC using a mobile phase consisting of 4% solvent B_m (toluene:ethyl acetate:acetonitrile:methanol (30:40:30:2)) in solvent A_m (hexane:toluene (50:50)) for 30 min followed by a linear gradient over 50 min between 4% and 100% solvent B_m in solvent A_m. The more polar th-18:1 isomers (i.e. fractions f and g) formed after incubation of [1-^{14}C]18:2 with a particulate fraction from fetal calf aorta were chromatographed as their free acids (D). NP-HPLC was carried out using a linear gradient between 4 % solvent B (solvent B_m with 0.5 % acetic acid) and 100 % solvent B in solvent A (solvent A_m with 0.5 % acetic acid) over 50 min.

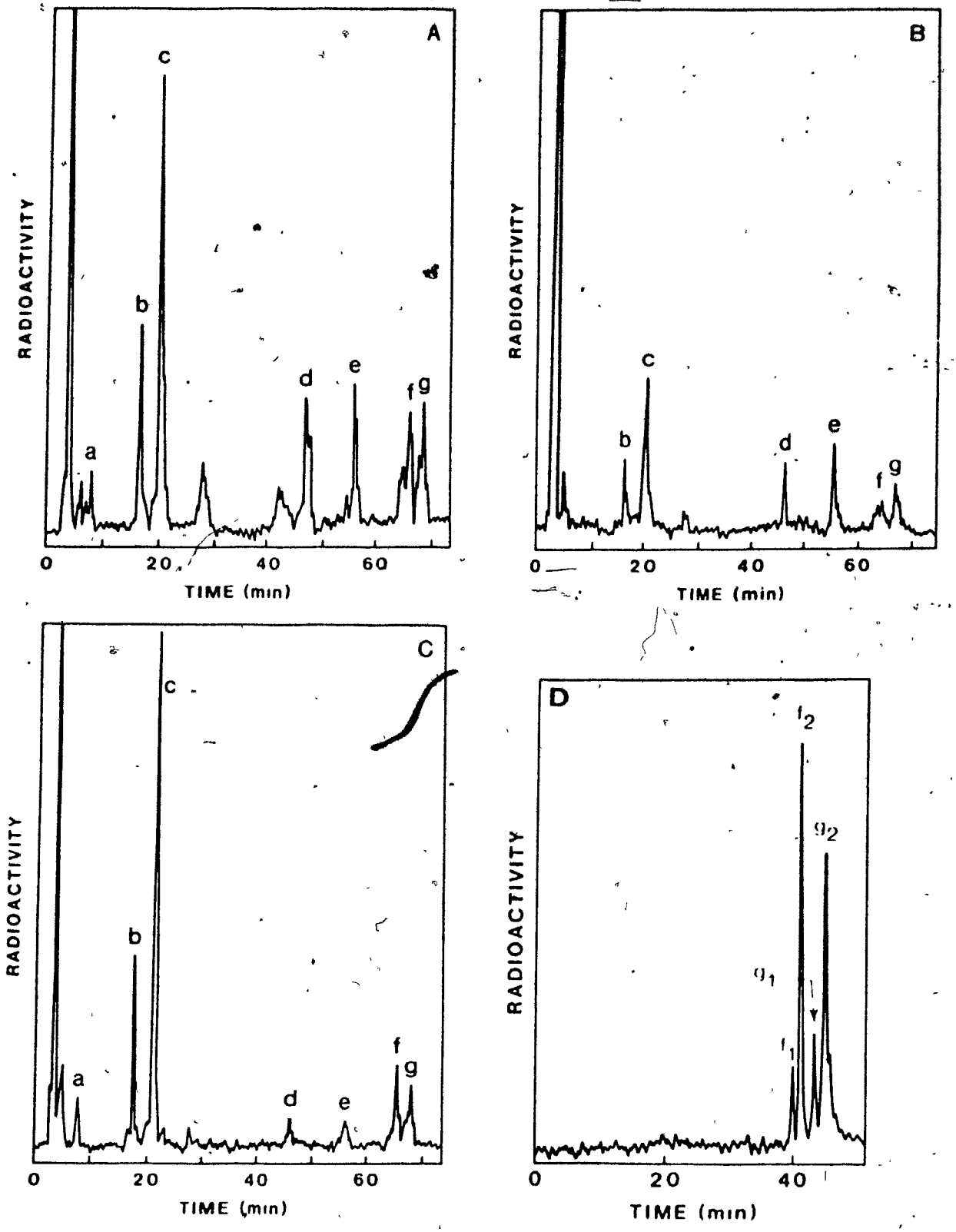

Fig. 12.

40 min at 37 °C. The NP-HPLC profile of the methylated products (Fig.12A) indicated that 18:2 had been converted to a large number of more polar metabolites (fractions a-g). The large peak with a t_R of 3.5 min was unconverted 18:2. Methemoglobin (10 μM), which can stimulate the decomposition of hydroperoxides, increased the amounts of all the metabolites by about 1.5-2 fold, with a preferential stimulation of the more polar products, e-g. Adrenaline, which can act as a peroxidase cofactor, on the other hand, stimulated the formation of the components of fractions b and c by 2-3 fold, but had little effect on the formation of the other products.

(a) Identification of Products

In order to identify the components of fractions a-g, particulate fractions from fetal calf aorta (40-100 g) were incubated with [1-^{14}C]18:2 (1.4 μCi, 500 μg) for 40 min at 37 °C in the presence of methemoglobin. The products were methylated with diazomethane and purified by NP-HPLC as shown in Fig.12A. Each product was then further purified by argentation HPLC or a second step of NP-HPLC. After conversion to their Me_3Si ether derivatives they were analyzed by GC-MS.

Fraction a

The radioactive material in fraction a (t_R, 24 min) was further purified by NP-HPLC using 1 % solvent B_m (toluene:ethyl acetate:acetonitrile:methanol (30:40:30:2)) in solvent A_m (hexane:toluene (50:50)). This product did

not form a Me_3Si ether derivative and had a mass spectrum (Fig.13) with major fragment ions at m/z 308 (M), 277 (M-31), 237 (M-71, loss of terminal pentyl group), 185 ($+CO-(CH_2)_7-CO_2CH_3$), 166 (loss of methyl 6-heptenoate due to a McLafferty rearrangement) and 151 (base peak, loss of $(CH_2)_7-CO_2CH_3$). The ions at m/z 151 and 185 were formed by cleavage on either side of an oxo group, indicating that a is 9-oxo-10,12-octadecadienoic acid (9o-18:2). Except for variations due to the position of the oxo group, this mass spectrum is similar to that reported in the literature for the methyl ester of 13-oxo-9,11-octadecadienoic acid (271).

Fractions b and c

Fractions b (t_R, 13 min) and c (t_R, 15 min) were purified further by argentation HPLC using methylene chloride:methanol (98:2) at a flow rate of 1 ml/min as the mobile phase. The Me_3Si ether derivatives of the two compounds had similar C values (19.9) and mass spectra (Figs.14A and 15A) with major fragment ions at m/z 382 (M), 367 (M-15), 351 (M-31), 311 (M-71, loss of terminal pentyl group) and 225 (M-157, loss of $(CH_2)_7-CO_2CH_3$). The base peak in the mass spectrum of b was at m/z 311, whereas that for c was at m/z 225. Hydrogenation of b and c revealed that each compound had two double bonds. The base peaks for the methyl ester-Me_3Si derivatives of the hydrogenated products were at m/z 173 (b: $Me_3SiO+=CH-(CH_2)_4-CH_3$) and 259 (c:$Me_3SiO+=CH-(CH_2)_7-CO_2CH_3$) (Figs.14B and 15B). These spectra are similar to those reported (272) for the corresponding derivatives of 13-

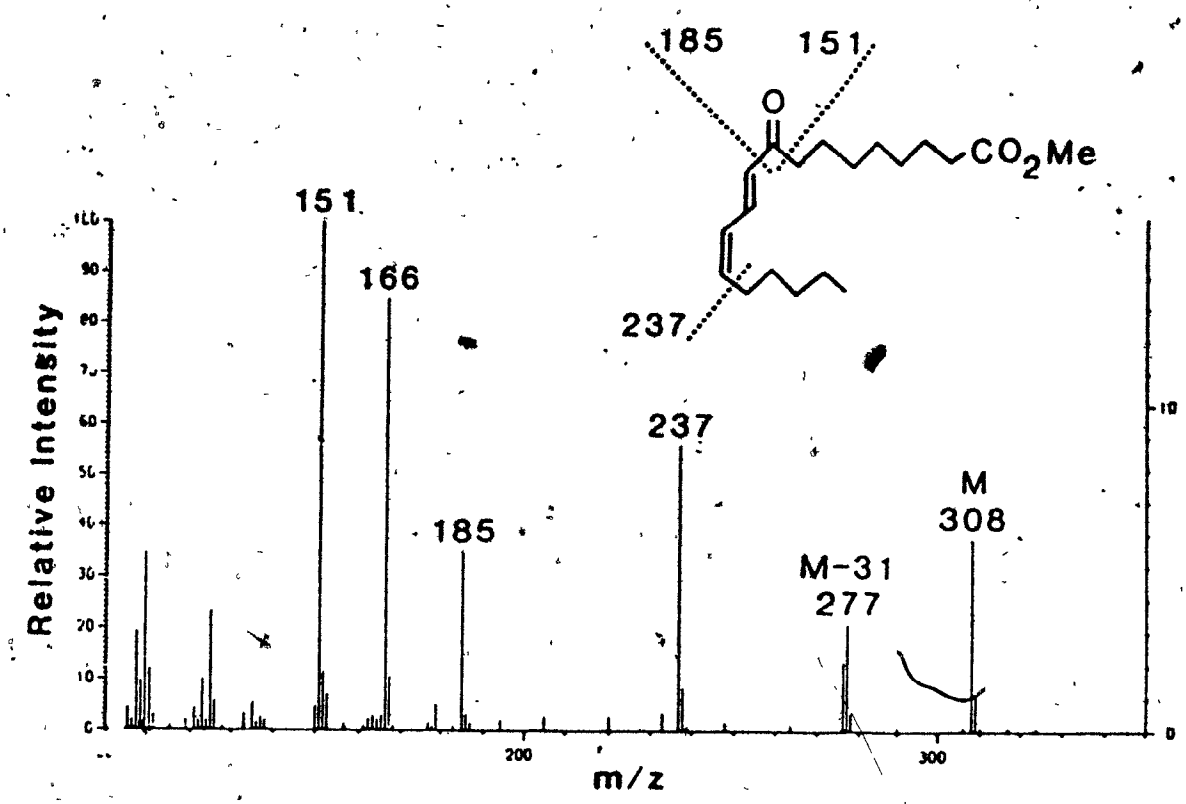

Fig.13. Mass spectrum of the trimethylsilyl ether derivative of the methyl ester of 9-oxo-10,12-octadecadienoic acid (fraction a).

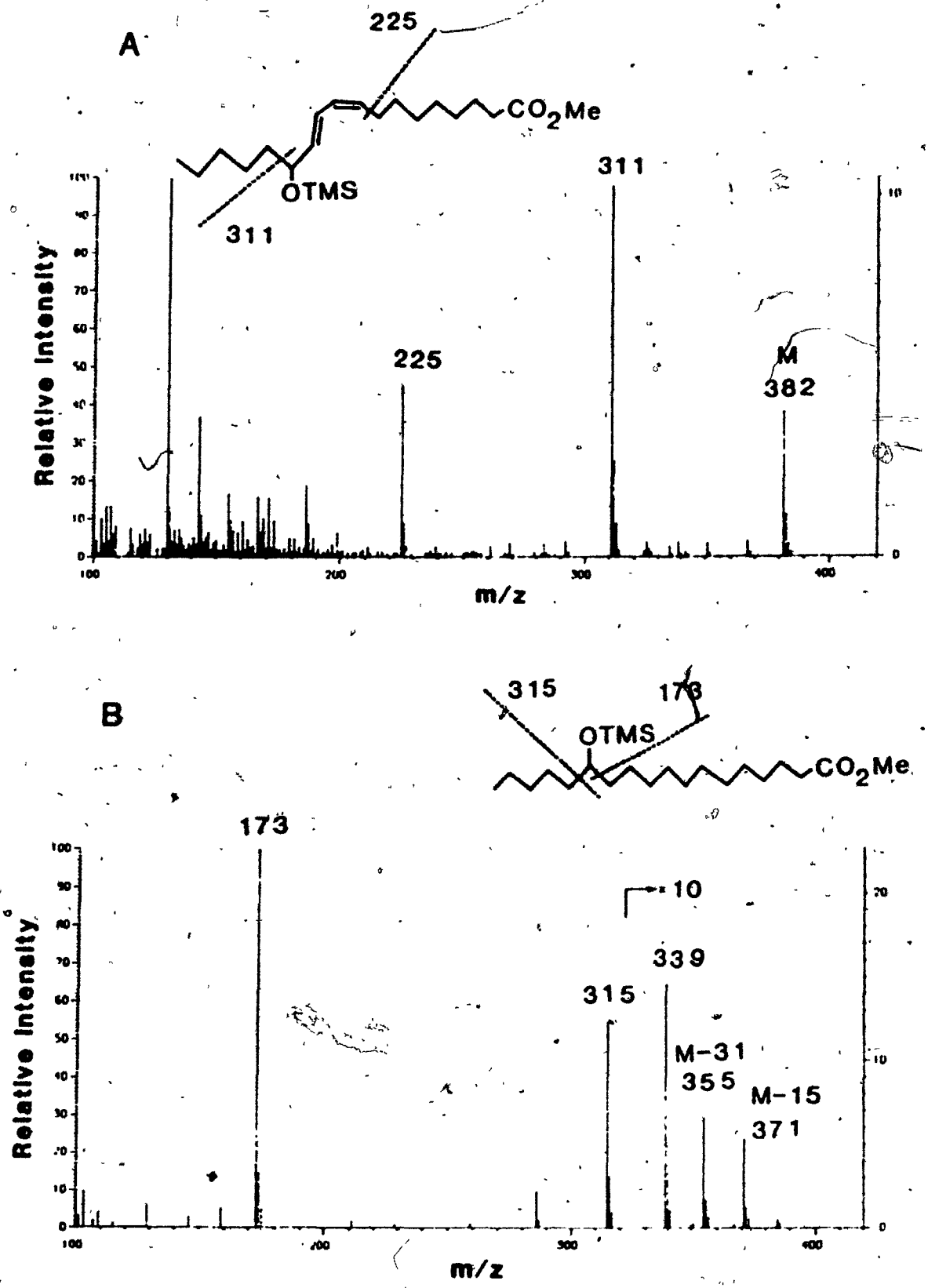

Fig.14. Mass spectra of the trimethylsilyl ether derivatives of the methyl esters of 13-hydroxy-9,11-octadecadienoic acid (fraction b) before (A) and after (B) hydrogenation.

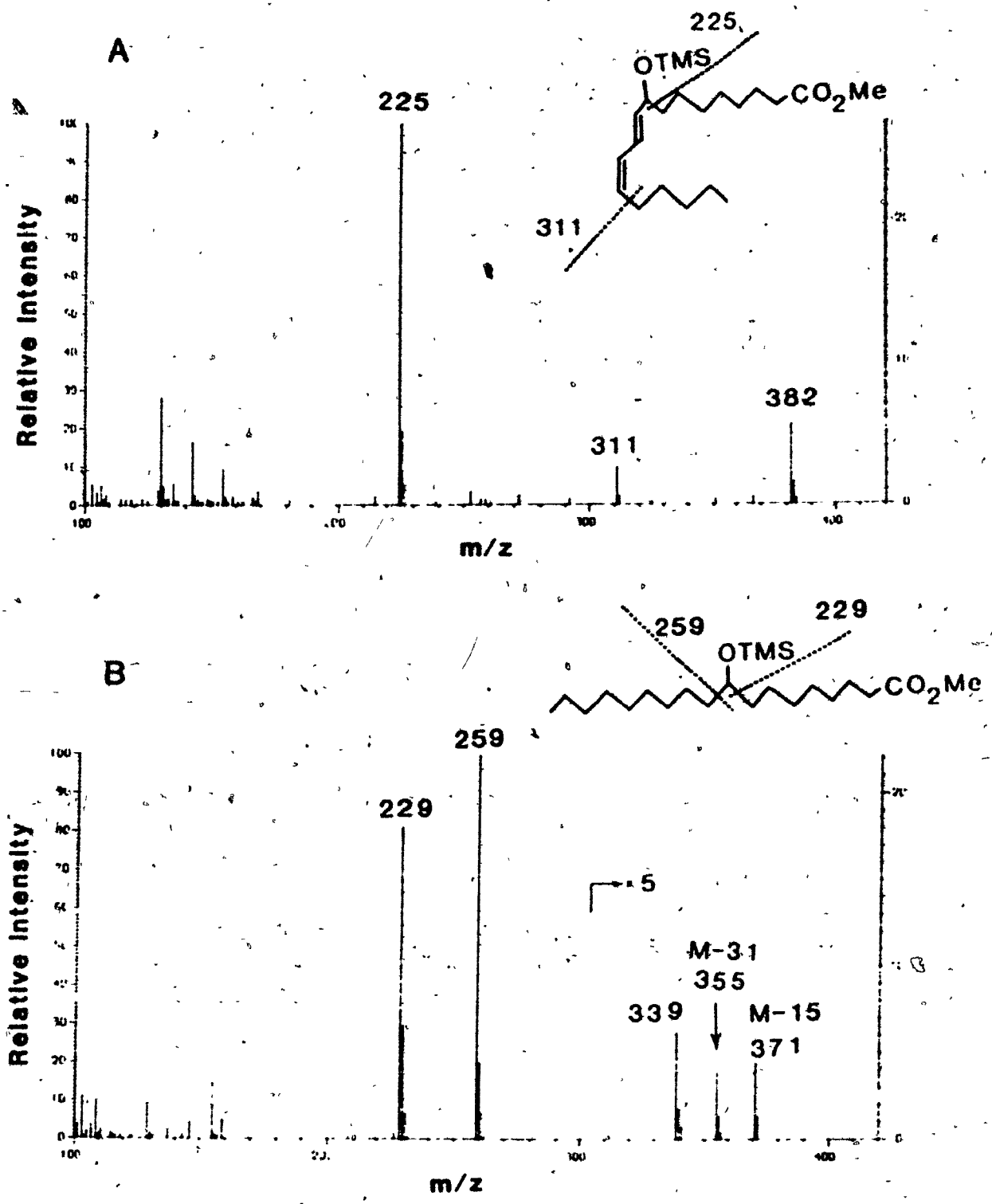

Fig.15. Mass spectra of the trimethylsilyl ether derivatives of the methyl esters of 9-hydroxy-10,12-octadecadienoic acid (fraction c) before (A) and after (B) hydrogenation.

hydroxy-9,11-octadecadienoic acid (13h-18:2) (b) and 9-hydroxy-10,12-octadecadienoic acid (9h-18:2) (c).

Fraction d

Fraction d was further purified by NP-HPLC (t_R, 19 min) using a gradient from 3 to 40 % solvent B_m in solvent A_m over 50 min. The mass spectrum (Fig.16A) of the Me_3Si ether, methyl ester derivative of this compound (C value, 20.7) had major fragment ions at m/z 398 (M), 383 (M-15), 285 (cleavage between the epoxy and Me_3SiO substituents of 12,13-epoxy-11-hydroxy-9-octadecenoic acid (12,13e-11h-18:1)), 241 (M-157, loss of $(CH_2)_7-CO_2CH_3$ from 9,10-epoxy-11-hydroxy-12-octadecenoic acid (9,10e-11h-18:1)) and 199 (base peak, cleavage between the epoxy and Me_3SiO groups of 9,10e-11h-18:1).

After hydrogenation, the methyl ester, Me_3Si derivative of d had major fragment ions at m/z 385 (M-15), 301, 287 (cleavage between epoxy and Me_3SiO groups of 12,13e-11h-18:0), 257 (cleavage between carbons 7 and 8 in 9,10e-11h-18:0), 215 and 201 (base peak, cleavage between epoxy and Me_3SiO groups of 9,10e-11h-18:0.) (Fig.16B).

The mass spectrum of the nonhydrogenated derivative of d is similar to that reported in the literature (273) for a mixture of 12,13e-11h-18:1 and 9,10e-11h-18:1 obtained after incubation of a 4:1 mixture of 13-hydroperoxy-9,11-octadecadienoic acid (13hp-18:2) and 9hp-18:2 with Fe^{3+}/cysteine. The base peak in the mass spectrum of the above mixture was at m/z 285, indicating that 12,13e-11h-

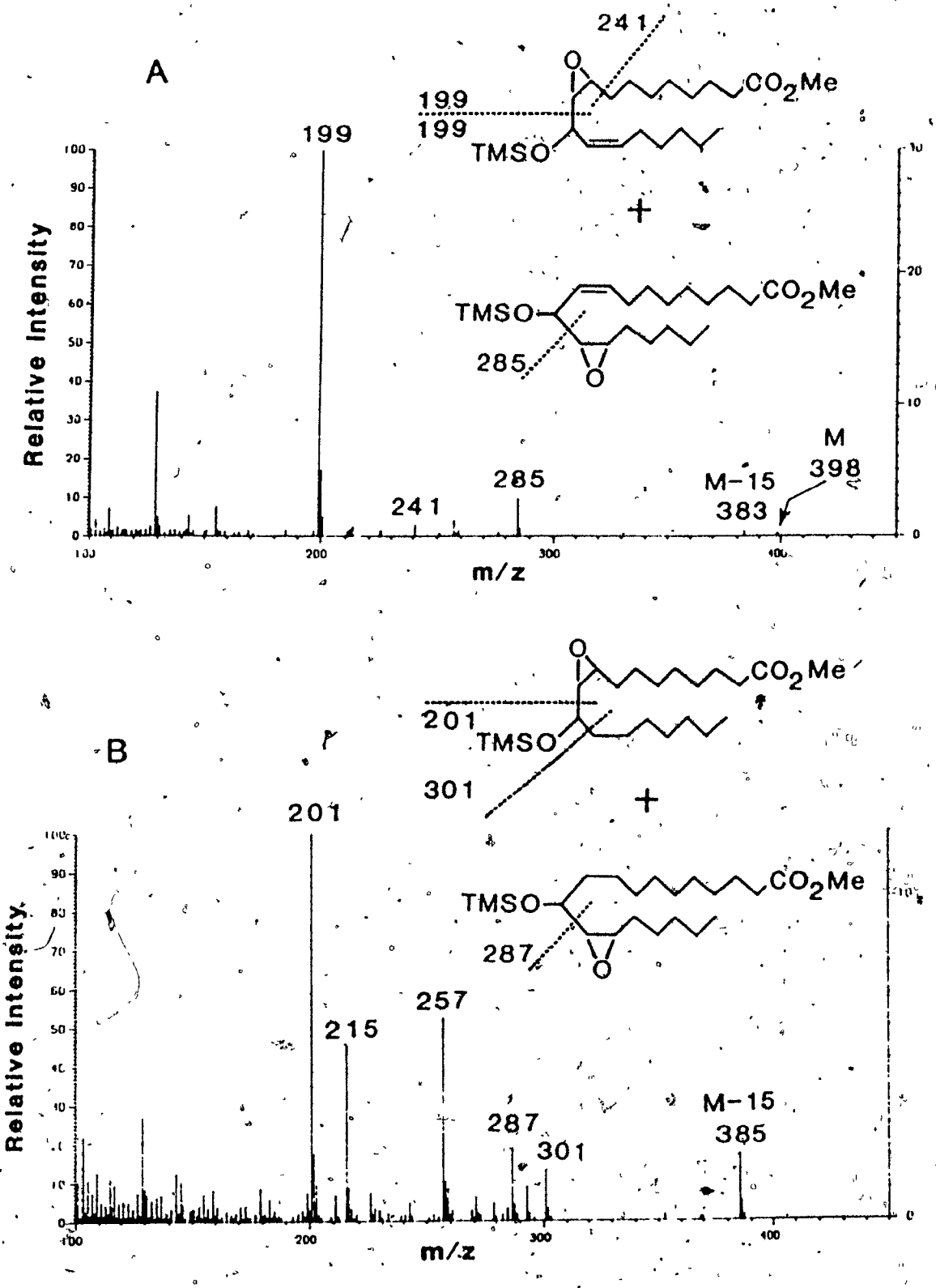

Fig.16. Mass spectra of the trimethylsilyl ether derivatives of the methyl esters of 9,10-epoxy-11-hydroxy-12-octadecenoic acid, along with a small amount of 12,13-epoxy-11-hydroxy-9-octadecenoic acid (fraction d) before (A) and after (B) hydrogenation.

18:1 was the major component. The ratio of the intensity of the fragment with m/z 199 to that with m/z 285 was about 10:1 for fraction d, however, indicating that aorta converts 18:2 predominantly to the 9,10-epoxy isomer.

Fraction e

Fraction e was purified further by argentation HPLC (t_R, 17 min) using a mobile phase consisting of methylene chloride:methanol:acetonitrile (90:9.75:0.25) and a flow rate of 1 ml/min. The mass spectrum (Fig.17A) of the methyl ester, Me_3Si ether derivative of e (C value, 22.4) had major fragment ions at m/z 560 (M), 470 (M-90), 361 (cleavage between vicinal Me_3SiO groups at carbons 10 and 11 of 9,10,11-trihydroxy-12-octadecenoic acid (9,10,11th-18:1)), 301 (cleavage between vicinal Me_3SiO groups at carbon 9 and 10), 271 (361-90), 259 (M-301), 211 (301-90) and 199 (M-361). This mass spectrum bears some similarity to that reported for a mixture of 11,12,13th-18:1 (major component) and 9,10,11th-18:1 (minor component) obtained after hydrolysis of the epoxyhydroxy compounds formed from a mixture of 13hp-18:2 and 9hp-18:2 by soybean lipoxygenase (263). Fraction e contains very little, if any, 11,12,13th-18:1, however.

The mass spectrum of the methyl ester, Me_3Si ether derivative of e after hydrogenation had major fragment ions at m/z 547 (M-15), 531 (M-31), 457 (M-90-15), 361 (cleavage between vicinal Me_3SiO groups at carbons 10 and 11), 271 (361-90), 259 (base peak, cleavage between vicinal Me_3SiO

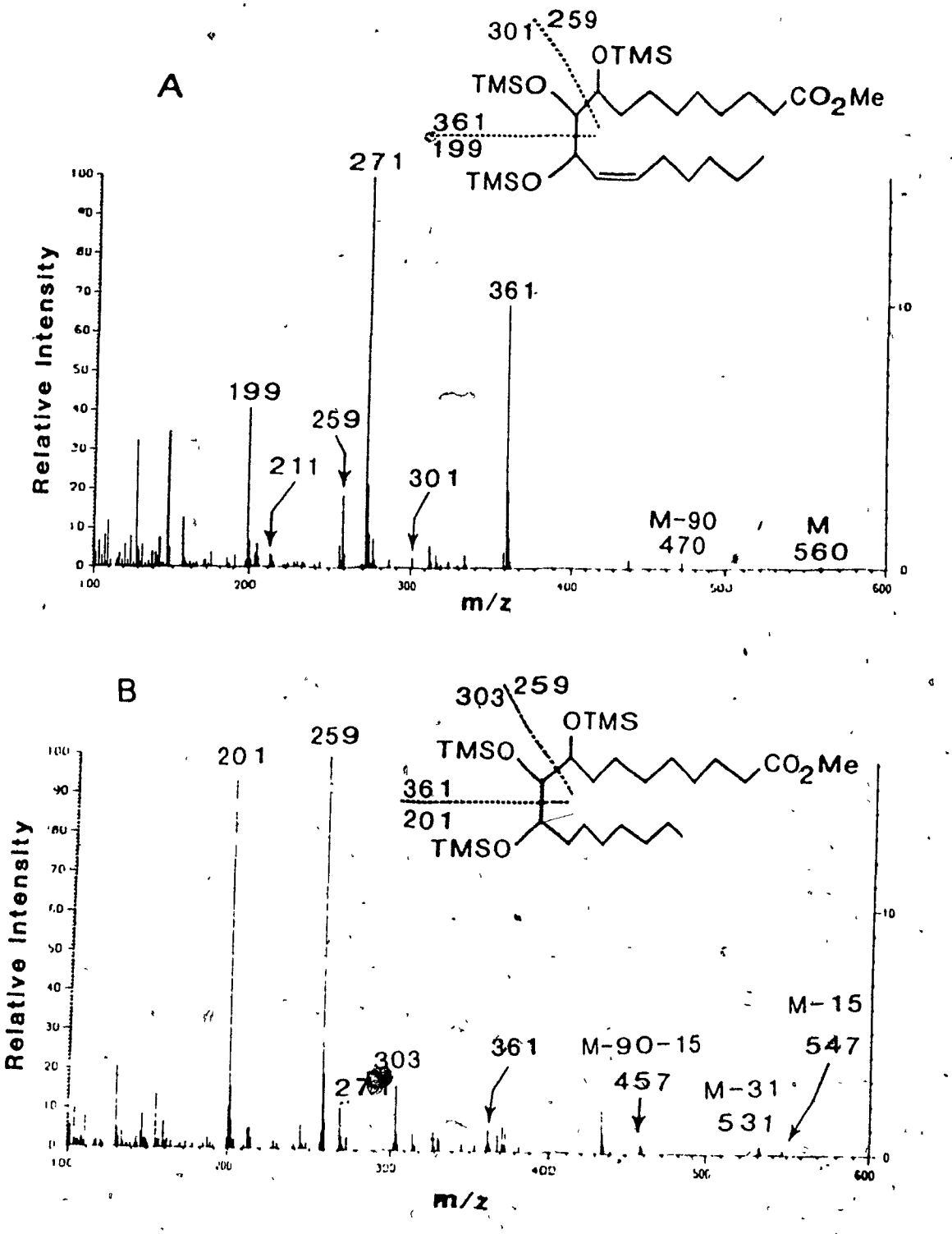

Fig. 17. Mass spectra of the trimethylsilyl ether derivatives of the methyl esters of 9,10,11-trihydroxy-12-octadecenoic acid (fraction e) before (A) and after (B) hydrogenation.

groups at carbons 9 and 10) and 201 (M-361) (Fig.17B). These results all indicate that e is 9,10,11th-18:1.

Fractions f and g

Fraction f was further purified by argentation HPLC (t_R, 16 min) as described above for fraction e. The mass spectrum (Fig.18A) of the methyl ester, Me_3Si ether derivative of f (C value, 22.5) had major fragment ions at m/z 545 (M-15), 460 (M-100, loss of hexanal with transfer of an Me_3Si group to the carboxyl group), 455 (M-90-15), 387 (cleavage between the vicinal Me_3SiO groups at carbons 12 and 13 of 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13th-18:1)), 301 (cleavage between the vicinal Me_3SiO groups at carbons 9 and 10 of 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13th-18:1)), 259 (M-301), 211 (301-90) and 173 (M-387).

The mass spectrum of the hydrogenated derivative of f had major fragment ions at m/z 547 (M-15), 531 (M-31), 457 (M-90-15), 441 (M-90-31), 389 (cleavage between vicinal Me_3SiO groups at carbons 12 and 13 of 9,12,13th-18:0), 303 (cleavage between vicinal Me_3SiO groups at carbons 9 and 10 of 9,10,18th-18:0), 299, 259 ($Me_3SiO^+=CH-(CH_2)_7-CO_2CH_3$, mainly cleavage between the vicinal Me_3SiO groups at carbons 9 and 10 in 9,10,13tb-18:0), 213 (base peak, 303-90) and 173 ($Me_3SiO^+=CH-(CH_2)_4-CH_3$) (Fig.18B).

The mass spectra of the methyl ester, Me_3Si ether derivatives of f, both before and after hydrogenation, are similar to those reported in the literature for mixtures of

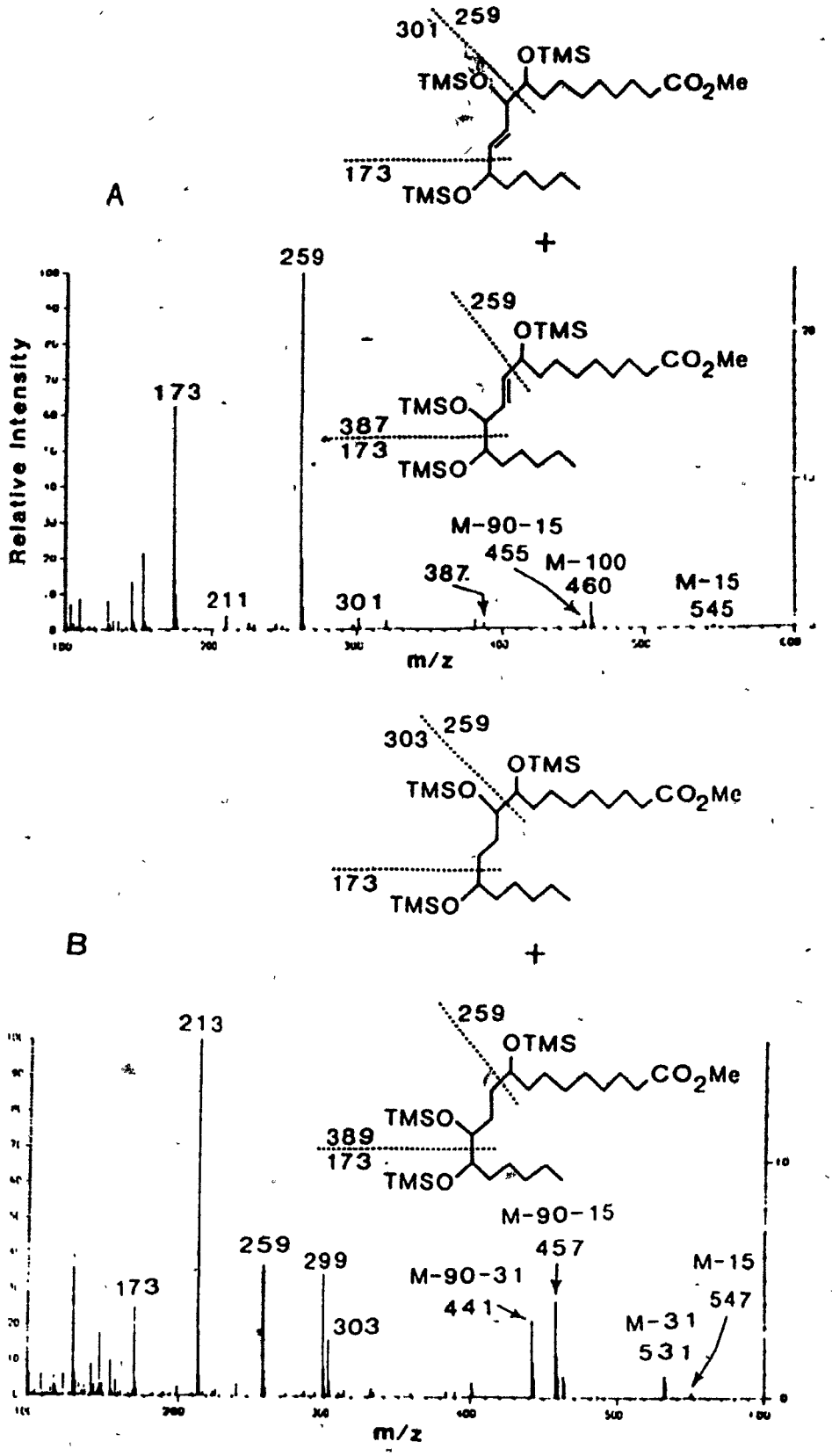

Fig.18. Mass spectra of the trimethylsilyl ether derivatives of the methyl esters of 9,10,13-trihydroxy-11-octadecenoic acid and 9,12,13-trihydroxy-10-octadecenoic acid (fraction f) before (A) and after (B) hydrogenation.

9,10,13th-18:1 and 9,12,13th-18:1, isolated after incubation of mixtures of 13hp-18:2 and 9hp-18:2 with either soybean lipoxygenase (263) or $FeCl_3$/cysteine (273). Thus, f would appear to be a mixture of the above two isomers, with the 9,10,13-trihydroxy isomer predominating.

Fraction g was further purified by argentation HPLC (t_R, 18 min) as described above. The mass spectrum and C value of the methyl ester, Me_3Si ether derivatives of g both before and after hydrogenation, were identical to those of f. Thus, g is also a mixture of different stereoisomers of 9,10,13th-18:1 and 9,12,13th-18:1.

In order to learn more about the relative amounts of the trihydroxyoctadecenoic acid isomers present in fractions f and g, the n-butylboronate derivatives were prepared. In this case, fractions f and g were chromatographed initially as the free acids (Fig.12D), giving four peaks of radioactivity (f_1, f_2, g_1 and g_2). The materials in each fraction were methylated with diazomethane, reduced with hydrogen in the presence of platinum dioxide and converted to their n-butylboronate derivatives. The n-butylboronates were then converted to their Me_3Si ether derivatives and analyzed by GC-MS. For comparison, the corresponding derivative of 9,12,13th-18:1 was prepared by incubation of [1-^{14}C]18:2 with wheat flour (274). A mixture consisting predominantly of 9,12,13th-18:1, along with a smaller amount of 9,10,13th-18:1, was also prepared by treating [1-^{14}C]18:2 with soybean lipoxygenase in the presence of guaiacol (263).

The mass spectrum of the hydrogenated n-butylboronate derivative of 9,12,13th-18:1 (C value, 24.0; Fig.19A) obtained from wheat flour had major fragment ions at m/z 469 (M-15), 453 (M-31), 327 (M-157, loss of $(CH_2)_7$-CO_2Me), 259 (Me_3SiO^+=CH-$(CH_2)_7$-CO_2Me), 237 (327-90) and 129. The mass spectrum of the product from aorta was similar (Fig.19B), except that additional fragment ions were observed at m/z 413 (M-71, loss of $(CH_2)_4$-CH_3), 323 (413-90) and 173 (Me_3SiO^+=CH-$(CH_2)_4$-CH_3). The mass spectrum of the soybean lipoxygenase product was similar to that of the product from aorta, except that the ions at m/z 413, 323, and 173 were less intense than those at 327, 237, and 259, respectively.

Each trihydroxyoctadecenoic acid isomer gives rise to three major fragment ions after methylation and hydrogenation and conversion to its n-butylboronate, Me_3Si ether derivative. The ions are derived from cleavage on either side of the Me_3Si group. For the derivative of 9,10,13th-18:1 major fragment ions are obtained at m/z 413, 323 (413-90), and 173, whereas the corresponding ions for the derivative of 9,12,13th-18:1 are at m/z 327, 237 (327-90), and 259. The ratios of the sums of these ions will give an approximation of the relative amounts of each of these isomers in the mixture (see Table 4). Using these criteria to distinguish between 9,10,13th-18:1 and 9,12,13th-18:1, aorta fractions f_2 and g_2 contained mixtures of these isomers with the former product predominating. Wheat flour conversion of 18:2 resulted in exclusive synthesis of 9,12,13th-18:1. Treatment of 18:2 with soybean

Fig.19. Mass spectra of th-18:1 isomers after hydrogenation and conversion to their trimethylsilyl ether, n-butylboronate, methyl ester derivatives. (A) 9,12,13-trihydroxy-10-octadecenoic acid formed by incubation of 18:2 with wheat flour. (B) th-18:1 isomers (9,10,13-trihydroxy-11-octadecenoic acid + 9,12,13-trihydroxy-10-octadecenoic acid) formed by incubation of 18:2 with a fetal calf aorta particulate fraction (compound $\underline{f}_2$).
(C) th-18:1 isomers formed by incubation of 18:2 with soybean lipoxygenase and guaiacol.

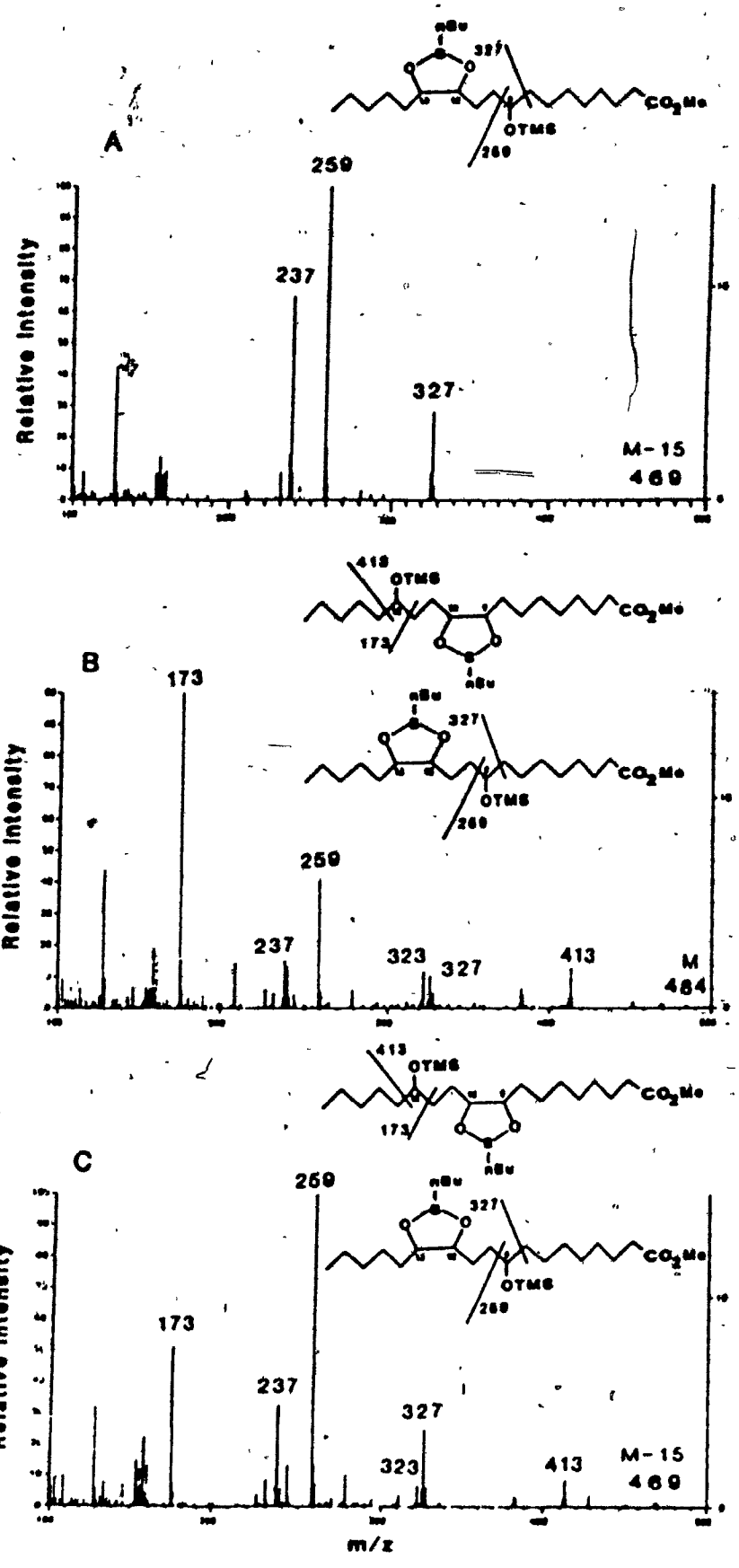
A
259
237
327
M-15
469
Relative Intensity
B
173
259
237
323
327
413
M
484
Relative Intensity
C
259
173
237
327
323
413
M-15
469
Relative Intensity
m/z

Fig. 19.

TABLE 4

GC-MS Characteristics of Trihydroxyoctadecenoic Acid Isomers

Mixtures of 9,10,13th-18:1 and 9,12,13th-18:1 were prepared from wheat flour, soybean lipoxygenase and fetal calf aorta as described in the text. After methylation and hydrogenation, the trihydroxy compounds were converted to their n-butylboronate-Me_3Si ether derivatives and analyzed by GC-MS. The major fragment ions for this derivative of 9,10,13th-18:1 are at m/z 413, 323 and 173, whereas the corresponding ions for 9,12,13th-18:1 are at m/z 327, 237 and 259, respectively. The ratios of the sums of these ions will give an approximation of the relative amounts of each of these isomers in the mixture.

Fraction	C value	Ratio of ion intensities of (9,10,13th-18:1/ 9,12,13th-18:1)*
9,12,13th-18:1 (wheat flour)	24.0	0.03
9,10,13th-18:1 + 9,12,13th-18:1 (soybean lipoxygenase)	24.0	0.50
f_2 (aorta)	24.1	2.40
g_2 (aorta)	24.1	3.00

* The sum of intensities of ions at m/z 413, 323 and 173 (9,10,13th-18:1) was divided by the sum of the intensities of ions at m/z 327, 259 and 237 (9,12,13th-18:1).

lipoxygenase in the presence of guaiacol yielded mainly 9,12,13th-18:1, along with a smaller amount of 9,10,13th-18:1. The amounts of materials in fractions f_1 and g_1 were insufficient to obtain good mass spectra. However, on the basis of the relative intensities of the fragment ions at m/z 173 and 259, fraction f_1 appeared to consist mainly of 9,12,13th-18:1, whereas fraction g_1 appeared to consist mainly of 9,10,13th-18:1.

3.2.2 Metabolism of 18:2 by Homogenates

Homogenates of fetal calf aorta in the absence of any added cofactors converted [1-^{14}C]18:2 to products similar to those formed by particulate fractions, except that the more polar products (d-g) were formed in relatively small amounts (Fig.12B).

3.2.3. Metabolism of 18:2 by Other Blood Vessels

(a) Ductus arteriosus

A chromatographic pattern similar to that shown in Fig.12A was obtained after incubation of [1-^{14}C]18:2 with particulate fractions from fetal calf ductus arteriosus, except that the amounts of 13h-18:2 (b) and 9h-18:2 (c) were about 1.5 times greater than with aorta.

(b) Adult aorta

Particulate fractions (7.5 mg protein/ml) from adult bovine aorta were less active than those from fetal aorta in converting [1-^{14}C]18:2 (2 µCi, 57 µM) to oxygenated products under identical conditions (Fig.12C). With adult aorta, 9o-

18:2 was not detected, whereas the amounts of 9h-18:2, 13h-18:2, and the trihydroxy products in fractions f and g were only about one-third of those observed for fetal aorta. The formation of the products in fractions d (mainly 9,10e-11h-18:1) and e (mainly 9,10,11th-18:1) by adult aorta was reduced to a lesser extent than the other products.

3.2.4. Time Courses for the Formation of Oxygenated 18:2 Metabolites

Particulate fractions from fetal calf aorta were incubated with [1-^{14}C]18:2 for various times in the absence of any added cofactors (Fig.20). Only small amounts of fraction a were formed, and it was not possible to quantitate it very accurately. The amounts of fraction b (13h-18:2), c (9h-18:2) and f+g (9,10,13th-18:1 and 9,12,13th-18:1) increased nearly linearly with time up to 5 min. The rates of formation of these products then declined and after 20 min their concentration changed only slightly. The material in fraction d (mainly 9,10e-11h-18:1) was formed more rapidly in the early stages of the reaction, but its concentration declined after 10 min (Fig.20, inset). Product e (9,10,11th-18:1), on the other hand, was formed very slowly initially, but its concentration increased linearly with time up to 20 min, and continued to increase, although more slowly, up to 60 min. These results suggest that product d is a precursor of product e.

Fig.21 shows the time course for oxygenation of 18:2 compared to 20:4. In this experiment the total amounts of

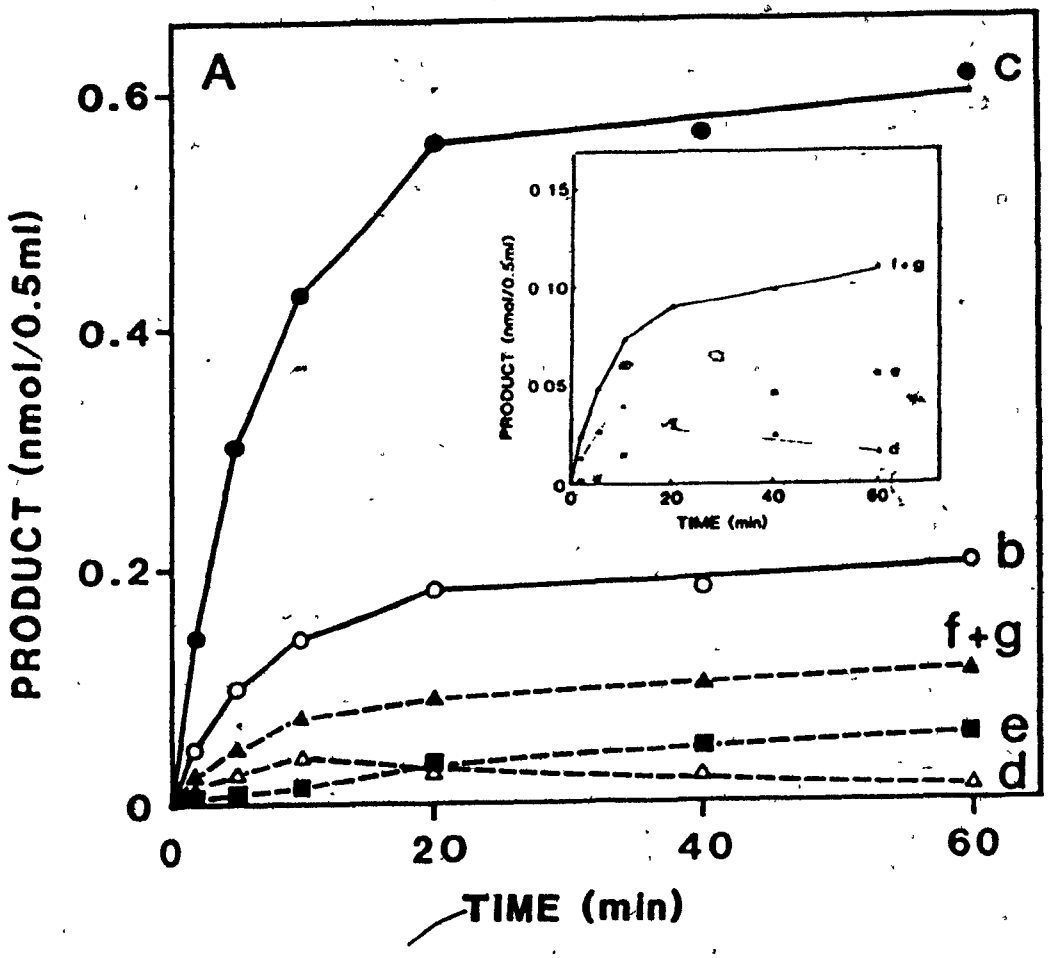

Fig.20. Time courses for the formation of b (O, 13h-18:2), c (●, 9h-18:2), d (Δ, 9,10e-11h-18:1), e (■, 9,10,11th-18:1) and f+g (▲, isomers of 9,10,13th-18:1 and 9,12,13th-18:1) from 18:2. Product a was not formed in sufficient amounts for quantitation. Particulate fractions (0.5 ml) from fetal calf aorta were incubated at 37 °C with [1-^{14}C]18:2 (0.7 μCi, 30.0 nmol) for various time periods. Products were extracted and analyzed as described in the legend to Fig.12. The radioactivity in fractions collected every minute was determined by liquid scintillation counting. All values have been corrected for recovery and are the means of duplicates. The inset shows the time courses for the formation of the polar products (d-g) to illustrate the probable precursor-product relationship between product d (9,10e-11h-18:1) and product e (9,10,11th-18:1).

Fig.21. Time courses for the formation of total 18:2 (●) and 20:4 (○) metabolites by fetal calf aorta particulate fractions (0.5 ml). After preincubation of the particulate fractions for 2 min at 37 °C, either [1-^{14}C]18:2 (0.5 μCi, 9.1 nmol) or [1-^{14}C]20:4 (0.17 μCi, 10.3 nmol) was added and incubations were continued for various time intervals. Incubations were terminated with ethanol and [9β-^{3}H]PGF$_{1\alpha}$ (500,000 dpm, 1 μg) was added. Products were extracted using ODS silica and the residue was dissolved in hexane/toluene/methanol/acetic acid (95:5:0.002:0.1) and applied to an open column (0.5 g) of silicic acid. Unconverted substrate was eluted with 22 ml hexane/diethyl ether/ acetic acid (98:5:1.5:0.1). The oxygenated metabolites were then eluted as a single fraction with 8 ml methyl formate/methanol (90:10). After evaporation of the solvent under nitrogen the radioactivity was measured by liquid scintillation counting. All values are the means of duplicates and have been corrected for recovery.

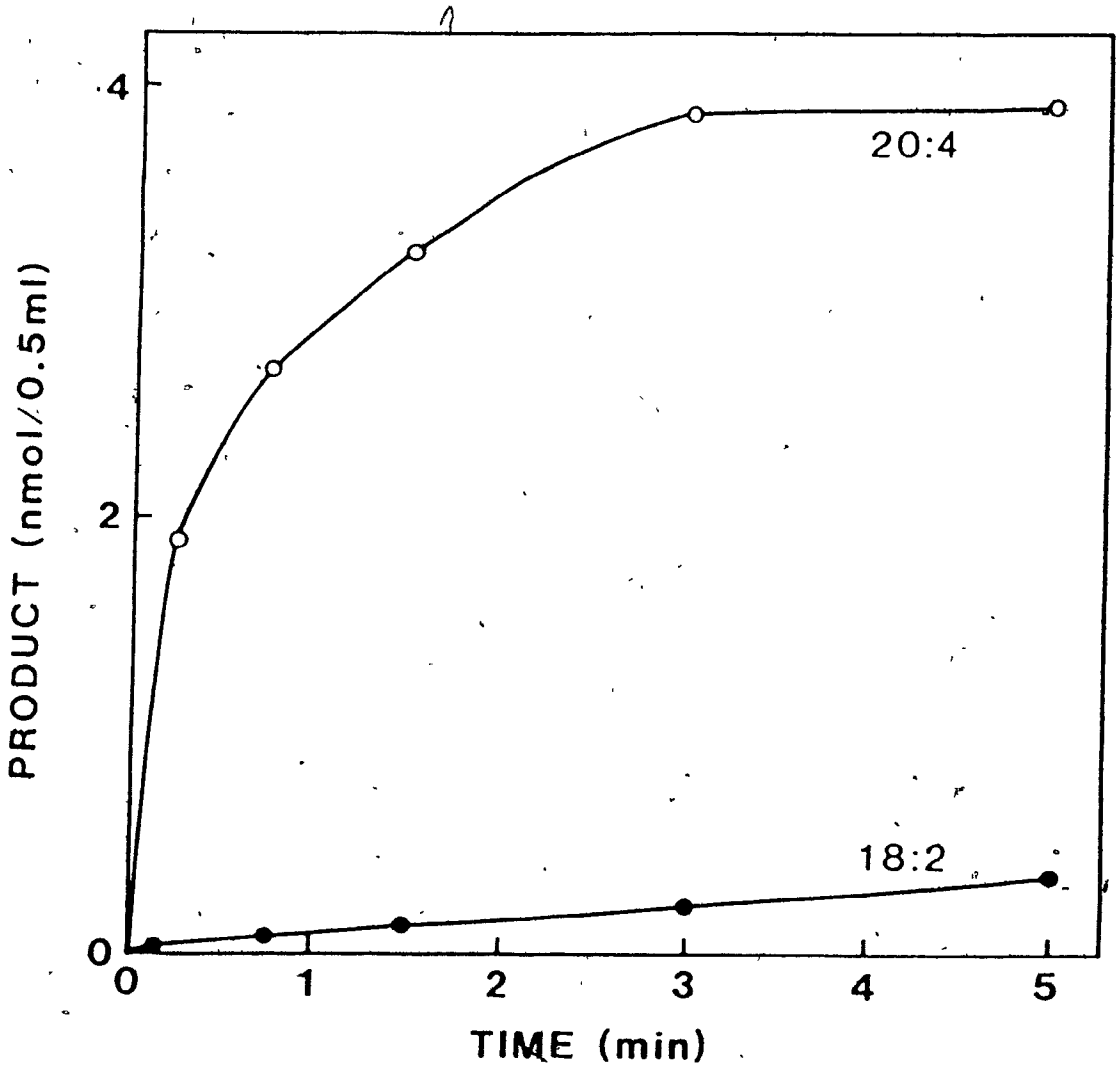

Fig.21.

^{14}C-labeled oxygenated metabolites formed from the corresponding ^{14}C labeled substrates were measured. The substrates were separated from their more polar monohydroxy, epoxyhydroxy and trihydroxy metabolites by chromatography on an open column of silicic acid. The formation of products from 18:2 was linear with time up to at least 5 min, whereas the formation of products (mainly 6-oxo$PGF_{1\alpha}$ and 6,15-dioxo$PGF_{1\alpha}$) from 20:4 was linear for no more then 15 s, presumably due to the more rapid formation of inhibitory peroxides or hydroperoxides from 20:4. As shown in Fig.21 20:4 is a much better substrate for prostaglandin endoperoxide synthase than 18:2 (approximately 10 times more oxygenated products formed after 5 min incubation).

3.2.5. Mechanism for the Formation of Oxygenated 18:2 Metabolites

Four main types of products are synthesized from 18:2 by aorta. They are: oxo, monohydroxy, epoxyhydroxy and trihydroxy products (Fig.22). Presumably all of these products are derived from hydroperoxide intermediates, formed enzymatically by either cyclooxygenase or lipoxygenase. The ensuing metabolism of the hydroperoxides to oxo, monohydroxy, epoxyhydroxy and trihydroxy products may be the result of enzymatic and/or non-enzymatic processes.

(a) Conversion of 18:2 to hp-18:2

In order to determine the nature of the enzyme catalyzing the initial oxygenation of 18:2 to the putative 9 and 13

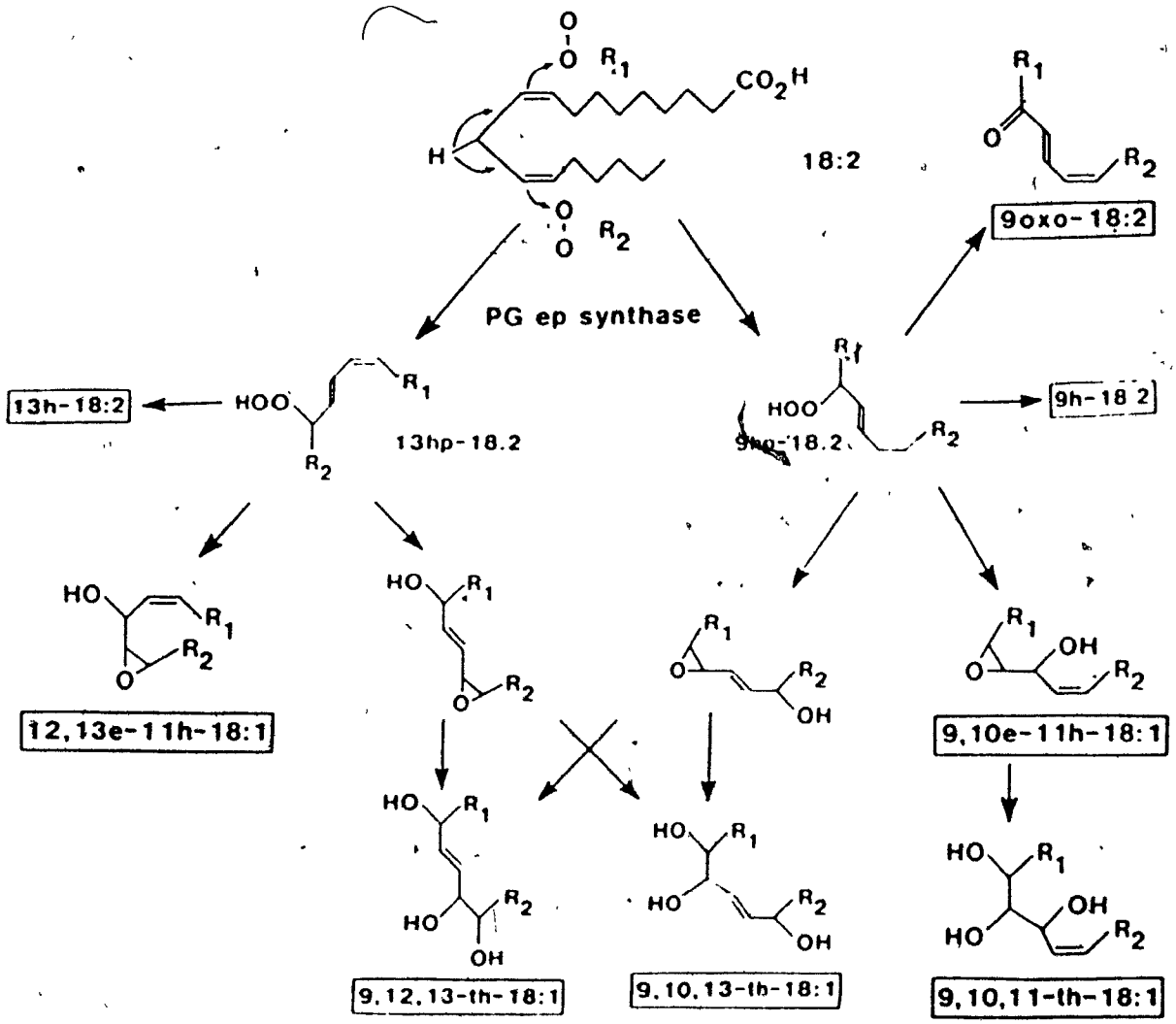

Fig.22. Metabolism of 18:2 by particulate fractions from aorta. The products enclosed in boxes were isolated and identified by GC-MS. ep, endoperoxide.

hydroperoxy intermediates, particulate fractions containing adrenaline (1 mM) were pre-incubated for 2 min with nordihydroguaiaretic acid, a lipoxygenase inhibitor, or with acetylsalicylic acid or indomethacin, prostaglandin endoperoxide synthase inhibitors. [1-^{14}C]18:2 (9.2 µM) was then added and the incubations were continued for a further 20 min and the products analyzed by NP-HPLC. Under these conditions, 9h-18:2 and 13h-18:2 accounted for 93 % of the oxygenated products, whereas epoxyhydroxy and trihydroxy products made up the remaining 7 %. The amount of 9h-18:2 and 13h-18:2 formed should therefore reflect the amount of the corresponding hydroperoxy products initially formed. Similar incubations with [1-^{14}C]20:4 (9 µM) in the presence and absence of inhibitors were carried out for comparison. Preincubation of particulate fractions with indomethacin inhibited the formation of both 9h-18:2 and 13h-18:2 (ID_{50}, approx. 10^{-7} M), as well as 6-oxo$PGF_{1\alpha}$ (ID_{50}, 4 x 10^{-7} M), in a dose dependent fashion (Fig.23). Acetylsalicylic acid also inhibited the formation of all three products but was much less potent (Fig.24). On the other hand, nordihydroguaiaretic acid had no effect on the formation of either the two monohydroxy metabolites of 18:2 or 6-oxo$PGF_{1\alpha}$, except at very high concentrations (10^{-4} M), when the formation of all the products was inhibited quite markedly (Fig.25).

The formation of trihydroxy products from 18:2 was also inhibited by indomethacin and acetylsalicylic acid. Since the amounts of these products formed under the conditions of

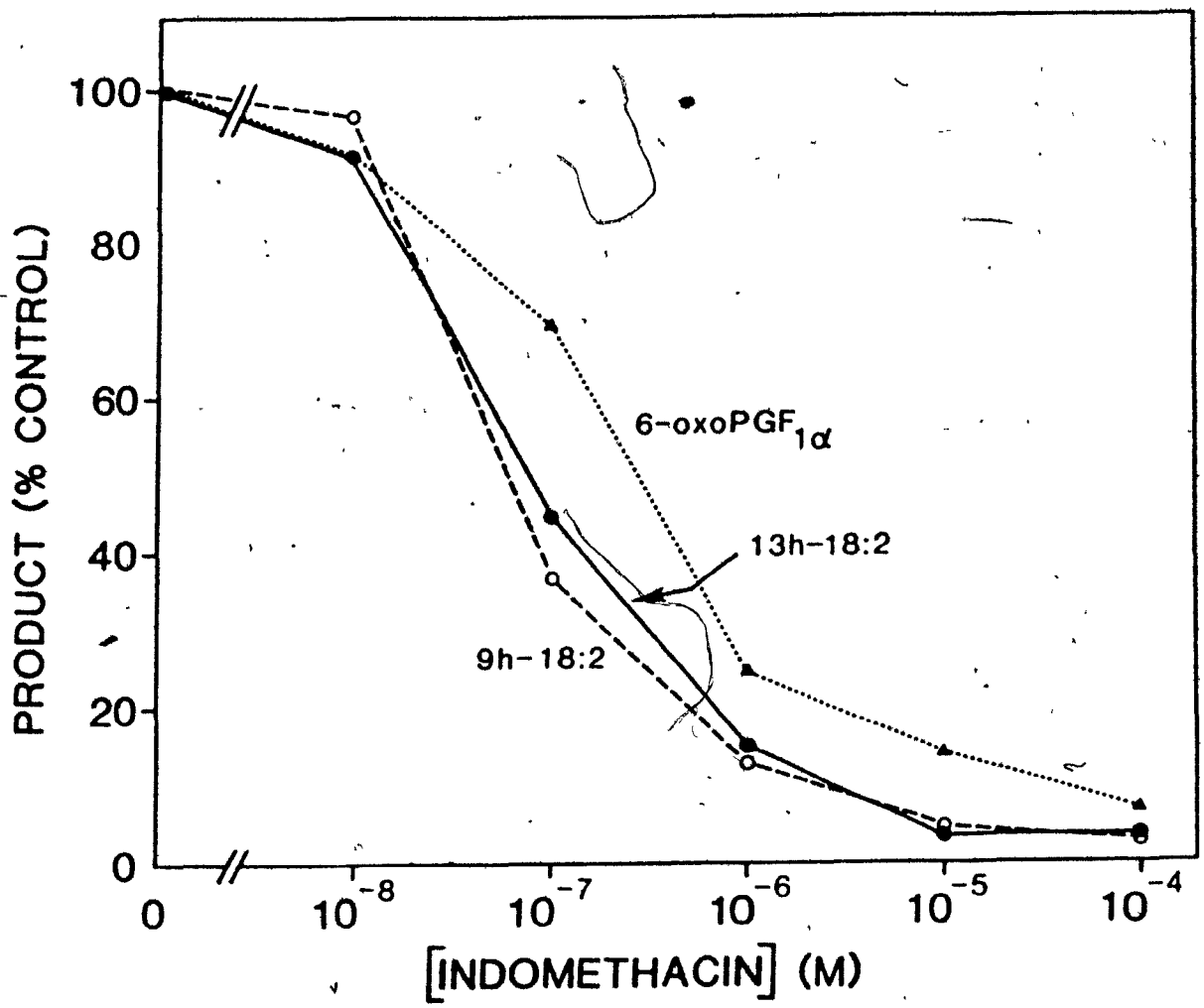

Fig.23. Effects of indomethacin on the formation of 6-oxoPGF$_{1\alpha}$ (▲), 13h-18:2 (●) and 9h-18:2 (○) by particulate fractions from fetal calf aorta in the presence of adrenaline (1 mM). Particulate fractions (0.5 ml) were preincubated for 2 min at 37 °C with 2.5 µl of ethanol containing various amounts of indomethacin. [1-^{14}C]18:2 (0.25 µCi, 4.6 nmol) or [1-^{14}C]20:4 (0.25 µCi, 4.5 nmol) was then added and the incubations were continued for a further 20 min. The products were extracted and analyzed by normal-phase HPLC using 4% solvent B$_m$ in solvent A$_m$ for monohydroxy 18:2 metabolites and 60% solvent B in solvent A for 6-oxoPGF$_{1\alpha}$. The flow rates were 2 ml/min. All values are the mean of duplicates and have been corrected for recovery. The amounts of radioactivity in the control incubations were as follows: 6-oxoPGF$_{1\alpha}$, 188,000 dpm; 13h-18:2, 4300 dpm; 9h-18:2, 13,700 dpm.

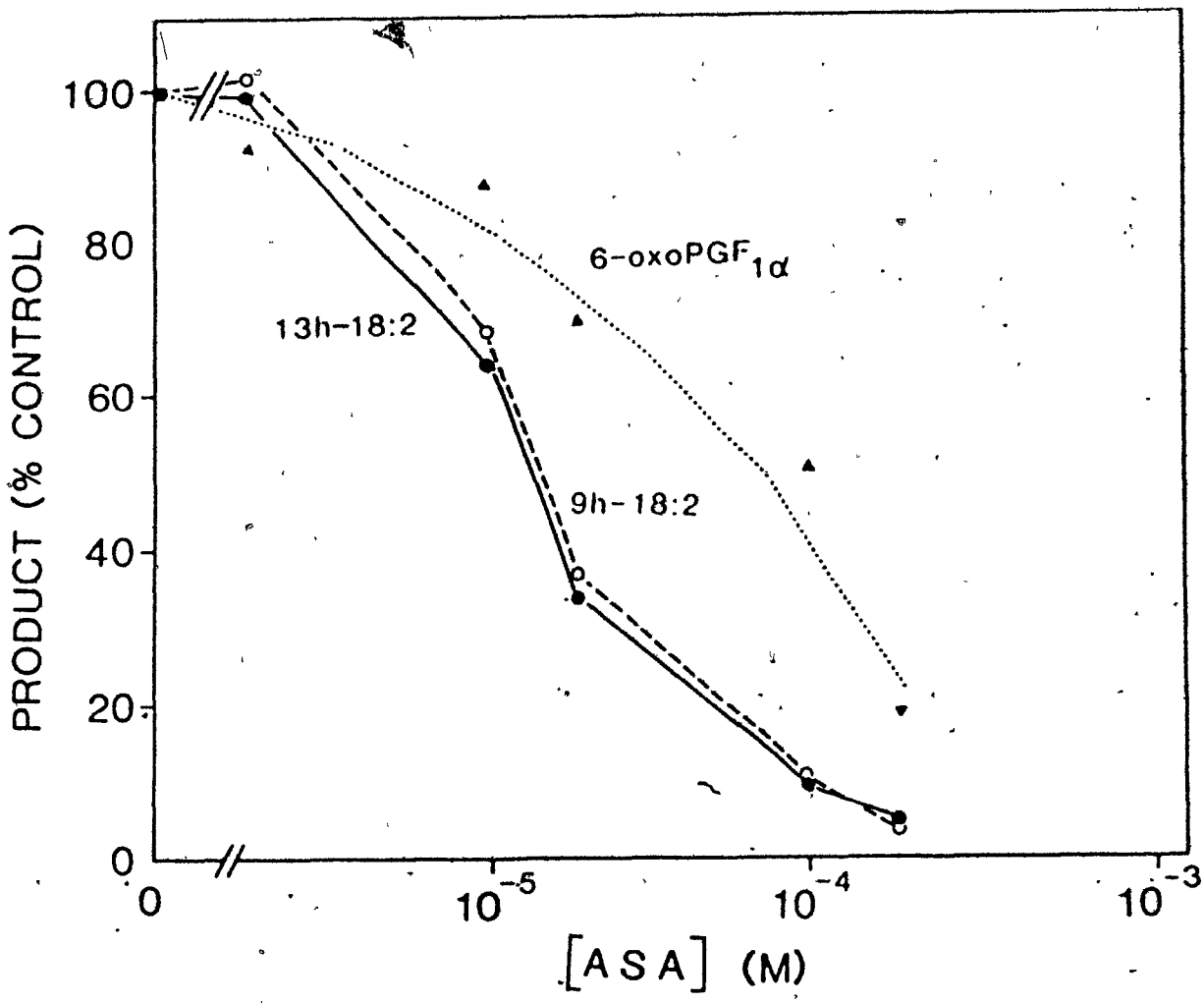

Fig.24. Effects of acetylsalicylic acid (ASA) on the formation of 6-oxoPGF$_{1\alpha}$ (▲), 13h-18:2 (●) and 9h-18:2 (○) by particulate fractions from fetal calf aorta in the presence of adrenaline (1 mM). Particulate fractions (0.5 ml) were preincubated for 2 min at 37 °C with 2.5 µl of ethanol containing various amounts of ASA. [1-^{14}C]18:2 (0.35 µCi, 6.4 nmol) or [1-^{14}C]20:4 (0.20 µCi, 6.1 nmol) was then added and the incubations were continued for a further 20 min. Extraction and chromatographic conditions were identical to those described in the legend to Fig.23. The amounts of radioactivity in the control incubations were as follows: 6-oxoPGF$_{1\alpha}$, 179,900 dpm; 13h-18:2, 16,300 dpm; 9h-18:2, 50,750 dpm.

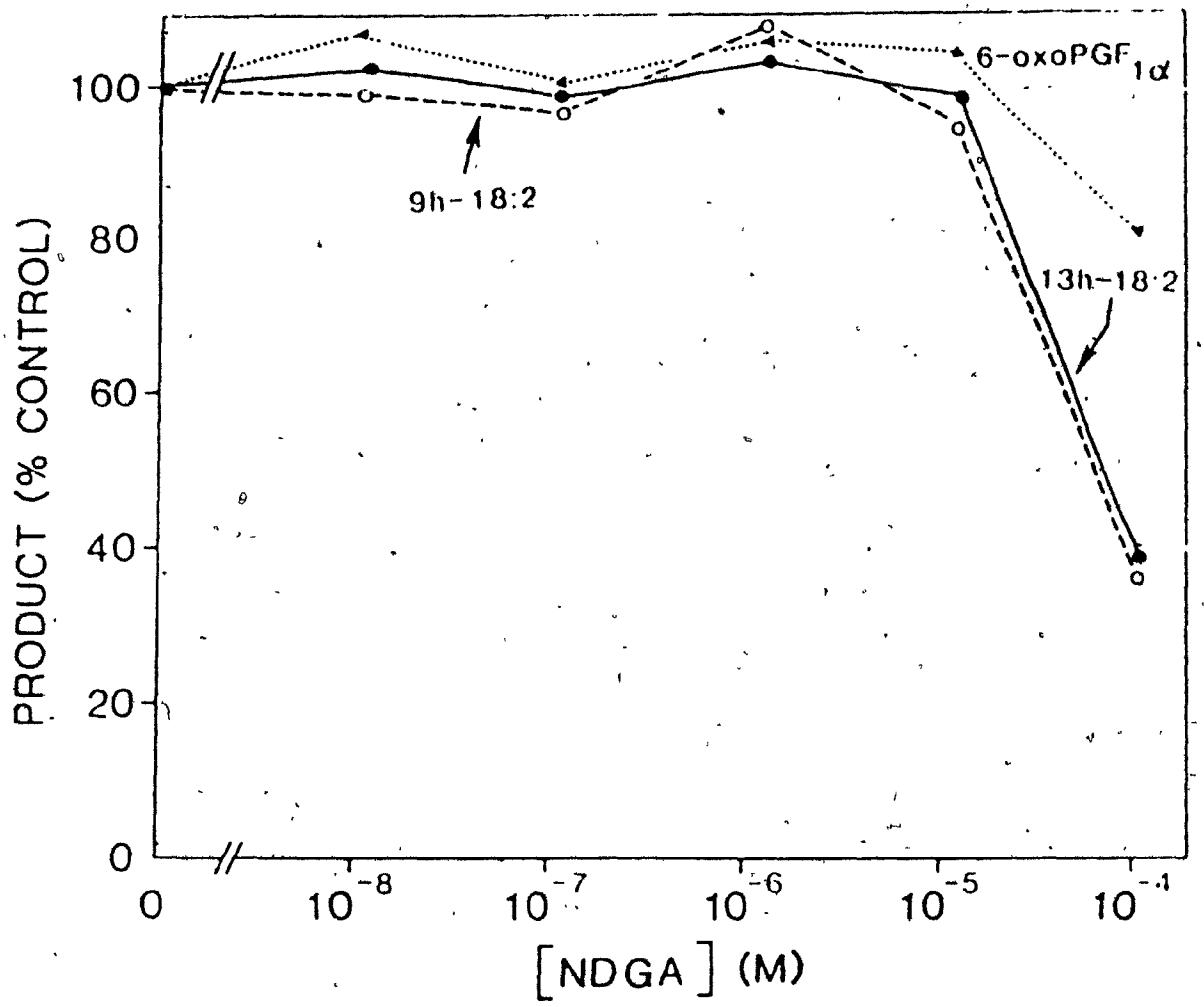

Fig.25. Effects of nordihydroguaiaretic acid (NDGA) on the formation of 6-oxoPGF$_{1\alpha}$ (▲), 13h-18:2 (●) and 9h-18:2 (○) by particulate fractions from fetal calf aorta in the presence of adrenaline (1 mM). Particulate fractions (0.5 ml) were preincubated for 2 min at 37 °C with 2.5 µl of actetone containing various amounts of NDGA. [1-^{14}C]18:2 (0.14 µCi, 2.5 nmol) or [1-^{14}C]20:4 (0.14 µCi, 2.6 nmol) was then added and the incubations were continued for a further 20 min. Extraction and chromatographic conditions were identical to those described in the legend to Fig.23. The amounts of radioactivity in the control incubations were as follows: 6-oxoPGF$_{1\alpha}$, 105,700 dpm; 13h-18:2, 8,100 dpm; 9h-18:2, 28,000 dpm.

the experiment were quite low (due to the presence of adrenaline and the low substrate concentrations) it was difficult to quantitate them accurately.

Indomethacin (10^{-5} M) inhibited the formation of both monohydroxy products (97 %) and trihydroxy products (59%) by particulate fractions which were incubated with [1-^{14}C]18:2 in the presence of methemoglobin (10 µM).

(b) Metabolism of 13hp-18:2

The metabolism of the hydroperoxy intermediates formed from 18:2 was investigated, but the results were rather inconclusive. In one experiment, 13hp-[1-^{14}C]18:2 (2 x 10^4 cpm, 10 µg), prepared from soybean lipoxygenase, was incubated for 2 min at 37 °C under varying conditions. Incubation in the presence of a particulate fraction from fetal calf aorta, without any cofactors, resulted in conversion to 13h-18:2 (major product) and 13o-18:2, as well as epoxyhydroxy and trihydroxy products (Fig.26A). A peak (approximately 15 % of added substrate) eluted in the position of 13hp-18:2, on NP-HPLC, which may represent unconverted substrate. The chromatographic profile (Fig.26B) obtained after incubation of a boiled particulate fraction with 13hp-[1-^{14}C]18:2 was almost identical, however. Since methemoglobin had been used in some incubations (see 3.2.1.) its effect on the metabolism of 13hp-18:2 was also investigated. Methemoglobin (10 µM), in buffer, in the absence of a particulate fraction, catalyzed the formation of oxo, monohydroxy, epoxyhydroxy and

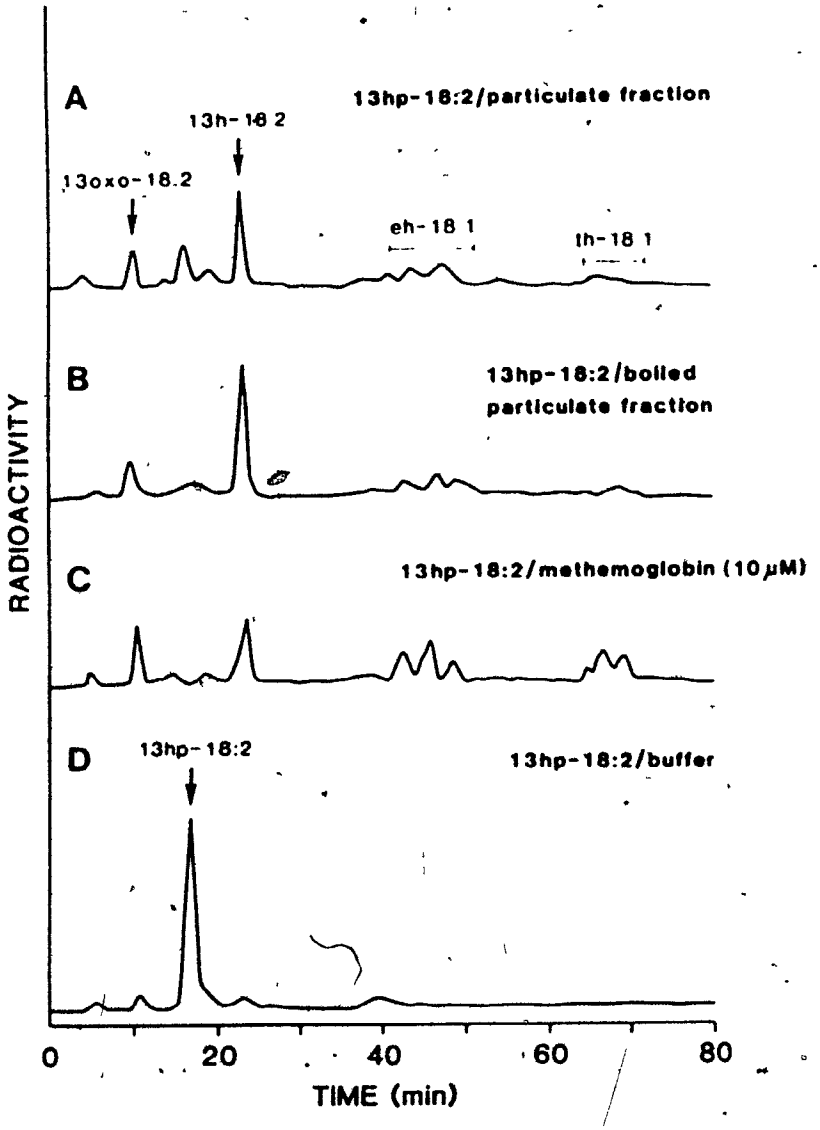

Fig.26. Metabolism of 13-hydroperoxy-9,11-octadecadienoic acid. 13hp-[1-^{14}C]18:2 (0.011 μCi, 32 nmol) was incubated at 37 °C for 2 min with (A) a fetal calf aorta particulate fraction, (B) a boiled fetal calf aorta particulate fraction, (C) buffer containing 10 μM methemoglobin, and (D) with buffer alone. The incubations were terminated with ethanol (2 vol) and products were extracted using ODS silica. The residues from the extracts were analyzed by normal-phase HPLC using a mobile phase consisting of 4% solvent B in solvent A for 30 min followed by a linear gradient over 50 min between 4% and 100% solvent B in solvent A.

trihydroxy products from 13hp-18:2 (Fig.26C). The profile of products was quite different from that of particulate fraction, however, with considerably larger amounts of epoxyhydroxy and trihydroxy products being formed. In contrast, when 13hp-18:2 was incubated in buffer alone (Fig.26D) very little degradation occurred. Most of the hydroperoxide (81 %) was recovered intact after incubation and extraction. Thus it would appear that 13hp-18:2 can be converted non-enzymatically to oxo, monohydroxy, epoxyhydroxy and trihydroxy products by particulate fractions from aorta. However, we cannot rule out the possibility that some of these reactions also occur enzymatically. As discussed earlier (see 1.4.1), for example, prostaglandin endoperoxide synthase has peroxidase activity, and could be partially responsible for the conversion of hp-18:2 to monohydroxy products.

(c) Conversion of eh-18:1 to th-18:1

In order to investigate the mechanism for the conversion of epoxyhydroxy products to trihydroxy products, 9,10e-11h-[1-^{14}C]18:1 was synthesized by incubating [1-^{14}C]18:2 with particulate fractions from fetal calf aorta. 9,10e-11h-[1-^{14}C]18:1 was then incubated with either boiled or unboiled particulate or 100,000 x g supernatant fractions from fetal calf aorta for various times (Fig.27). Both the particulate (6 pmol/h/mg protein) and the supernatant (27 pmol/h/mg protein) fractions converted 9,10e-11h-18:1 to 9,10,11th-18:1. When the fractions were boiled prior to the

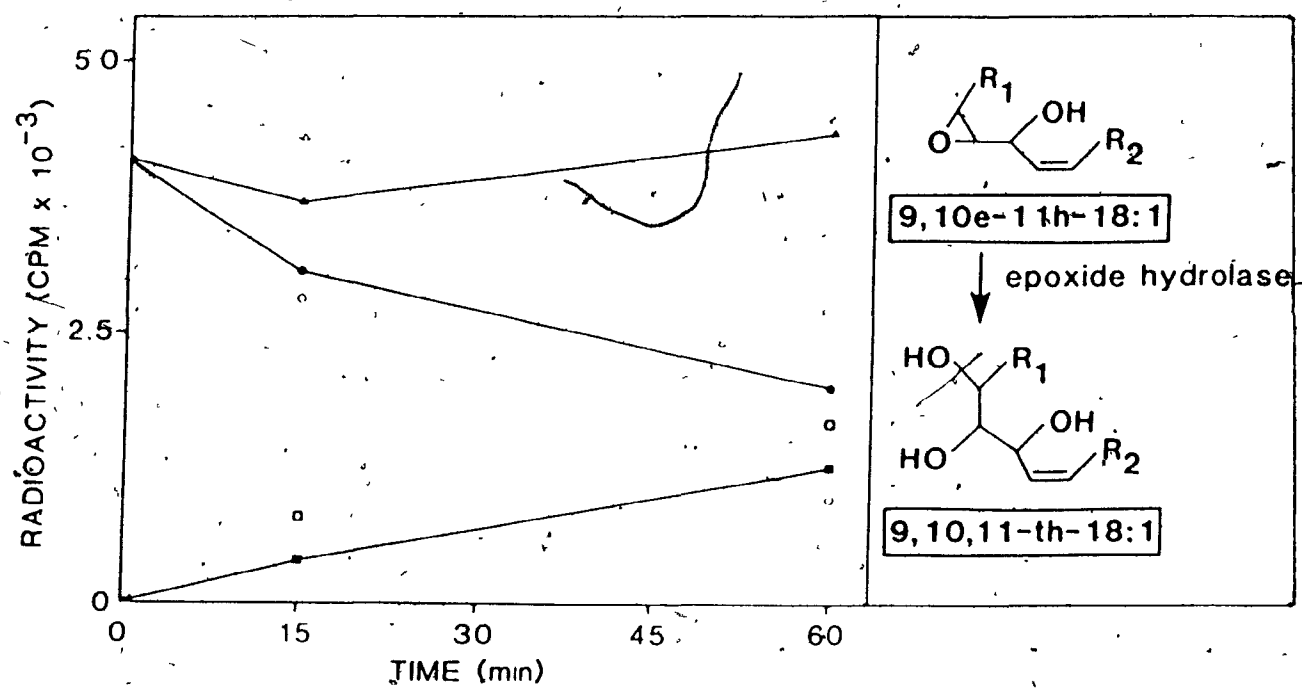

Fig.27. Conversion of product d (9,10e-11h-18:1; ○, ●, △ and ▲) to product e (9,10,11th-18:1; □ and ■) in the presence of particulate (___;●;■) and cytosolic (---;○;□) fractions (0.5 ml) from fetal calf aorta. No conversion of product d was observed in the presence of boiled particulate (▲) or cytosolic (△) fractions. 9,10e-11h-[1-^{14}C]18:1 (4100 cpm, 57 pmol), prepared as described in the text, was incubated with the above fractions for various times at 37 °C. The products were extracted and analyzed by normal-phase HPLC. The protein concentrations of the particulate and cytosolic fractions were 4.1 and 1.7 mg/ml, respectively. All values are means of duplicates and have been corrected for recovery.

incubation, however, 9,10e-11h-18:1 was not metabolized, suggesting that its conversion to 9,10,11th-18:1 is catalyzed enzymatically by an epoxide hydrolase in aorta.

3.2.6. Effects of Substrate Concentration on the Formation of 18:2 Metabolites

At low concentrations (18 μM) of 18:2, the amounts of products formed were in the order, c (9h-18:2) > b (13h-18:2) > f+g (9,10,13th-18:1 + 9,12,13th-18:1) > d (9,10e-11h-18:1) = e (9,10,11th-18:1) (Fig.28). The proportion of 18:2 converted to each of the products declined as its concentration was raised. Some products were more affected than others, however, and at higher concentrations of 18:2 (125 μM) the amounts of products formed were in the order, c > f+g > b > d > e. Raising the concentration of 18:2 from 18 to 125 μM increased the combined amount of epoxyhydroxy and trihydroxy products from 23 to 40 % of the total products. This may be presumably due to saturation of the peroxidase component of prostaglandin endoperoxide synthase, resulting in decreased conversion of 9hp-18:2 and 13hp-18:2 to monohydroxy products, and increased formation of epoxyhydroxy products (Fig.22).

The amount of e (9,10,11th-18:1) formed does not increase when the concentration of 18:2 is increased from 125 to 374 μM, whereas the amount of its precursor, d, continues to increase. This is probably due to saturation of the epoxide hydrolase which converts d to e.

The K_m and V_{max} values for the conversion of 18:2 and

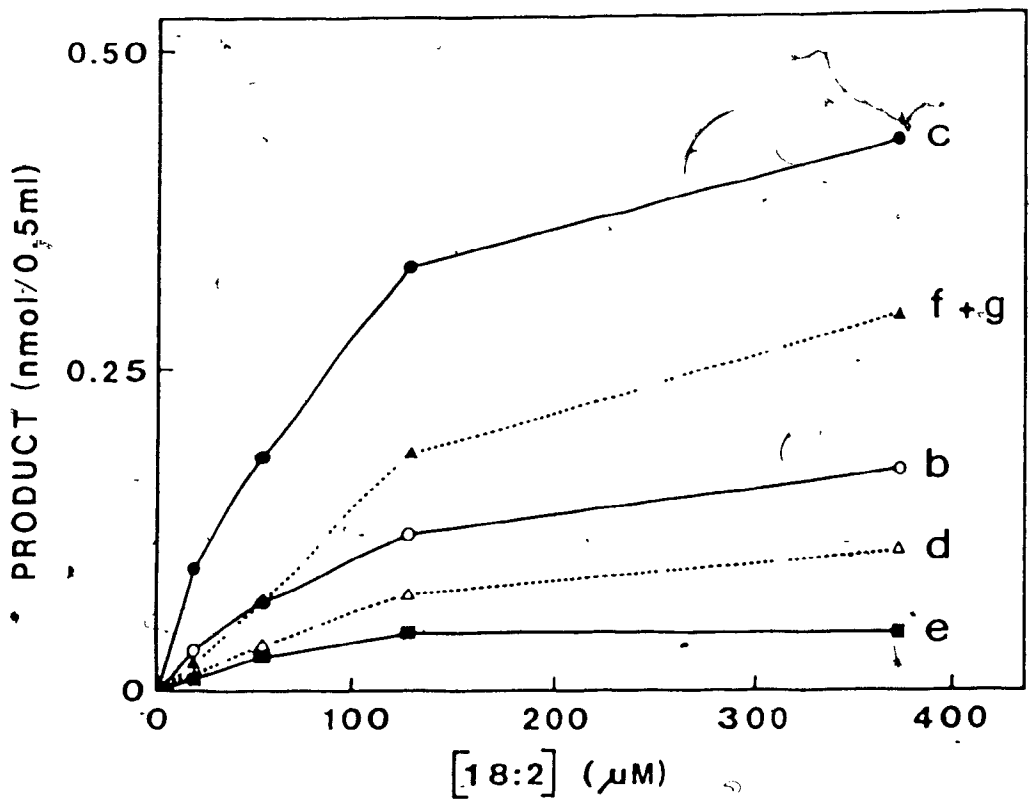

Fig.28. Effects of substrate concentration on the formation of 18:2 metabolites. Particulate fractions (0.5 ml) from fetal calf aorta were incubated at 37 °C with [1-^{14}C]18:2 (0.48 μCi) and varying amounts of unlabeled 18:2 for 20 min. The amounts of products were determined by NP-HPLC as described in the legend to Fig. 12. All values are means of duplicates and have been corrected for recovery.

20:4 to products were determined from Lineweaver-Burk plots (Fig.29). In this experiment the total amounts of polar metabolites formed from each substrate were measured. It was possible to use a longer incubation time for 18:2 (1.5 min) than for 20:4 (15 s), since the rate of oxygenation of 18:2 remained linear for a much longer period of time (see Fig.21). The apparent K_m values obtained for the oxygenation of 18:2 and 20:4 were 442 and 48 μM, respectively. The apparent V_{max} for 18:2 was 0.67 nmol/min/mg protein, whereas that for 20:4 was 2.7 nmol/min/mg protein.

3.2.7. Competitive Inhibition of 18:2 and 20:4 Oxygenation

Enrichment of endothelial cells with 18:2 has been reported to result in inhibition of PGI_2 production by these cells (178,179). In order to determine whether 18:2 could compete with 20:4 for prostaglandin endoperoxide synthase in aorta, [1-^{14}C]20:4 (0.2 μCi, 13.8 μM) was incubated with a particulate fraction from fetal calf aorta in the presence of various amounts of unlabeled 18:2 (Fig.30). Under these conditions, 18:2 was not a very potent inhibitor of the metabolism of 20:4, the highest concentration (357 μM) resulting in 43 % inhibition of the formation of 6-oxo$PGF_{1\alpha}$. On the other hand, 20:4 (ID_{50}, 10 μM) was a much more potent inhibitor of the formation of 9h-18:2 and 13h-18:2 from [1-^{14}C]18:2 (15 μM). The formation of the trihydroxy 18:1 isomers was inhibited to a much lesser extent, probably due to the saturation of peroxidase by PGG_2 and 15hp-PGI_2 formed

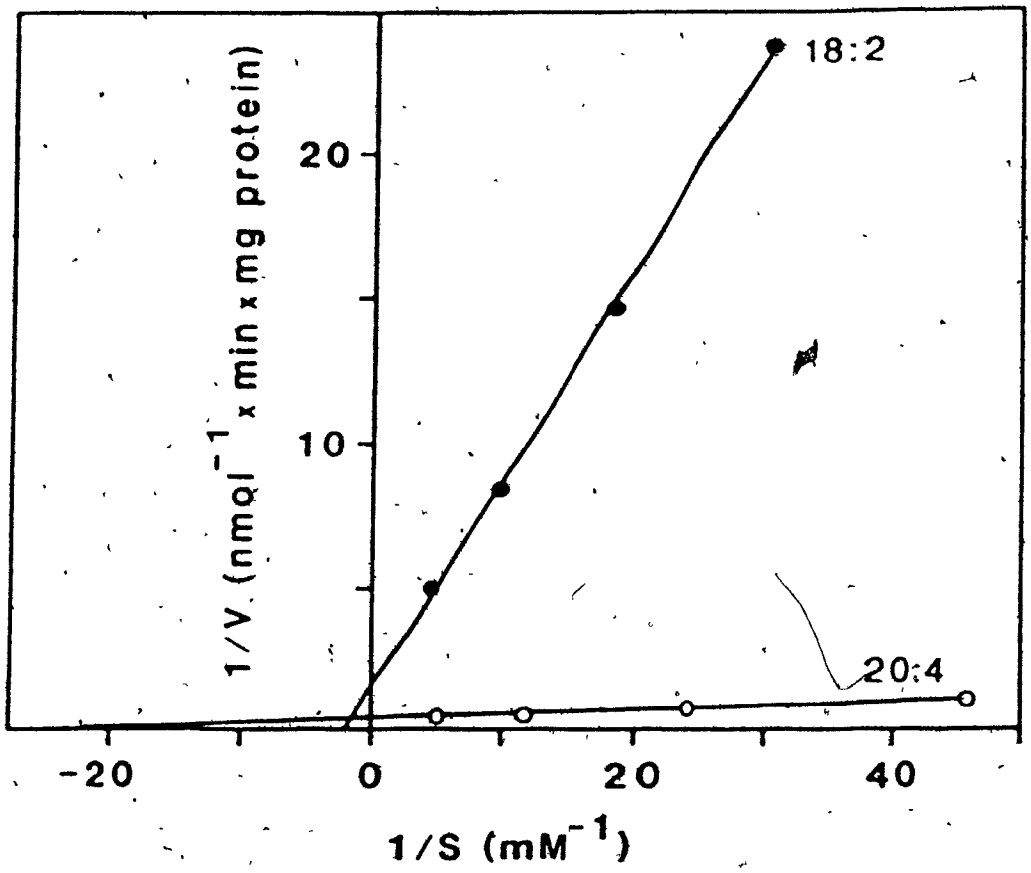

Fig.29. Lineweaver-Burk plots for the conversion of 18:2 (●) and 20:4 (○) to oxygenated metabolites by particulate fractions from fetal calf aorta. The total amounts of metabolites formed from each substrate were determined as described in the legend to Fig.21. The incubation times were 15 s for 20:4 and 1.5 min for 18:2.

Fig.30. Effects of 18:2 on the metabolism of [1-^{14}C]20:4 and of 20:4 on the metabolism of [1-^{14}C]18:2 by particulate fractions from fetal calf aorta. [1-^{14}C]18:2 (0.41 μCi, 7.5 nmol) along with various amounts of unlabeled 20:4 (●,■) or [1-^{14}C]20:4 (0.20 μCi, 6.9 nmol) along with various amounts of unlabeled 18:2 (○) were incubated with particulate fractions (0.5 ml) from fetal calf aorta for 20 min at 37 °C. 6-oxoPGF$_{1\alpha}$ (○) was measured by NP-HPLC using a linear gradient between 4% solvent B in solvent A and 100% solvent B over 40 min. The monohydroxy (b+c, ■) and trihydroxy (e+f+g, ●) metabolites of 18:2 were measured by NP-HPLC as described in the legend to Fig.12. All values have been corrected for recovery using [^{3}H]PGF$_{1\alpha}$ as an internal standard and are the mean of duplicates. The amounts of metabolites formed in the controls were: 6-oxoPGF$_{1\alpha}$ (1.0 nmol), monohydroxy metabolites of 18:2 (0.17 nmol) and trihydroxy metabolites of 18:2 (0.011 nmol).

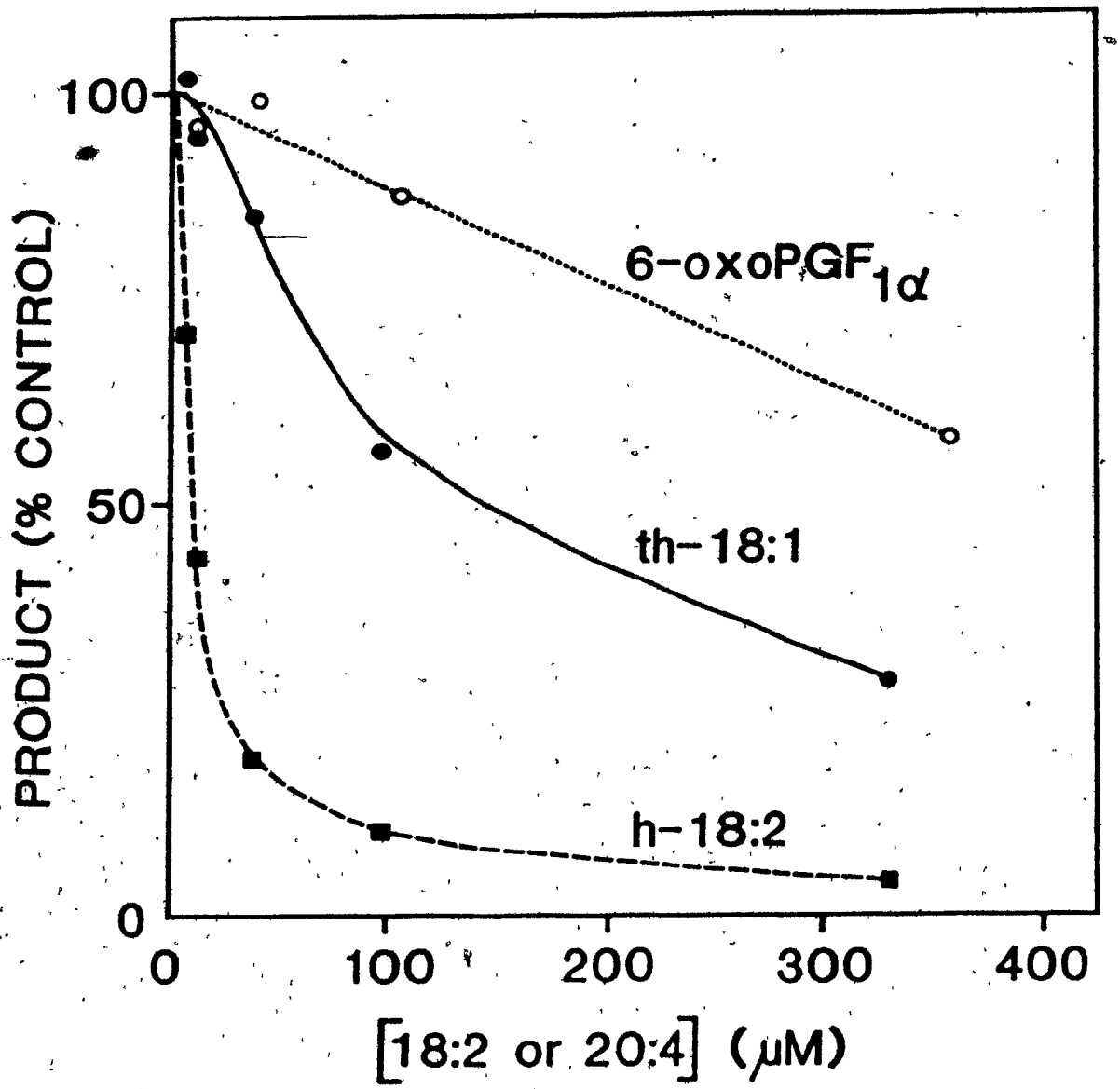
100
50
0
PRODUCT (% CONTROL)
6-oxoPGF1α
th-18:1
h-18:2
0
100
200
300
400
[18:2 or 20:4] (μM)

Fig.30.

from 20:4. Thus, as the concentration of 20:4 was increased, the proportion of 9hp-18:2 and 13hp-18:2 metabolized to the corresponding monohydroxy products decreased, resulting in diversion to the epoxyhydroxy-trihydroxy pathway (see Fig.22).

3.2.8. Identification of Metabolites formed from Endogenous 18:2 Released by Slices of Fetal Calf Aorta

Slices of fetal calf aorta were incubated in Krebs-Ringer medium, containing 15 mM Tris-HCl, pH 7.4, with [1-^{14}C]18:2 (2.6 µCi, 0.048 µmol) for 60 min at 37 °C. This amount of radioactive substrate was not sufficient to interfere with the mass spectra of products formed from endogenous unlabeled 18:2, and made it possible to detect the 18:2 metabolites during HPLC. The radioactive products were extracted and purified by normal-phase and argentation HPLC as described above for products isolated from particulate fractions. Each fraction was converted to its methyl ester, Me_3Si ether derivative and analyzed by GC-MS using a Hewlett Packard 5985B instrument which repetitively scanned the column effluent. As shown in Table 5, all the products isolated from particulate fractions, with the exceptions of 9o-18:2, 12,13e-11h-18:1 and 9,10e-11h-18:1, were identified by GC-MS. The mass spectra of the products confirmed that the carbons of their carboxyl groups were primarily ^{12}C rather than ^{14}C, indicating that they were derived mainly from endogenous substrate rather than from the negligible amount of [1-^{14}C]18:2 added (of which the carbon of the carboxyl group was nearly 100 % ^{14}C). In each case the gas

TABLE 5

Oxygenated Metabolites of Endogenous 18:2 Synthesized by Slices of Fetal Calf Aorta

Slices of fetal calf aorta were incubated in Krebs-Ringer Tris medium with [1-^{14}C]18:2 (3 μCi, 54.7 Ci/mol) as described in the text. The products were purified by HPLC and identified by GC-MS.

Product	m/z values of major ions detected*
13h-18:2 (b)	382,311,225
9h-18:2 (c)	382,367,351,311,225
9,10,11th-18:1 (e)	560,470,361,271,259,173
9,10,13th-18:1 + 9,12,13th-18:1 (f & g)	545,460,387,301,259,173

* The base peak is underlined.

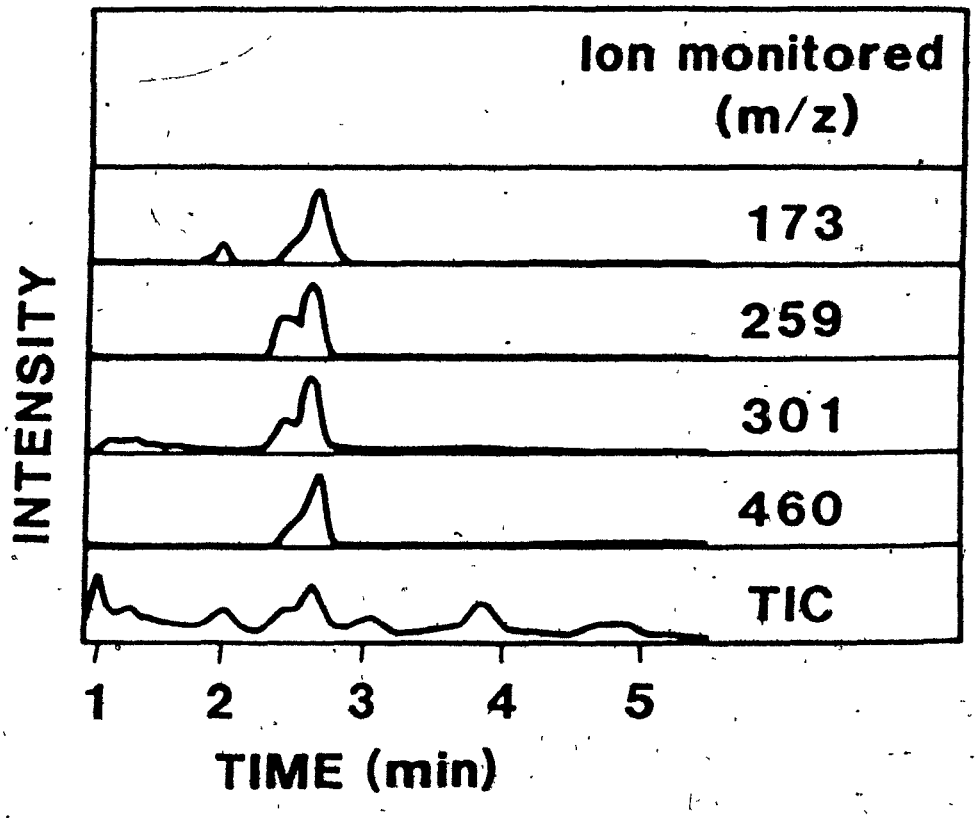

Fig.31. Mass fragmentogram of fraction g (9,10,13th-18:1 + 9,12,13th-18:1) formed from endogenous substrate. Slices of fetal calf aorta (20 g) were incubated with [1-^{14}C]18:2 for 60 min at 37 °C in Krebs-Ringer Tris medium. The products were purified by HPLC and analyzed by GC-MS using a Hewlett-Packard 5985B instrument. TIC, total ion current.

chromatographic profile of the major ions of each of the compounds in Table 5 showed a peak with a t_R identical to that for the corresponding compound formed from exogenous substrate. This is illustrated by Fig.31, which shows the profiles for ions with m/z values of 173, 259, 301 and 460, as well as the total ion current for the methyl ester, Me_3Si ether derivative of the material in fraction g, which was formed from endogenous substrate. The identification of the endogenous products as a mixture of 9,10,13th-18:1 and 9,12,13th-18:1 was further supported by the complete mass spectra which were recorded at 2.4 and 2.6 min.

Oxygenated metabolites formed from endogenous 20:4 and 20:3 by aorta have previously been detected in our laboratory using a similar method (254,256).

3.3. Quantitation of Oxygenated PUFA Metabolites Released by Aorta

Previous work from our laboratory (254,256,257) and data presented above have demonstrated that a variety of fatty acid hydroperoxides are formed during the metabolism of PUFA by aorta. These products could have inhibitory effects on the synthesis of PGI_2, if not rapidly metabolized. By measuring the monohydroxy and trihydroxy products derived from fatty acid hydroperoxides, a reliable assessment of their amounts within vascular tissue may be obtained.

It was not possible to quantitate the endogenous monohydroxy and trihydroxy products described above by GC-MS because appropriate internal standards were not available.

To this end, we decided to establish a suitable GC-MS assay for quantitation of the major oxygenated metabolites of 20:4, 20:3 and 18:2 produced from slices of vascular tissue.

To attempt to measure approximately 10 different oxygenated PUFA metabolites from a single incubation of vascular tissue slices in a reproducible manner poses quite a formidable task. Obviously, there are many ways of approaching this problem. The assay system that has been developed here has tried to minimize workup and analysis time while at the same time attempting to maximize sensitivity and specificity and to minimize potential sources of interference.

3.3.1. Development of a Method for the Quantitation of Oxygenated PUFA Metabolites

A brief step by step procedure of the assay is outlined below (see Methods and Fig. 5 for further details).

1) incubation of vascular slices in physiological medium
2) removal of medium and division into parts A and B in a 1:3 ratio
3) addition of internal standards to part A (deuterated PGE_2, deuterated 6-oxo$PGF_{1\alpha}$) and part B (14h-19:2, 7h-19:1)
4) extraction of samples with ODS silica
5) methylation of carboxylic acids in part B
6) HPLC separation of products in part A (PGE_2 and 6-oxo$PGF_{1\alpha}$) and part B (groups containing (i) monohydroxy and (ii) trihydroxy products)
7) derivatization of samples
8) GC-MS with selected ion monitoring

3.3.2. Quantitation of Prostaglandins in Part A

Quantitation of the products from part A (6-oxo$PGF_{1\alpha}$ and PGE_2) was quite straightforward, since the appropriate deuterium-labeled internal standards were available. The GC stationary phase (SP2250 3 %) used in these studies was not capable of separating 6-oxo$PGF_{1\alpha}$ and PGE_2. Therefore, a RP-HPLC step was required to separate these prostaglandins prior to GC-MS analysis. After suitable derivatization (methyl ester, Me_3Si ether, O-methyloxime derivatives) ions at M-31 were monitored for each compound (598 and 602 for 6-oxo$PGF_{1\alpha}$ and its tetradeuterated analogue (Fig.32A); and 508 and 512 for PGE_2 and d_4-PGE_2 (Fig.32B)). PGE_2 eluted as two peaks (syn and anti O-methyloxime isomers) upon GC analysis (275). The second larger peak was used for quantitation purposes.

3.3.3. Quantitation of Products in Part B

In order to quantitate monohydroxy and trihydroxy PUFA metabolites in part B, corresponding analogues synthesized from nonadecadienoic acid (described in Methods) were used as internal standards.

NP-HPLC was used to separate monohydroxy and trihydroxy products into two groups (i.e. resolution of individual products was not optimized). At the same time enough separation of the monohydroxy products from the solvent front and early eluting contaminants (e.g. free fatty acids and BHT) had to be maintained.

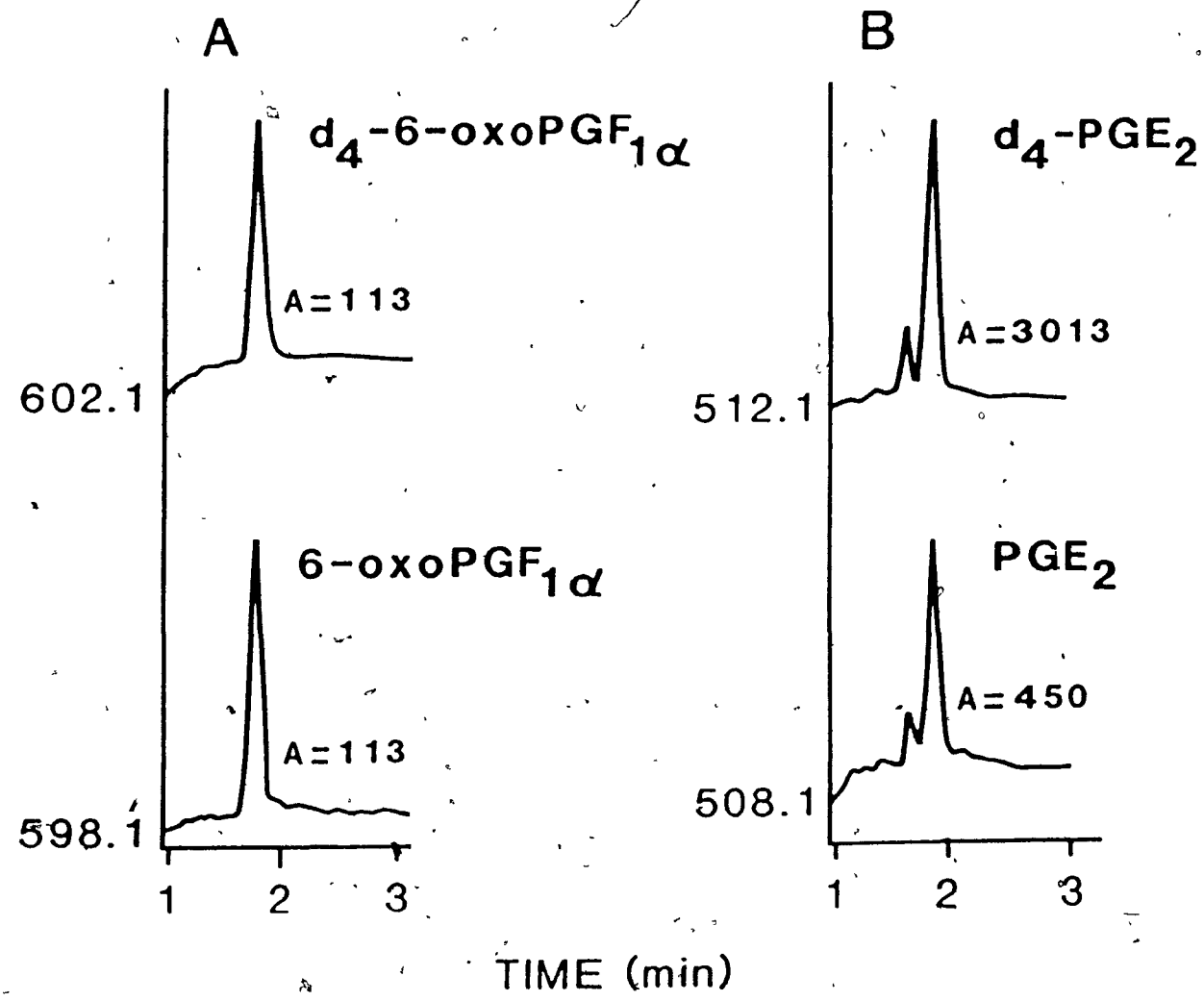

Fig.32. Selected ion chromatograms of the trimethylsilyl ether, O-methyloxime, methyl ester derivatives of (A) 6-oxoPGF$_{1\alpha}$ and (B) PGE$_2$, along with their tetradeuterated analogues.

(a) Monohydroxy PUFA metabolites

The products analyzed and quantitated from the monohydroxy region (t_R's between 11 and 17 min on NP-HPLC) were: 12h-17:2, the major oxygenated metabolite from 20:3 in bovine aorta, 12h-17:3, 9h-18:2, 13h-18:2, 11h-20:4, 12h-20:4 and 15h-20:4, along with the internal standard, 14h-19:2. These products were hydrogenated and partially separated by gas chromatography during analysis by GC-MS (Fig.33). As a result of the hydrogenation there was little or no interference between the different hydroxy-20:0 isomers, or the hydroxy-18:0 isomers. For each compound a pair of major ions compatible with a fragmentation on either side of the carbon atom bearing the OTMS substituent, resulted. The only problem was that 12h-17:2 and 12h-17:3 could not be distinguished from one another by this procedure. In preliminary experiments, in which the monohydroxy fatty acids were not hydrogenated, the amount of 12h-17:2 was estimated by the intensity of the molecular ion at m/z 368, after subtracting the calculated intensity of the M+2 peak at m/z 368 for 12h-17:3. In this way, the ratio of 12h-17:2 to 12h-17:3 was determined to be about 1:2 for fetal calf aorta, and about 1:8 for rabbit aorta. All data presented in this study represent a combined total of 12h-17:2 and 12h-17:3.

(b) Trihydroxy PUFA metabolites

The trihydroxy region (t_R's between 43 and 44 min on NP-HPLC) contained th-18:1 and the internal standard, th-19:1.

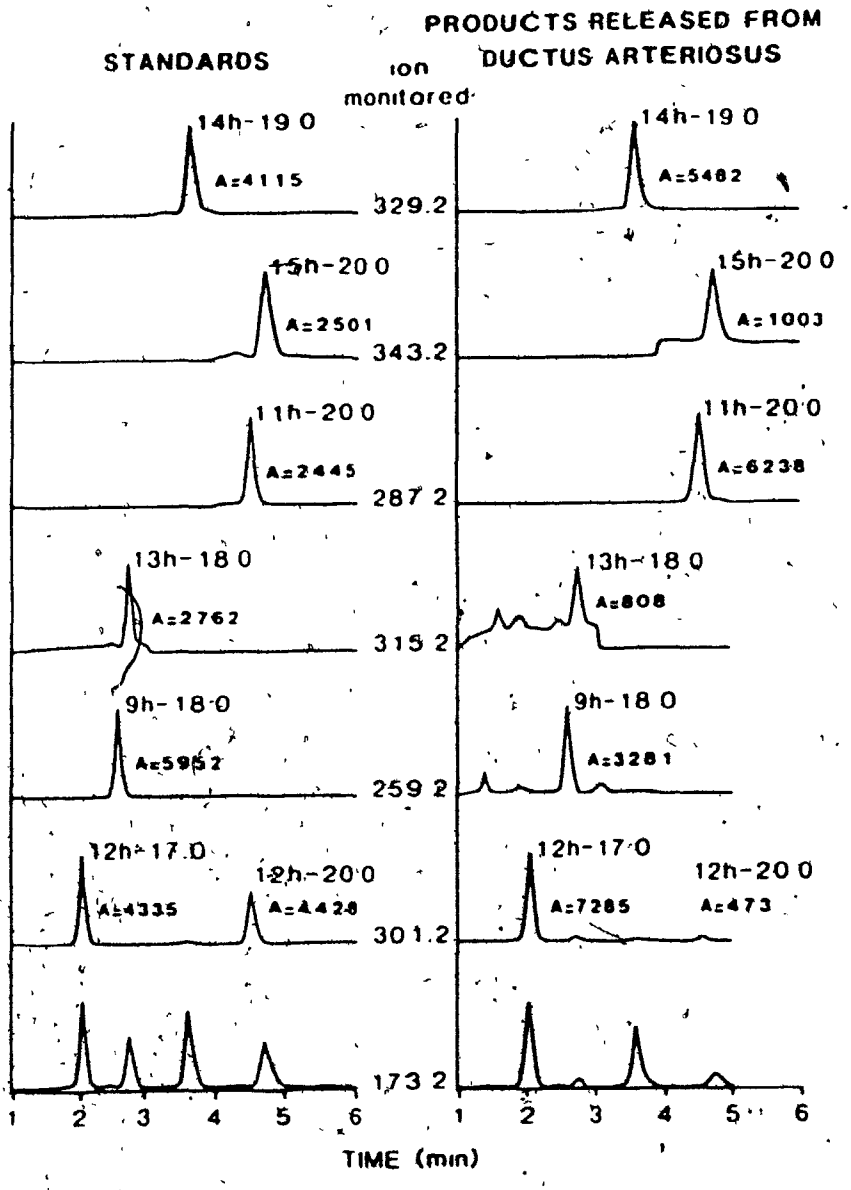

Fig.33. Selected ion chromatograms of derivatized (hydrogenated, trimethylsilyl ether, methyl ester) monohydroxy fatty acids. A mixture of standards (100 ng of each; left side) and monohydroxy products released from an incubation of fetal calf aorta ductus arteriosus slices (right side), along with the internal standard, 14h-19:2 (200 ng), are displayed. A, peak area.

The th-18:1 which was measured was actually a mixture of 9,10,13th-18:1 and 9,12,13th-18:1, while the internal standard was a mixture of 10,11,14th-19:1 and 10,13,14th-19:1 (approximately 1:1.25) (Fig.34). The th-18:1 employed for the standard curve was synthesized from fetal bovine aorta and contained 9,10,13th-18:1 and 9,12,13th-18:1 in a ratio of approximately 2.5:1. For these studies, it has been assumed that this ratio remains fairly constant. If synthesis by aorta differs significantly from the 2.5:1 ratio of 9,10,13th-18:1 to 9,12,13th-18:1 there could be a significant error in the absolute amounts calculated since the fragment with a m/z value of 259 is much more abundant for 9,10,13th-18:1 than 9,12,13th-18:1. For example, if much more 9,10,13th-18:1 is synthesized than 9,12,13th-18:1, the ratio of the intensity of the ion at m/z 259 to 273 is increased with respect to the ratio obtained from the standards used to construct the standard curve, and there will be, therefore, an overestimation of the th-18:1 products synthesized by aorta. Preliminary experiments indicated that bovine and rabbit aortic lipids contained an approximate 2.5:1 ratio of 9,10,13th-18:1 to 9,12,13th-18:1.

Both th-18:1 and th-19:1 gave somewhat assymetrical peaks during GC analysis due to the presence of different isomers.

3.3.4. Standard Curves for Quantitation of PUFA Metabolites

Standard curves were constructed after analyzing mixtures of internal standard (200 ng) and different amounts (0-200 ng) of either monohydroxy (Fig.35A), trihydroxy (Fig.35B)

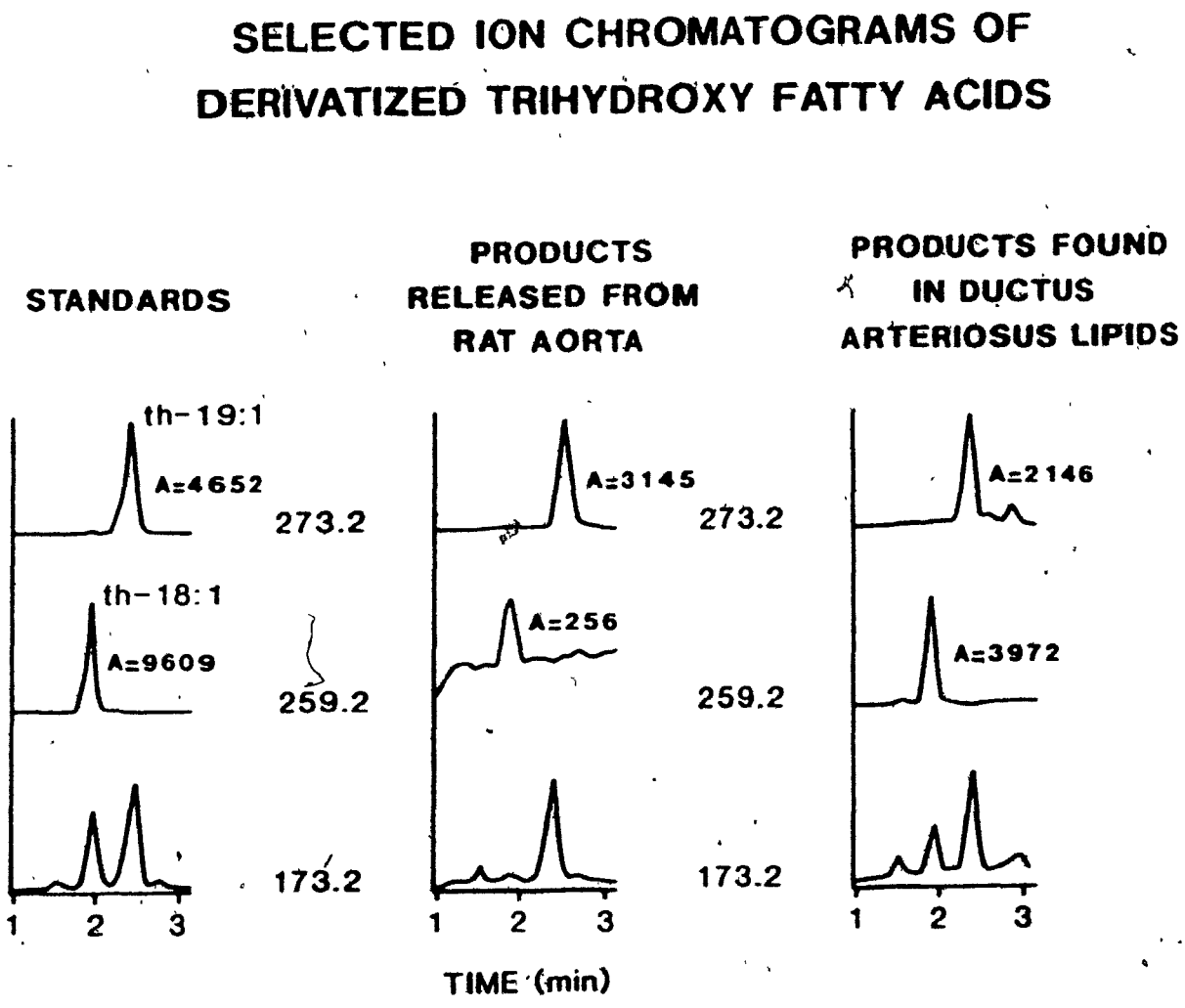

Fig.34. Selected ion chromatograms of derivatized (trimethylsilyl ether, methyl ester) trihydroxy fatty acids. Left side: standard th-18:1 (200 ng). Centre: trihydroxy products released from aorta. Right side: trihydroxy products in fetal calf ductus arteriosus after alkaline hydrolysis of a chloroform/methanol extract. The internal standard th-19:1 (200 ng) was added to each sample. A, peak area.

Fig.35. (A) Standard curves for the quantitation of monohydroxy metabolites of 18:2 and 20:4 by GC-MS using selected ion monitoring. Mixtures of the internal standard, 14h-19:2 (200 ng) and different amounts of 9h-18:2 (▲---▲), 13h-18:2 (●---●), 11h-20:4 (x——x), 12h-20:4 (O——O), 15h-20:4 (Δ—Δ) and HHT (▣---▣) were treated as described in Fig.5. The ratio of the areas for the ions at m/z 259 (9h-18:2), 315 (13h-18:2), 287 (11h-20:4), 301 (12h-20:4), 343 (15h-20:4) and 301 (HHT) to that for the ion at m/z 329 (14h-19:2) were plotted against the amounts of monohydroxy 18:2 or 20:4 products in each of the samples.

(B) Standard curve for the quantitation of th-18:1. Mixtures of the internal standard, th-19:1 (200 ng), and different amounts of th-18:1 (synthesized using fetal calf aorta) were treated as described in Fig.5. The ratios of the intensities of the ions at m/z 259 (th-18:1) to those at m/z 273 (th-19:1) were plotted against the amounts of th-18:1 in the mixture.

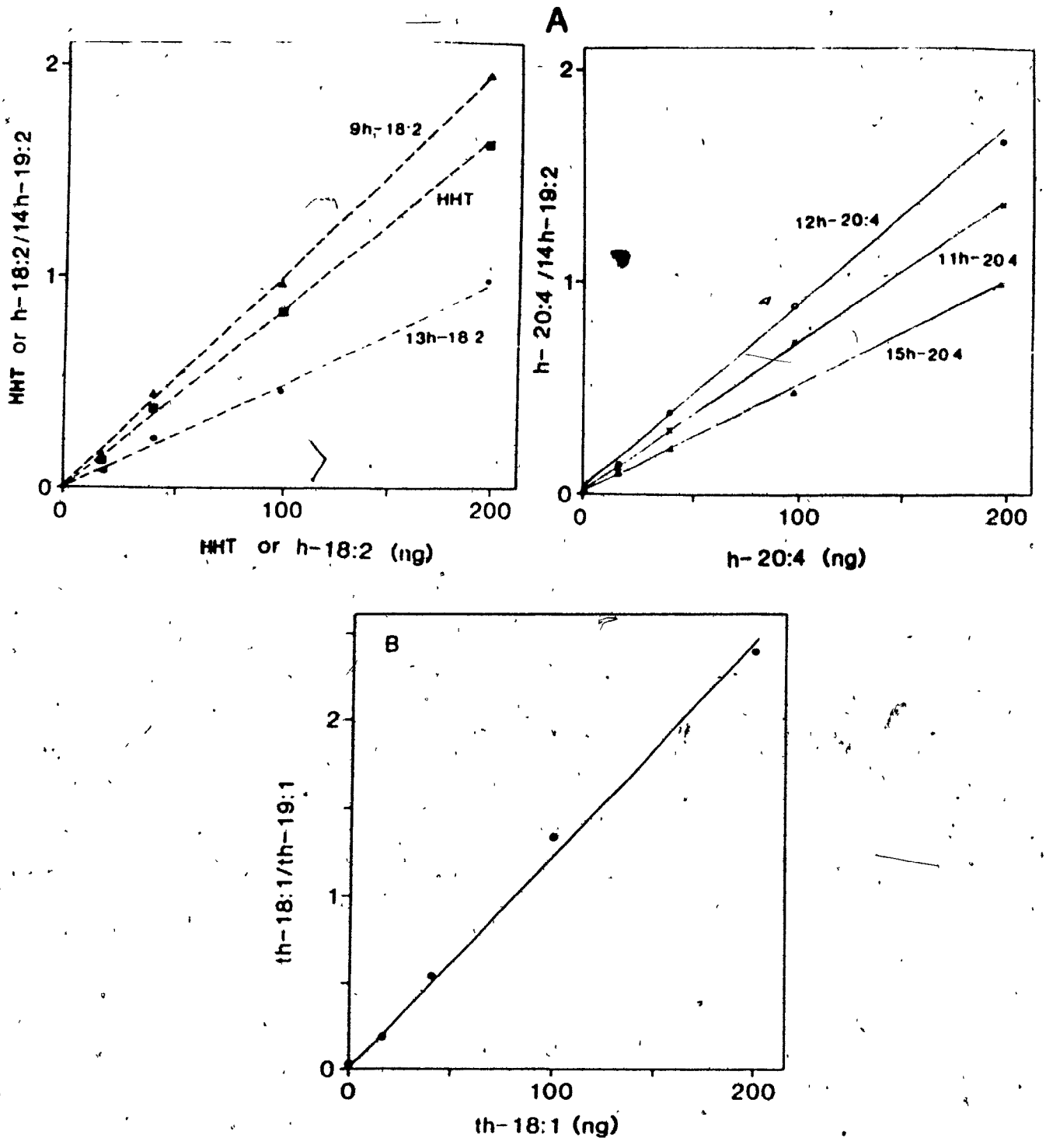
A
9h-18:2
HHT
13h-18:2
HHT or h-18:2/14h-19:2
HHT or h-18:2 (ng)
12h-20:4
11h-20:4
15h-20:4
h-20:4/14h-19:2
h-20:4 (ng)
B
th-18:1/th-19:1
th-18:1 (ng)

Fig. 35.

or PG standards. Each of the mixtures was subjected to the extraction, HPLC and derivatization procedures used for regular samples to confirm that this step was reproducible. Standard curves were linear over the range tested (0-200 ng for monohydroxy and trihydroxy products and 0-500 ng for 6-oxo$PGF_{1\alpha}$). Peak areas were computed using the Hewlett-Packard computer and the ratio of monohydroxy product, trihydroxy product or PG to internal standard was calculated and plotted against the amount of added monohydroxy, trihydroxy or PG standard.

3.3.5. Quantitation of Other PUFA Metabolites

In preliminary experiments with slices of rat, rabbit and bovine aorta, an attempt was made to detect and to quantitate other oxygenated PUFA metabolites of 20:3 and 20:4, in addition to the aforementioned ones. The 20:4 metabolites sought were: 5h-20:4, 8h-20:4, 9h-20:4 and th-20:3 (both 11,12,15th-20:3 and 11,14,15th-20:3). The metabolites of 20:3 which were measured were PGE_1 and th-17:1 (isomers of 8,11,12th-17:1).

In order to quantitate 5h-20:4 produced by rat aortic slices, octadeuterated 5h-20:4 was used as an internal standard. The deuterated and non-deuterated 5h-20:4 was then purified by NP-HPLC. The methyl ester, TMS ether derivatives were formed and ions at 305 and 313, corresponding to 5h-20:4 and deuterated 5h-20:4, respectively, were monitored. The amount of 5h-20:4 produced by rat aorta was below the limits of the GC-MS

assay (< 3 ng/sample).

To quantitate 8h-20:4 and 9h-20:4 produced by rat and rabbit aorta slices, 10h-19:2 (prepared as described in Methods section for 9h-18:2) was added as an internal standard. A fraction corresponding to this group of monohydroxy products was obtained after NP-HPLC. Ions at m/z 255, 265, and 396 were monitored for the methyl ester, TMS ether derivatives of 9h-20:4, 8h-20:4 and 10h-19:2, respectively. 8h-20:4 (2-5 ng/g tissue) was detected in some samples obtained from rabbit aorta. 9h-20:4 was not detected in any samples (detection limit, ca. 2 ng/sample).

In our laboratory, both 11h-20:4 and 15h-20:4 had been previously found to be synthesized from endogenous 20:4 by fetal calf aorta (254). Their putative intermediates, 11hp-20:4 and 15hp-20:4, respectively, might also degrade to th-20:3 metabolites. Therefore an attempt was made to determine if these trihydroxy products are synthesized by aorta. Incubation of slices of rat, rabbit and bovine aorta and workup of samples were carried out as described in sections 3.3.1. and 3.3.3.(b). Ions at m/z 173 and 273 (internal standard, th-19:1), 173, 283 (11,12,15-trihydroxy-5,8,13-eicosatrienoic acid and 11,14,15-trihydroxy-5,8,12-eicosatrienoic acid) and 211 and 301 (11,12,15th-20:3) were monitored. During GC-MS analysis no peak corresponding to the methyl ester, TMS ether derivative of either 11,12,15th-20:3 (ions at m/z 173, 211, 283, 301) or 11,14,15th-20:3 (ions at m/z 173, 283) was observed at a C-value between 24 and 25, where these products would be expected to

chromatograph. Since we did not have any standard th-20:3 the detection limit could not be ascertained. It appears, then, that aorta synthesizes little, if any, th-20:3 from endogenous 20:4.

Small amounts (2-3 ng/g tissue) of trihydroxy metabolites of 20:3 (isomers of 8,11,12th-17:1) were detected in incubations from fetal bovine aorta slices. These isomers were isolated in the same manner as th-18:1 isomers and ions at 173 and 446 were monitored. The ratio of the ion at 446 (th-17:1) to 273 (th-19:1 internal standard) was computed and compared to a standard curve. Standard th-17:1 was prepared from fetal bovine aorta particulate fractions incubated with exogenous 20:3.

We were unable to detect PGE_1 from incubations of slices of fetal bovine ductus arteriosus, and rat and rabbit aorta slice incubations (detection limits, ca. 10 ng/sample). Ions at m/z 470 and 297 were monitored for PGE_1, whereas ions at 474 and 301 were monitored for the tetradeuterated PGE_1 (Upjohn Co., Kalamazoo, MI) internal standard.

Since the metabolites mentioned above were either undetectable or were found in very small amounts approaching the limits of assay sensitivity it was decided not to pursue their quantitation in further experiments.

3.3.6. Validation of Assay

Two major problems had to be dealt with before a routine analysis of oxygenated PUFA metabolites from vascular tissue slices could be initiated. The first problem was to assure

a reliable and reproducible assay and the second was to determine the extent of autoxidation during sample workup and to take precautionary means to minimize this process.

The reproducibility of the assay was tested as follows: fetal calf aorta slices (6 g) were incubated for 20 min at 37 °C in Krebs-Ringer Tris medium. The medium was separated from the tissue slices and separated into fractions A to D as shown in Fig.5. Fractions A (PGs-medium), B (monohydroxy and trihydroxy products-medium), C (monohydroxy and trihydroxy products-free within tissue) and D (monohydroxy and trihydroxy products-total products within tissue) were each further divided into 4 equal parts before extraction with ODS silica SepPaks. All 16 fractions were carried separately through the workup procedure (extraction, HPLC separation, derivatization and GC-MS analysis). Since all fractions were derived from a single tissue slice incubation, biological variations between samples were not possible, so differences in the final values should accurately reflect intra-assay variance and thus the reproducibility of the assay. Table 6 shows the amounts of products released into the medium, as well as the amounts of free and total (free + esterified) products in the tissue slices. Virtually all values fall within a range of ±10 % of the mean, indicating reproducibility from a methodological standpoint is quite satisfactory.

To minimize autoxidation during sample workup, a number of precautionary measures were taken, especially for

TABLE 6

Reproducibility of GC-MS Assay for Oxygenated PUFA Metabolites

Fetal calf aorta slices (6 g) were incubated for 20 min at 37 °C in Krebs-Ringer Tris buffer. The medium and tissue slices were separated into fractions A to D as shown in Fig.5 and then each fraction was further divided into four equal parts before extraction. All 16 fractions were then carried through the workup procedure separately. All values are presented as ng/g tissue (mean ± S.E.M.).

	Medium Products (Fractions A and B)	Free Tissue Products (Fraction C)	Total Tissue Products (Fraction D)
12h-17:3	246±3	317±8	386±8
9h-18:2	57±1	80±2	371±2
13h-18:2	25±0.5	45±1	164±7
11h-20:4	137±6	230±5	359±5
12h-20:4	8.3±0.3	15±0.2	43±1
15h-20:4	28±0.5	50±2	96±1
th-18:1	33±2	19±2	268±4
6-oxoPGF$_{1\alpha}$	6030±95	--	--

measurement of oxygenated PUFA metabolites contained within the tissue slices. These measures included:

1) freezing the tissue slices in liquid nitrogen immediately after incubation prior to workup
2) pulverizaton of the slices using a tissue pulverizer in an acetone-dry ice bath
3) homogenization of the resulting powder at approximately -20 °C in argon-bubbled chloroform/methanol (2:1) containing the antioxidant, butylated hydroxytoluene (0.05 %)
4) carrying out the hydrolysis step in an argon atmosphere in 95 % EtOH that had been degassed and bubbled with argon
5) storage of samples at -20 °C in toluene containing 0.05 % BHT, under argon
6) extraction and purifying the products by NP-HPLC as soon as possible (3 h maximum time before extraction and hydrolysis; 48 h before NP-HPLC).

The procedure was validated by measuring the amounts of monohydroxy and trihydroxy metabolites of 18:2 present in rabbit aortic tissue in the presence and absence of exogenous 18:2 (1 mg), added prior to homogenization of the tissue in chloroform/methanol (2:1) and hydrolysis. Similar amounts of mono- and tri-hydroxy metabolites of 18:2 were detected in the presence and absence of exogenous 18:2, indicating that it was not substantially autoxidized during the workup procedure (Table 7). When 18:2 (1 mg) alone, in the absence of tissue, was subjected to the above workup

TABLE 7

Total Amounts (free + esterified) of 18:2 Oxygenation Products in Rabbit Aorta in the Presence and Absence of Exogenous 18:2.

Rabbit aortae were rapidly removed, cleared of adhering fat, and frozen in liquid nitrogen, either immediately or after incubation in Krebs-Ringer Tris buffer for 20 min at 37 °C. The frozen tissue was pulverized, extracted with chloroform:methanol (2:1) containing 0.05% butylated hydroxytoluene, and the internal standards, 14h-19:2 and th-19:1, and, in some cases, 18:2 (1 mg). The material in the extract was hydrolyzed and the oxygenated 18:2 metabolites were quantitated by GC-MS. The values are means ±S.E.M. The numbers of samples are given in brackets.

Sample	Incubation time (min)	Product (ng/g tissue)		
		9h-18:2	13h-18:2	th-18:1
18:2 (4)	0	77±12	58±8	41±10
Aorta (3)	0	2708±75	1993±35	523±68
Aorta + 18:2 (3)	0	2357±359	1929±66	583±57
Aorta (7)	20	2391±423	1995±302	509±61

procedure, only small amounts of products were detected by GC-MS.

3.3.7. Oxygenated PUFA Metabolites Formed by Slices of Vascular Tissue

The methods described above were used to determine the amounts of oxygenated metabolites of 18:2 and 20:4 produced by slices of adult rat, rabbit and bovine aorta, as well as fetal bovine aorta and ductus arteriosus.

(a) Oxygenation products of 18:2 and 20:4 formed by rat aorta

Slices of rat aorta were incubated in Krebs-Ringer Tris medium, and the products were quantitated by GC-MS (Fig.36). In some cases, the samples were preincubated for 30 min at 0 °C with indomethacin (10^{-4} M) (cross-hatched bars). Far more 6-oxo$PGF_{1\alpha}$ (5900 ng/g tissue) was released by rat aorta than any other PUFA metabolite (open bars). Relatively small amounts of PGE_2 and 12h-17:3 were also detected. Since 12h-17:3 was measured after hydrogenation to 12h-17:0, the amount shown in Fig.36 represents the sum of 12h-17:3 and 12h-17:2, with the former predominating (see section 3.3.3.(b)).

The major h-20:4 metabolite released by rat aorta was 11h-20:4 (332 ng/g tissue). Release of both the 11- and 15-hydroxy metabolites of 20:4 was inhibited by indomethacin, suggesting that they were formed by cyclooxygenase. Only a relatively small amount of the lipoxygenase product, 12h-20:4, was detected. The formation of this product was not

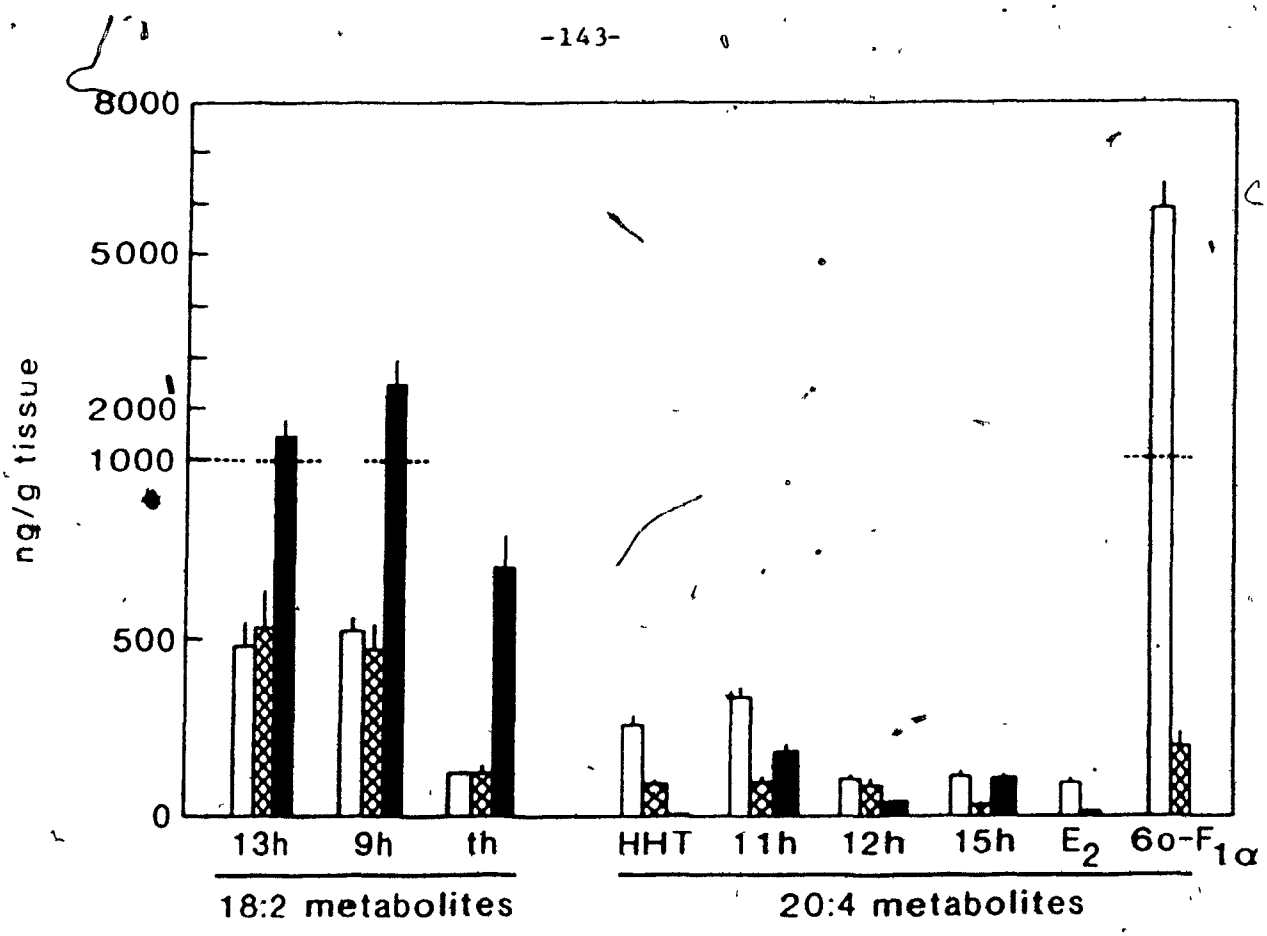

Fig.36. Amounts of oxygenated products of 18:2 and 20:4 synthesized by rat aorta. Slices of rat aorta were incubated in Krebs-Ringer-Tris medium for 20 min at 37 °C and the products were analyzed as described in Fig.5. Products released from rat aorta in the presence (cross-hatched bars) and absence (open bars) of indomethacin (10^{-4}) and esterified metabolites (solid bars) are displayed. The abbreviations are: 13h, 13h-18:2; 9h, 9h-18:2; th, th-18:1; HHT, 12h-17:3 (the values for HHT also include some 12h-17:2, as discussed in the text); 11h, 11h-20:4; 12h, 12h-20:4; 15h, 15h-20:4; E_2, PGE_2; 6o-$F_{1\alpha}$, 6-oxo$PGF_{1\alpha}$.

affected by indomethacin. The amounts of monohydroxy metabolites derived from 18:2 were considerably larger than those derived from 20:4. Rat aorta released approximately equal amounts (ca. 500 ng/g tissue) of 9h-18:2 and 13h-18:2. Smaller amounts of th-18:1 (ca. 122 ng/g tissue) were also detected.

In addition to the 9- and 13- monohydroxy metabolites of 18:2 detected by GC-MS after hydrogenation to the corresponding hydroxystearate derivatives, we also detected 12-hydroxystearate in these mixtures. Approximately equal amounts of this product and 9-hydroxystearate were present in the fraction of PUFA metabolites released by rat aorta, whereas the fraction derived from esterified products contained about one-quarter as much 12-hydroxystearate as 9-hydroxystearate. Similar results were obtained with rabbit aorta. Indomethacin did not have any significant effect on the release of any of the monohydroxy metabolites of 18:2.

Much larger amounts of monohydroxy and trihydroxy metabolites derived from 18:2 were found esterified to lipids (Fig.36, shaded bars). Smaller amounts of monohydroxy-20:4 isomers were present in this fraction, whereas prostaglandins and 12h-17:3 were virtually undetectable.

(b) Oxygenated PUFA metabolites formed by rabbit aorta

Rabbit aorta released much smaller amounts of cyclooxygenase products than rat aorta (Fig.37, open bars). Only about one-tenth as much 6-oxo$PGF_{1\alpha}$ was released by

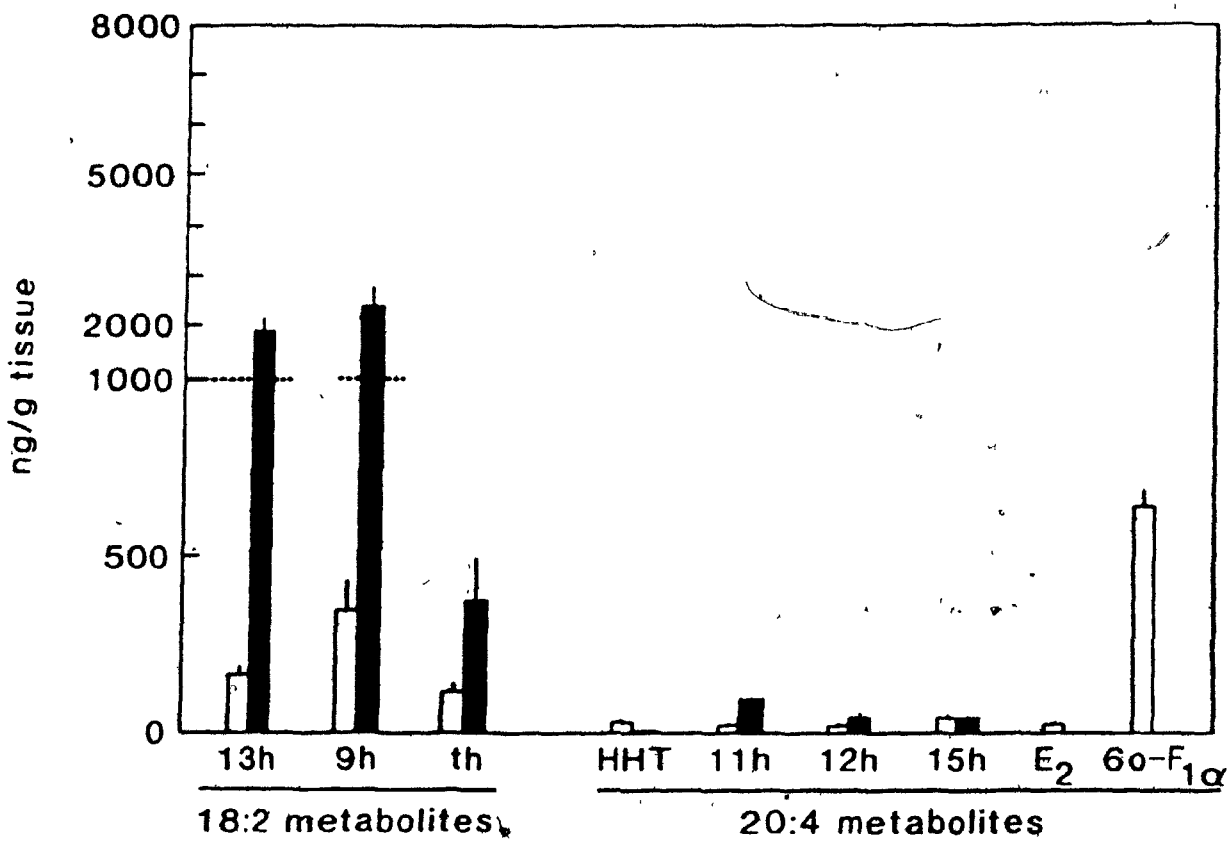

Fig.37. Amounts of oxygenated products of 18:2 and 20:4 synthesized by rabbit aorta. Incubations were carried out as described in the legend to Fig.36. Products released from rabbit aorta are shown by open bars and esterified products by solid bars.

rabbit aorta compared to rat aorta. Much smaller amounts of the cyclooxygenase products, PGE_2, 12h-17:3, 11h-20:4, and 15h-20:4 were also detected. Production of the lipoxygenase product, 12h-20:4, was also less in rabbit aorta than in rat aorta. On the other hand, comparable amounts of oxygenated metabolites of 18:2, both in the free and esterified (shaded bars) forms, were produced by aorta from the two species.

(c) Formation of oxygenated PUFA metabolites by bovine blood vessels

Adult bovine aorta

Adult bovine aorta was divided into two approximately equal layers: an inner layer including the intima and inner media, and an outer layer, including the outer media and the adventitia. Our results confirm previous findings (160,276) that the inner part (open bars) of the aorta is more active in synthesizing prostaglandins than the outer part (hatched bars) (Fig.38A). Approximately twice as much 6-oxo$PGF_{1\alpha}$ was released by the inner layer of the aorta compared to the outer layer. Similar results were obtained for PGE_2 and monohydroxy metabolites of 20:4 formed by cyclooxygenase. Bovine aorta exhibited very little 12-lipoxygenase activity.

Unlike cyclooxygenase products derived from 20:4, equal or greater amounts of both free (Fig.38A) and esterified (Fig.38B; solid bars, inner layer; striped bars, outer layer) oxygenated 18:2 metabolites were formed by the outer layer of bovine aorta, compared with the inner layer. This was also true for esterified products derived from 20:4, which were probably not formed by the action of

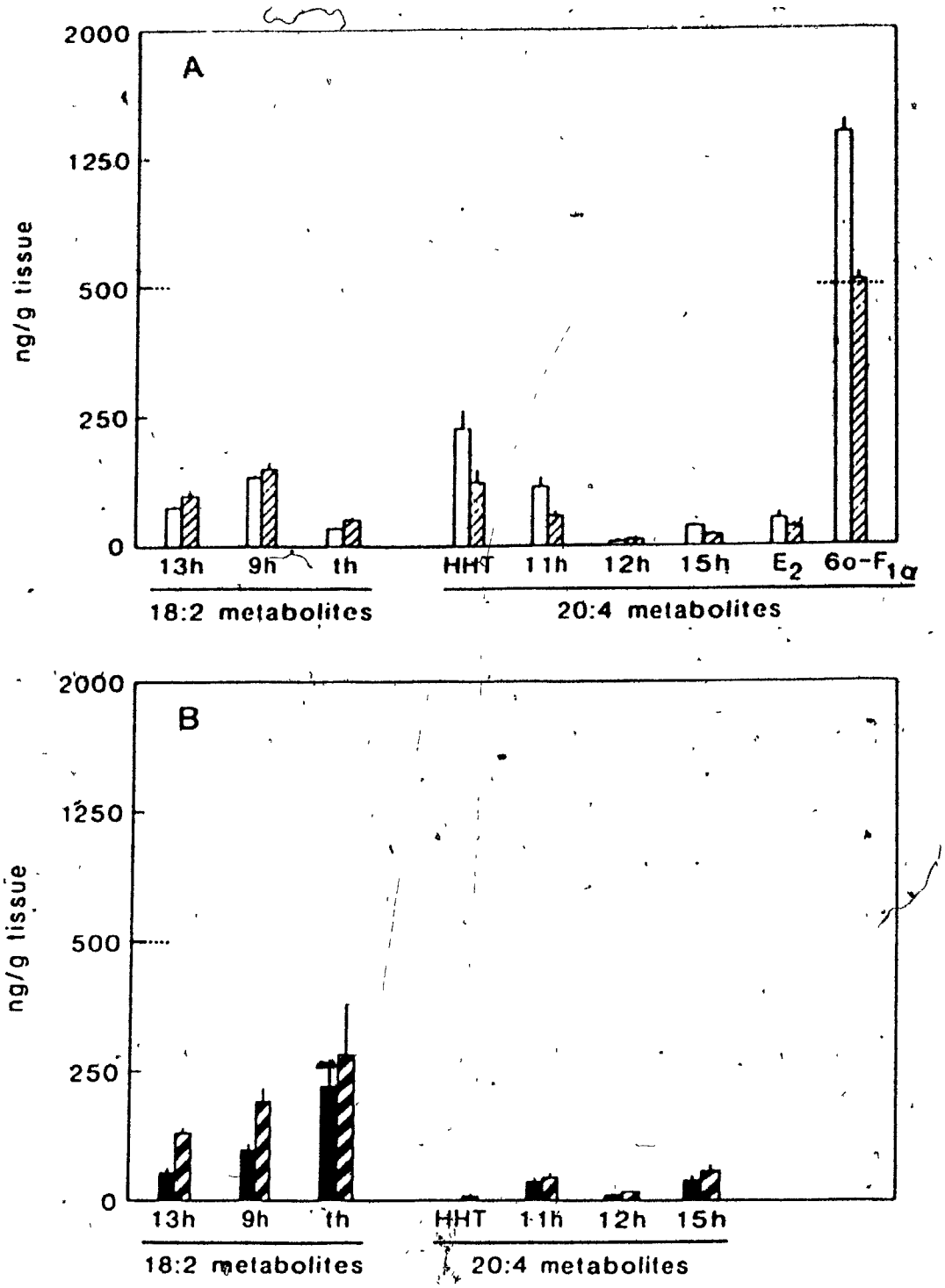

Fig.38. Amounts of oxygenated products of 18:2 and 20:4 synthesized by adult bovine aorta. Incubations were carried out as described in the legend to Fig.36. (A) Products released from the inner (open bars) and outer (hatched bars) segments of adult bovine aorta. (B) Esterified products detected in the inner (solid bars) and outer (striped bars) segments of adult bovine aorta.

cyclooxygenase. Much smaller amounts of oxygenated metabolites of 18:2 were formed by bovine aorta compared to rat and rabbit aorta. Unlike rat and rabbit aorta, the major esterified 18:2 metabolite detected in bovine aorta was th-18:1.

Fetal calf aorta

Fetal calf aorta was considerably more active than adult aorta in forming cyclooxygenase products (Fig.39). About 5 times as much 6-oxoPGF$_{1\alpha}$ was released by fetal aorta compared to adult aorta. Greater amounts of other cyclooxygenase products were also synthesized by fetal aorta, but the difference was not as great as with 6-oxoPGF$_{1\alpha}$. As with adult aorta, the inner layer (open bars) produced about twice the amounts of cyclooxygenase-derived metabolites as the outer layer (hatched bars). Although the amount of 12h-20:4 was somewhat greater than in adult aorta, 12-lipoxygenase activity was still very low. The amounts of released oxygenated metabolites of 18:2 were about the same for adult and fetal aorta. Similar amounts were produced by the inner and outer layers of the aorta. The amounts of esterified products present in fetal calf aorta (data not shown) were the same as in the ductus arteriosus (see below, Fig.40, solid bars) and somewhat greater than that found in the outer portion of adult aorta (Fig.38B).

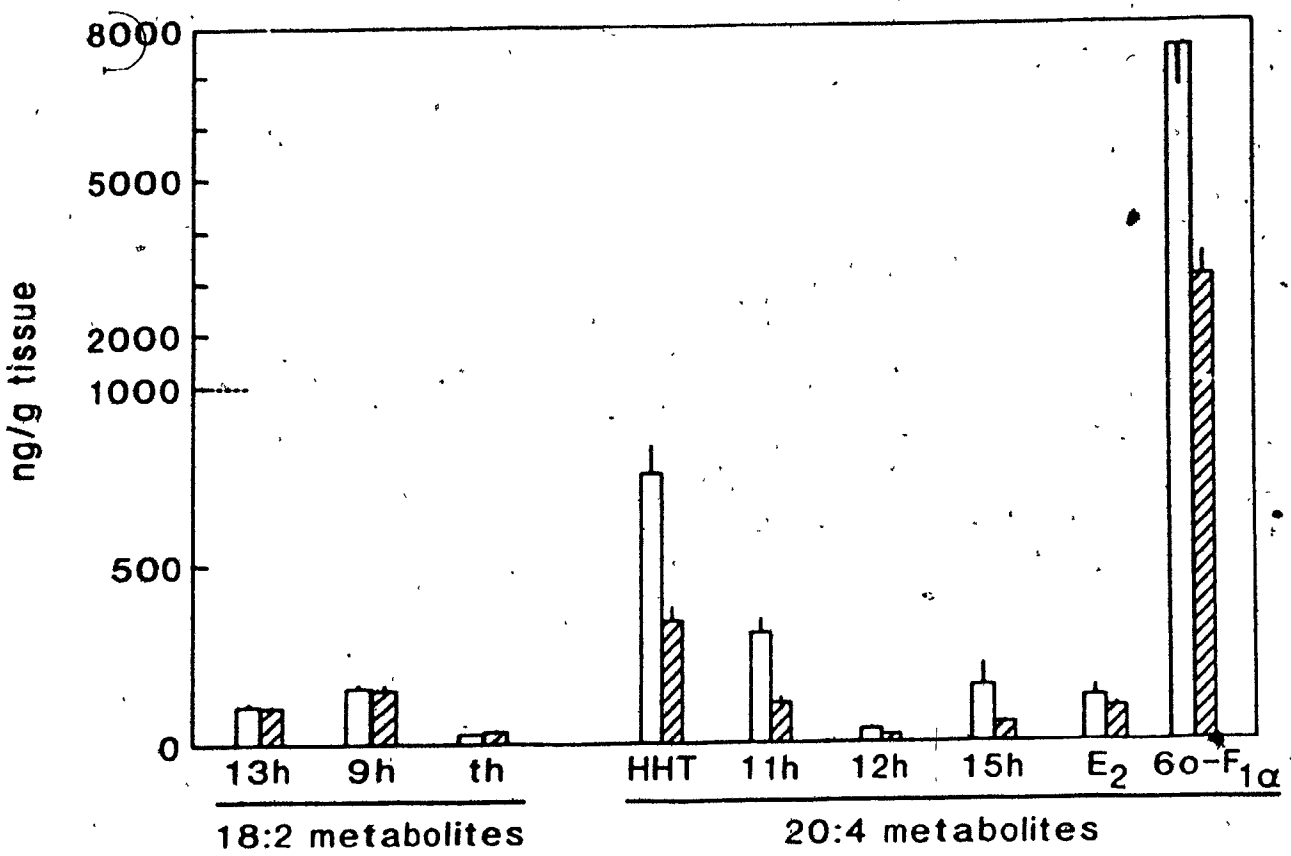

Fig.39. Amounts of oxygenated products of 18:2 and 20:4 synthesized by fetal calf aorta. Incubations were carried out as described in the legend to Fig.36. Products released from the inner (open bars) and outer (hatched bars) segments of fetal calf aorta are shown.

Fetal calf ductus arteriosus

The amounts of 20:4 and 18:2 metabolites released by the ductus arteriosus (which was not divided into inner and outer layers) were intermediate between the inner and outer layers of fetal calf aorta (Fig.40). As with fetal aorta, only a relatively small amount of PGE_2 (94 ng/g tissue) was released. This is only about 2-3% the amount of 6-oxo$PGF_{1\alpha}$ detected. The amounts of esterified monohydroxy metabolites of 18:2 in the ductus arteriosus were similar to those found in adult bovine aorta, but a smaller amount of th-18:1 was detected in the lipid fraction of ductus arteriosus.

3.3.8. Fatty Acid Content of Aorta.

There were considerable species differences in the relative amounts of 18:2 and 20:4 metabolites released by rat, rabbit, and bovine blood vessels. In order to determine whether these differences could be related to the tissue content of these PUFA, we measured the total amounts (free + esterified) of 18:2 and 20:4 in aortae from these three species, as well as in fetal calf ductus arteriosus (Table 8). Fig.41 shows the fatty acid profiles from fetal calf, rat and rabbit aortae. The content of 18:2 in rat and rabbit aorta (2-3 mg/g tissue) was about 10 times that in adult and fetal bovine aorta. Rat and rabbit aorta also had about 2-3 times as much 20:4 as bovine aorta. If the total amounts of oxygenated esterified 18:2 metabolites are expressed as percentages of the total amounts of 18:2 in the tissue, similar values are obtained for aortae from adults of the

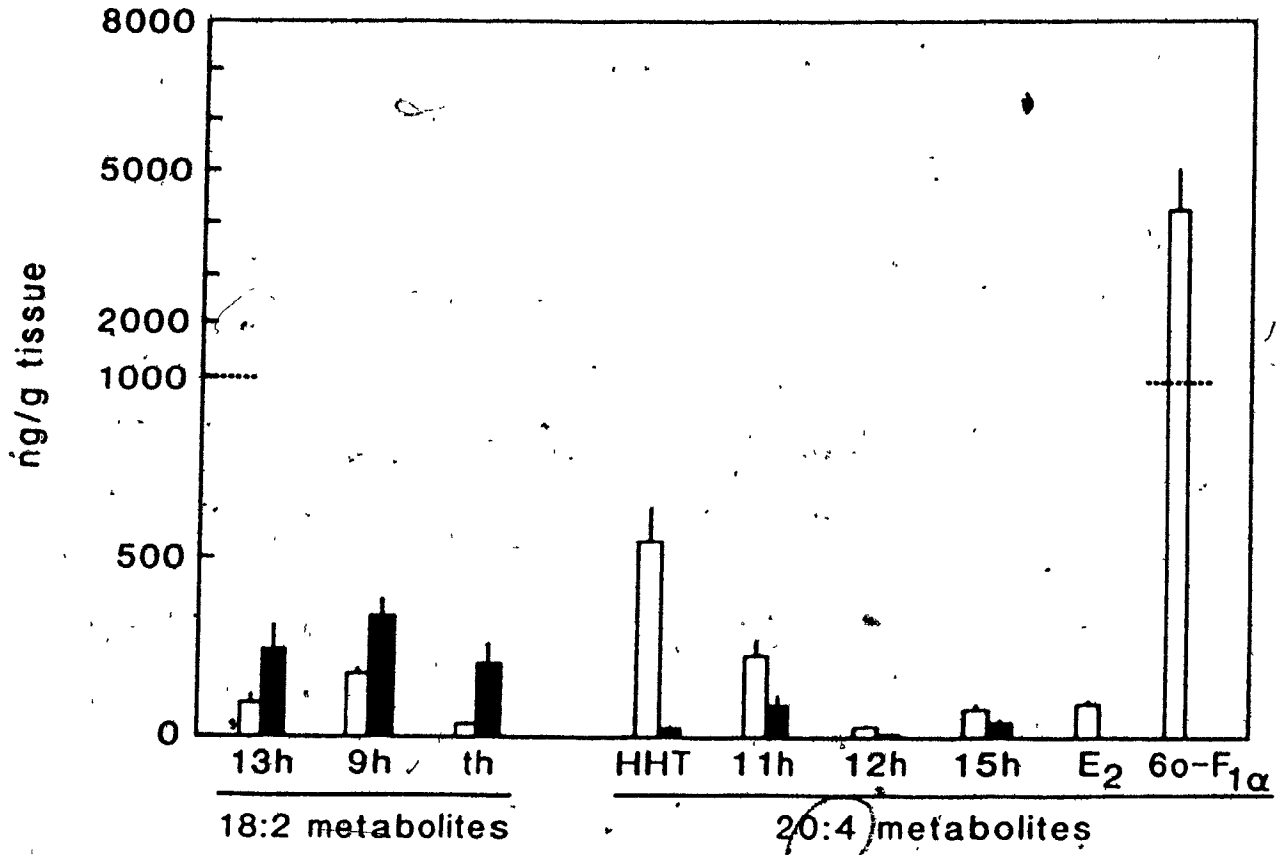

Fig.40. Amounts of oxygenated products of 18:2 and 20:4 synthesized by fetal calf ductus arteriosus. Incubations were carried out as described in the legend to Fig.36. Open bars represent released products and solid bars esterified products from fetal calf ductus arteriosus.

TABLE 8

Total Amounts of 18:2 and 20:4 and Their Esterified Oxygenation Products in Aorta and Ductus Arteriosus

Aortic lipids were extracted with chloroform:methanol. The material in the extract was hydrolyzed and 18:2 and 20:4 were quantitated by gas chromatography as shown in Fig.41, with arachidic acid (t_R, 12 min) as an internal standard. The values for 18:2 and 20:4 are means ±S.E.M. of determinations carried out on 4 different tissue samples. The values for h-18:2 (9h-18:2, 13h-18:2, and th-18:1) and h-20:4 (11h-20:4, 12h-20:4, and 15h-20:4) were calculated from the data in Figs. 36-40 for the esterified 18:2 and 20:4 metabolites. The numbers in brackets are the percentages of 18:2 and 20:4 which are found in the form of oxygenated metabolites.

	Product (ug/g tissue)			
Tissue	18:2	h-18:2+th-18:1	20:4	h-20:4
Rat aorta	2182±370	4.7 (0.21%)	1313±27	0.34 (0.026%)
Rabbit aorta	2813±820	4.8 (0.17%)	956±136	0.19 (0.020%)
Bovine				
-adult aorta (inner)	272±21	0.37 (0.14%)	428±35	0.086 (0.020%
-adult aorta (outer)	306±40	0.61 (0.20%)	407±38	0.12 (0.031%)
-fetal aorta	142±36	0.73 (0.51%)	423±72	0.22 (0.052%)
-ductus arteriosus	121±18	0.79 (0.65%)	415±98	0.17 (0.041%)

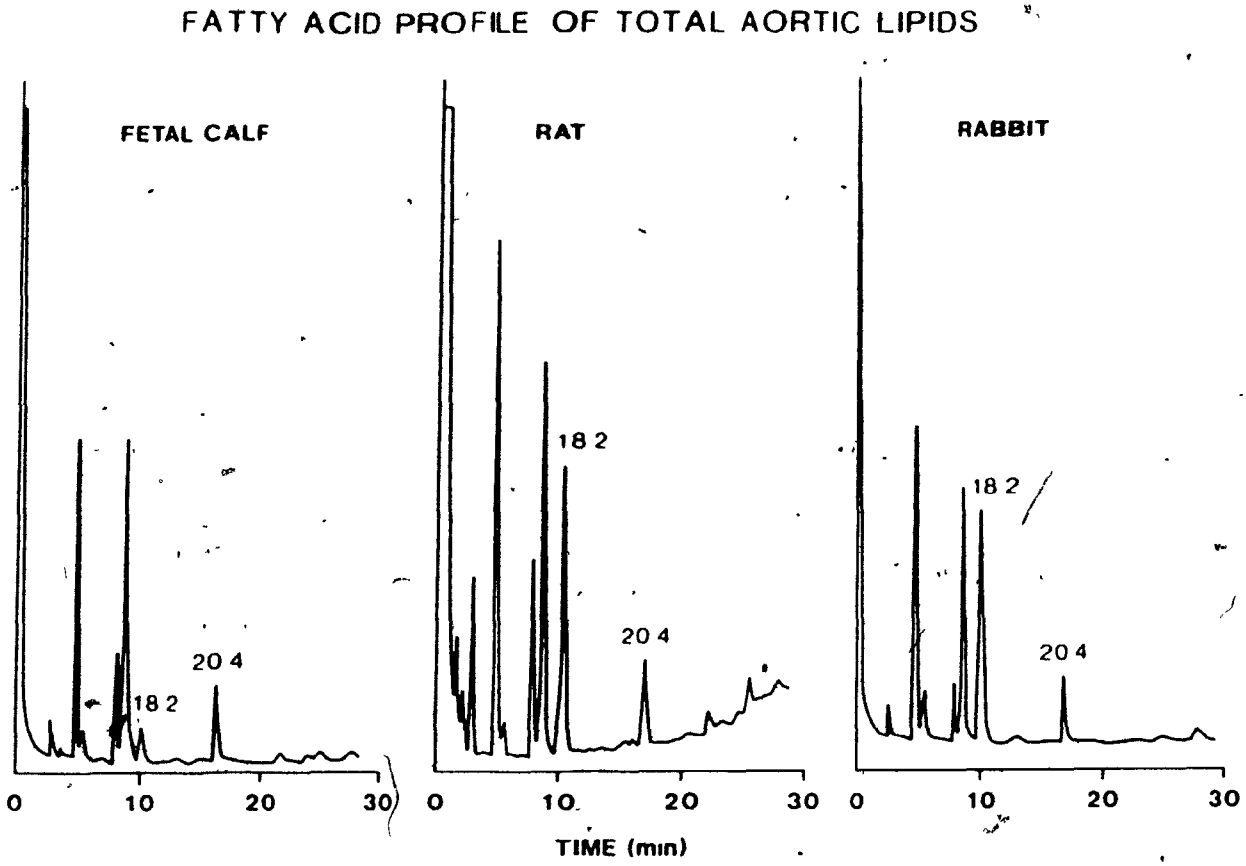

Fig.41. Fatty acid profile of total aortic lipids from fetal calf (left side), adult rat (centre), and adult rabbit (right side). Aortic lipids were extracted as described in the Methods section. An aliquot of the extract was analyzed by gas chromatography on a 10% Silar 10C column (2 x 0.015 m), employing a temperature program from 150 °C to 210 °C at 2 °C/min.

three species (0.14 to 0.21% of 18:2 oxidized). Similarly, the percentage of total aortic 20:4 which is found in the form of oxygenated, esterified metabolites is similar in all three species (about 0.02 to 0.03% of total 20:4). However, the percent of 20:4 in this form is much less than the percent of 18:2 which is oxygenated and esterified. The percentages of esterified oxygenated PUFA metabolites were about 2 to 3 times higher in fetal bovine aorta and ductus arteriosus than in adult aorta (Table 8).

3.3.9. Mechanism of Formation of Esterified Monohydroxy and Trihydroxy Products

The esterified mono- and tri-hydroxy metabolites could be formed either by direct oxygenation of lipids, or by incorporation of oxygenated products into lipids. For example, 5-hydroxy-6,8,11,14-eicosatetraenoic acid is rapidly incorporated into lipids in neutrophils (277). In order to determine whether this could also be the case for the h-18:2 and th-18:1 metabolites under investigation, both vascular endothelial and smooth muscle cells were incubated with 13h-[1-^{14}C]18:2 and th-[1-^{14}C]18:1 for 4 h at 37 °C. For comparison, similar incubations with [1-^{14}C]18:2 were carried out. The lipids were then extracted from the cells, separated into neutral and polar fractions, and analyzed by HPLC.

18:2 was well incorporated into both the neutral (Fig.42A) and polar lipid fractions from endothelial cells. The profile of neutral lipids into which 18:2 was incorporated consisted of various unidentified triglycerides (t_R's 25-33

Fig.42. Incorporation of 18:2 and 13h-18:2 into lipids from endothelial and smooth muscle cells. [1-^{14}C]18:2 (7.3 x 10^5 cpm) (A and C) and 13h-[1-^{14}C]18:2 (7.3 x 10^5 cpm) (B and D) were incubated for 4 h at 37 °C with cultured endothelial cells (A and B) or smooth muscle cells (C and D). The medium was removed and the cells were washed once with medium and the lipids were extracted as described in the Methods section. Neutral lipids were analyzed by reversed-phase HPLC using water:tetrahydrofuran (24:76) at a flow rate of 0.7 ml/min as the mobile phase. Polar lipids were analyzed by NP-HPLC with acetonitrile:methanol:sulfuric acid (100:6:0.05) at a flow rate of 1 ml/min as the mobile phase.

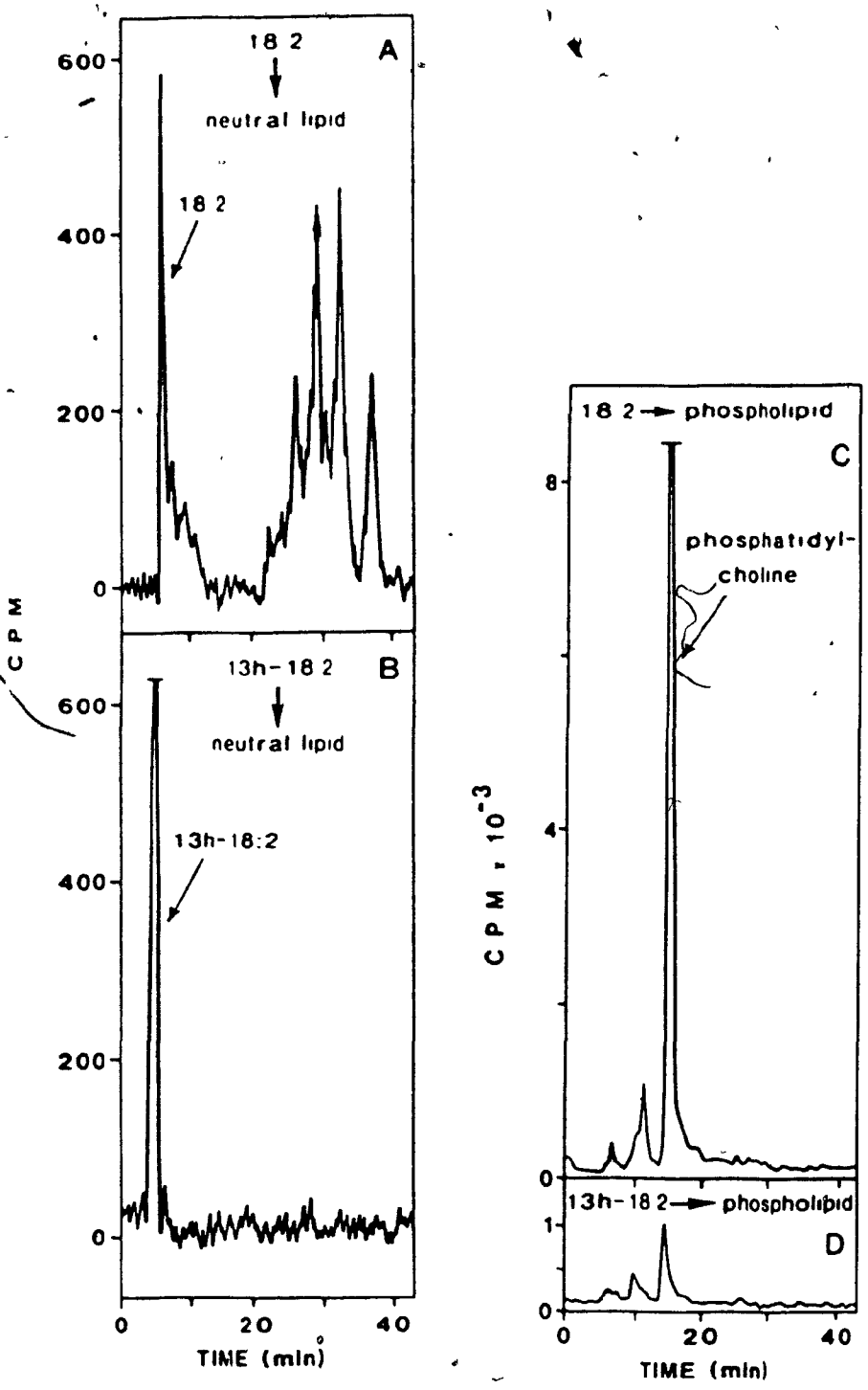
18 2
A
neutral lipid
18 2
600
400
200
0
CPM
13h-18 2
B
neutral lipid
13h-18:2
600
400
200
0
0
20
40
TIME (min)
18 2 → phospholipid
C
phosphatidyl-
choline
8
4
0
CPM x 10-3
13h-18 2 → phospholipid
D
1
0
0
20
40
TIME (min)

Fig. 42.

min) and cholesteryl linoleate (t_R, 38 min). Virtually no 13h-18:2 was incorporated into neutral (Fig.42B) or polar (not shown) lipid fractions from endothelial cells. Similar results were obtained with th-18:1 (data not shown).

Smooth muscle cells also incorporated 18:2 very well into the polar lipid fraction (Fig.42C). 18:2 was incorporated primarily into phosphatidylcholine (t_R, 17 min) as was the case for endothelial cells. A small amount of [^{14}C]18:2 was incorporated into a product with a t_R of 11 min, probably phosphatidylethanolamine, although this phospholipid was not completely resolved from phosphatidylserine. Incorporation of 18:2 into neutral lipids of smooth muscle cells was a very minor pathway under the conditions used (not shown). A small amount of 13h-[1-^{14}C]18:2 was incorporated into polar lipids (Fig.42D), but this was only about 11 % the amount of [1-^{14}C]18:2 incorporated into this fraction. There was no detectable incorporation of th-18:1 into lipids from smooth muscle cells (data not shown).

3.4. Effect of Selenium Deficiency on the Production of Oxygenated PUFA Metabolites

The synthesis of PGI_2, the major 20:4 metabolite in vascular tissue, can be inhibited by fatty acid hydroperoxides (174). Peroxidases are important enzymes involved in the reduction of these hydroperoxides to the corresponding hydroxy fatty acid, which do not affect PGI_2 formation. An impairment in peroxidase activity could result in inhibition of PGI_2 synthesis and might very well

be reflected in the accumulation of non-enzymatic decomposition products of hydroperoxides, such as th-18:1.

Glutathione peroxidase, one of these peroxidase enzymes, catalyzes the breakdown of hydrogen peroxide and a wide variety of organic hydroperoxides (278). Reduced glutathione is its specific hydrogen donor substrate, being continuously supplied by glutathione reductase and the hexose monophosphate shunt (110). Selenium is a tightly bound, integral component of one form of this enzyme with a stoichiometry of 4 g-atoms selenium/mol of glutathione peroxidase (259). The availability of this trace element, therefore, determines the activity of this form of glutathione peroxidase.

In order to test the hypothesis that a reduction in the ability of blood vessels to metabolize fatty acid hydroperoxides via glutathione peroxidase might result in decreased PGI_2 synthesis, the effects of a selenium-deficient diet on the synthesis of free and esterified oxygenation products of PUFA by rat and rabbit aorta was investigated. Male Sprague-Dawley weanling rats and male New Zealand White rabbits (6-8 weeks of age) were fed a selenium-deficient basal diet or a diet supplemented with 0.5 ppm selenium for 5 to 7 weeks. After this time period, animals were sacrificed and aortic glutathione peroxidase activity, as well as the production of oxygenated PUFA metabolites, were measured. There was no significant difference in weight gain between the control and selenium-deficient groups during the study (Table 9).

Aortic glutathione peroxidase activity was virtually absent in the selenium-deficient group of rats (Table 9). On the other hand, rabbit aortic glutathione peroxidase activity was inhibited by only 44 % in the selenium-deficient group when compared to controls after 7 weeks of feeding.

Figs.43 and 44 show the amounts of released and esterified oxygenated PUFA metabolites produced by control and selenium-deficient rat and rabbit aortae, respectively. The profile of products released from rat aorta (Fig.43; open and cross-hatched bars) is very much similar to what was presented in Fig.36. The synthesis of 6-oxo$PGF_{1\alpha}$, the major oxygenated PUFA metabolite, was marginally reduced in the selenium-deficient group (5300 ng/g tissue; cross-hatched bars) when compared to the control group (5800 ng/g tissue; open bars). This difference was not statistically significant, though. The other oxygenated metabolites of 18:2 and 20:4 released by slices of rat aorta were not affected by selenium deficiency. The profile of esterified products (shaded and striped bars) is somewhat different from that shown in Fig.36. However, there was no statistically significant difference in the amounts of esterified products between the control (shaded bars) and selenium-deficient (striped bars) groups.

The profile of released (open and cross-hatched bars) and esterified (shaded and striped bars) oxygenated PUFA metabolites from rabbit aorta (Fig.44) is essentially the

TABLE 9

Body Weights and Aortic Glutathione Peroxidase Activities of Control and Selenium-Deficient Rats and Rabbits

Rats and rabbits were fed a control or selenium-deficient diet (see Table 1) for 5-7 weeks, after which time aortic glutathione peroxidase activity (nmol NADPH oxidized/min/mg protein) was measured as described in the Methods section. Rat weights are in grams and rabbit weights are in kilograms. The values are means ± S.E.M.

	Initial Body Weight	Final Body Weight	GSH Peroxidase Activity
RAT			
Control	58±2 (n=17)	174±12 (n=17)	72.4±8.1 (n=7)
Selenium-Deficient	59±2 (n=18)	180±13 (n=18)	1.8±0.7 (n=7)
RABBIT			
Control	1.44±0.07 (n=6)	2.57±0.08 (n=6)	86.6±11.6 (n=6)
Selenium-Deficient	1.49±0.06 (n=6)	2.50±0.13 (n=6)	48.4±4.7 (n=6)

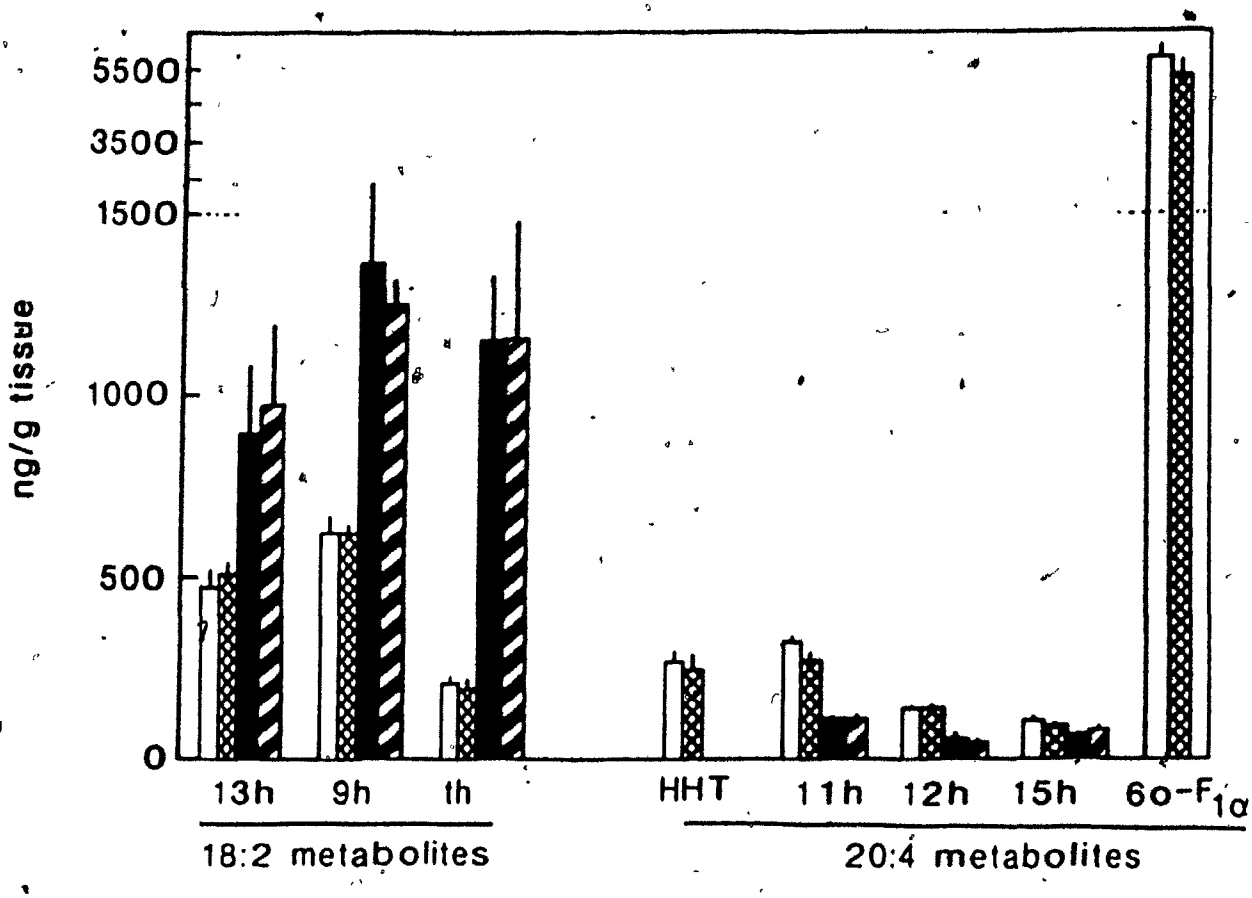

Fig.43. Amounts of oxygenated products of 18:2 and 20:4 synthesized by rat aorta of rats fed control and selenium-deficient diets. Incubations were carried out as described in the legend to Fig.36. Products released from aortae of control (open bars) and selenium-deficient (cross-hatched bars) rats. Esterified products from aortae of control (solid bars) and selenium-deficient (striped bars) rats are also shown.

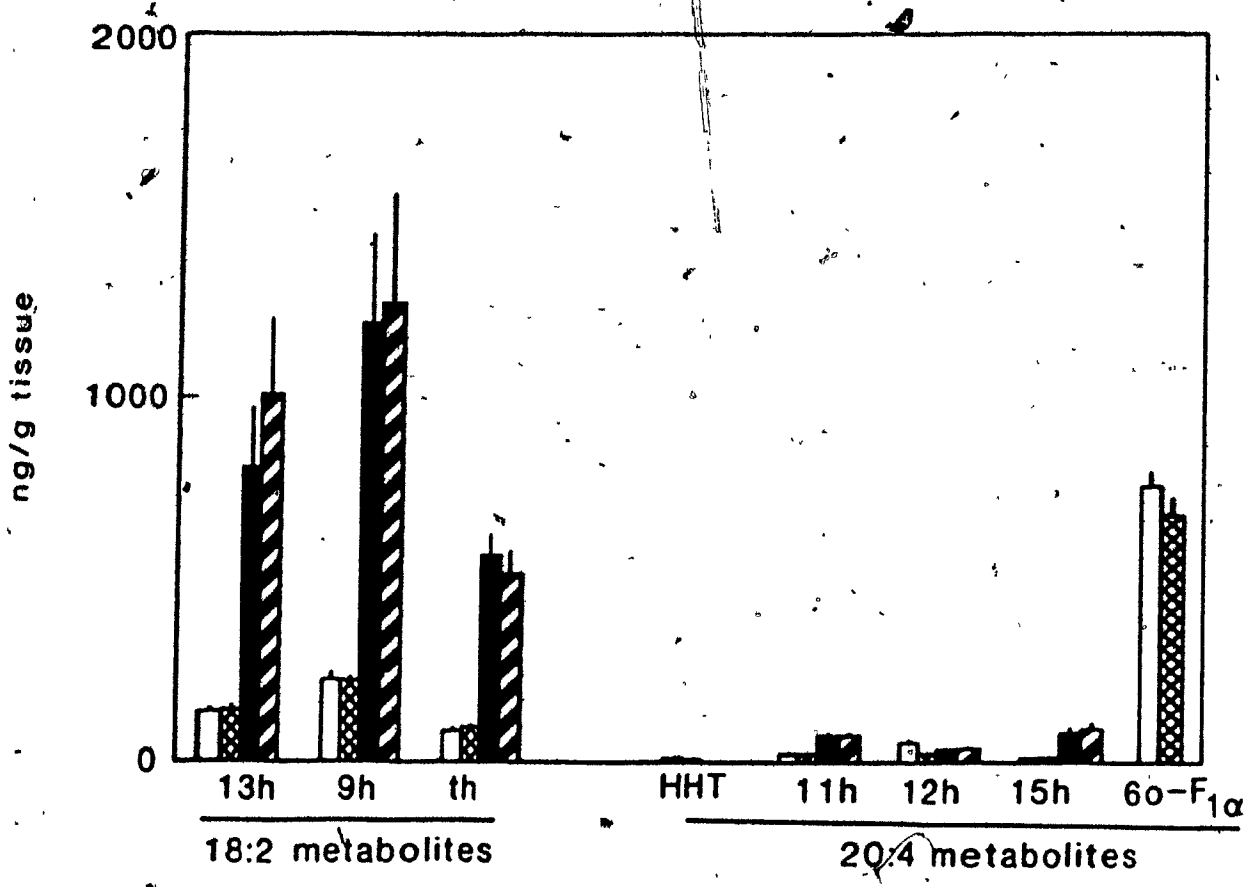

Fig.44. Amounts of oxygenated products of 18:2 and 20:4 synthesized by rabbit aorta of rabbits fed control and selenium-deficient diets. Incubations were carried out as described in the legend to Fig.36. Products released from aortae of control (open bars) and selenium-deficient (cross-hatched bars) rabbits. Esterified products from aorta of control (solid bars) and selenium-deficient (striped bars) rabbits are also shown.

same as presented in Fig.37. 6-oxoPGF$_{1\alpha}$ synthesis was reduced by 10 % (not statistically significant; T-test) in the selenium-deficient group (677 ng/g tissue; cross-hatched bars) when compared to the control group (756 ng/g tissue; open bars). All other released products, with the exception of the minor lipoxygenase product, 12h-20:4, were not affected by selenium deficiency. The latter product was 57 % lower in the selenium-deficient group when compared to the control group. The amounts of esterified products of rabbit aorta from the control group (shaded bars) were not statistically different from the selenium-deficient group (striped bars).

DISCUSSION

PGI_2 synthesis by vascular tissue is important in preventing or limiting the formation of thrombi and may be partially responsible for the thromboresistant properties of vascular endothelium (95). The control of its synthesis is, therefore, an essential requirement. Hydroperoxy PUFA can alter the activities of the enzymes that synthesize PGI_2 from 20:4 by vascular tissue (174). Although fairly extensive knowledge of 20:4 metabolism existed at the onset of this study (1980), much less was known about the metabolism of other PUFA to hydroperoxides. At that time a report appeared in the literature demonstrating that 18:2 could inhibit PGI_2 formation in cultured endothelial cells supplemented with 18:2 (178). This effect could have been due to competition with 20:4 for PG endoperoxide synthase or possibly to inhibitory hydroperoxy metabolites of 18:2, synthesized via lipoxygenase or PG endoperoxide synthase. Since 18:2 is found in cellular lipids in quantities similar or exceeding those of 20:4, we decided to investigate its metabolism in vascular tissue with a view of clarifying its inhibitory effect on PGI_2 formation.

4.1. Metabolism of 18:2 by Particulate Fractions from Bovine Aorta

Our study demonstrated that 18:2 is metabolized to oxo, monohydroxy, epoxyhydroxy and trihydroxy products by particulate fractions from aorta. All of these substances are derived from the putative intermediates, 9hp-18:2 and

13hp-18:2, which are synthesized from 18:2 by PG endoperoxide synthase (Fig.22). The major pathway of metabolism of the hydroperoxy intermediates is reduction to the corresponding hydroxy compounds. This step may be catalyzed by the peroxidase component of PG endoperoxide synthase or, by enzymes present in the cytosolic fraction, as discussed below. However, it was observed that this reduction step could take place even in the presence of boiled particulate fraction (section 3.2.5.(b), Fig.26B), indicating that it could be catalyzed by a factor in aorta which is not an enzyme (cf. factor in uterus responsible for reduction of PGH_2 to $PGF_{2\alpha}$; section 1.4.2.(c)).

4.2. Monohydroxy 18:2 Metabolites from Other Tissues

Linoleic acid is also converted to monohydroxy metabolites by some other mammalian tissues. Microsomal fractions from sheep seminal vesicles (78) and homogenates of VX_2 carcinoma tissues (272) convert 18:2 to 9h-18:2 along with a smaller amount of 13h-18:2. In the case of VX_2 carcinoma tissue homogenates, the formation of the two monohydroxy compounds was inhibited by indomethacin in a manner similar to PGE_2, suggesting that PG endoperoxide synthase was responsible for their formation. Rabbit peritoneal tissue has also been reported to convert 18:2 to a mixture of 9h-18:2 and 13h-18:2 (279,280). Only the formation of 9h-18:2 was clearly dependent on PG endoperoxide synthase activity. Lipoxygenase(s) appeared to be partly responsible for the formation of 13h-18:2 (280).

13h-18:2 was the major product formed by porcine leukocytes incubated with exogenous 18:2 (281), whereas 9h-18:2 did not appear to be formed. Both 13h-18:2 and 9h-18:2, with the former product predominating, have been found in psoriatic scale extracts (282).

The biological activity of the monohydroxy 18:2 products is unknown. They exhibit no chemokinetic activity as do some lipoxygenase metabolites of 20:4, such as 5-HETE and LTB_4 (151). Perhaps their hydroperoxy precursors possess biological activity.

4.3. Epoxyhydroxy PUFA Metabolites

In addition to the formation of monohydroxy metabolites, 9hp-18:2 and 13hp-18:2 can be converted by aorta to epoxyhydroxy products. This conversion may not be catalyzed enzymatically, since epoxyhydroxy products were formed after incubation of 13hp-18:2 with a boiled particulate fraction from fetal calf aorta (Fig.26B) and these products have been reported to be formed non-enzymatically by a number of systems, such as ferrous ions (273) and hemoglobin (271). The latter agent converts 13hp-18:2 mainly to 12,13e-9h-18:1 and 12,13e-11h-18:1 (271).

The mechanism of formation of these epoxyhydroxy products from their hydroperoxy precursors was not investigated. This would have required detailed studies employing labeled oxygen. However, a mechanism has recently been proposed that provides a rationale for the conversion of fatty acid hydroperoxides to such products in mammalian tissues. Dix

and Marnett (283) have studied the reaction of fatty acid hydroperoxides with hematin, the prosthetic group of prostaglandin endoperoxide synthase, which has peroxidase activity. They reported that hematin catalyzes the rearrangement of 13hp-18:2 to 12,13e-9h-18:1 and 12,13e-11h-18:1, in which both the hydroxyl and epoxide oxygens are derived from the hydroperoxide group. The apparent mechanism for this transformation involves an oxygen rebound whereby the iron-containing hematin complex reduces the hydroperoxide group to an alkoxyl radical and transfers one of the hydroperoxy oxygens to an intermediate carbon-centred radical generated by alkoxyl radical cyclization (Fig.45). This mechanism has been invoked to explain the formation of epoxyhydroxy products derived from 20:4 and 12hp-20:4 formed by subcellular fractions of rat lung (284).

We have also shown that epoxyhydroxy compounds are formed from 20:3 by aorta. Fetal calf aorta converted 20:3 to two stereoisomers of 11,12e-10h-17:1, which were isolated and identified by GC-MS.

The biological activity of the various epoxyhydroxy products synthesized by aorta is unknown. Arachidonic acid epoxides formed via the epoxygenase pathway are selective stimuli for the in vitro release of such peptide hormones as luteinizing hormone (285), somatostatin (286), insulin and glucagon (287). However, these epoxygenase products lack the additional hydroxyl group. 11,12e-8h-20:3 (termed Hepoxilin A) and 11,12e-10h-20:3 (Hepoxilin B) are formed

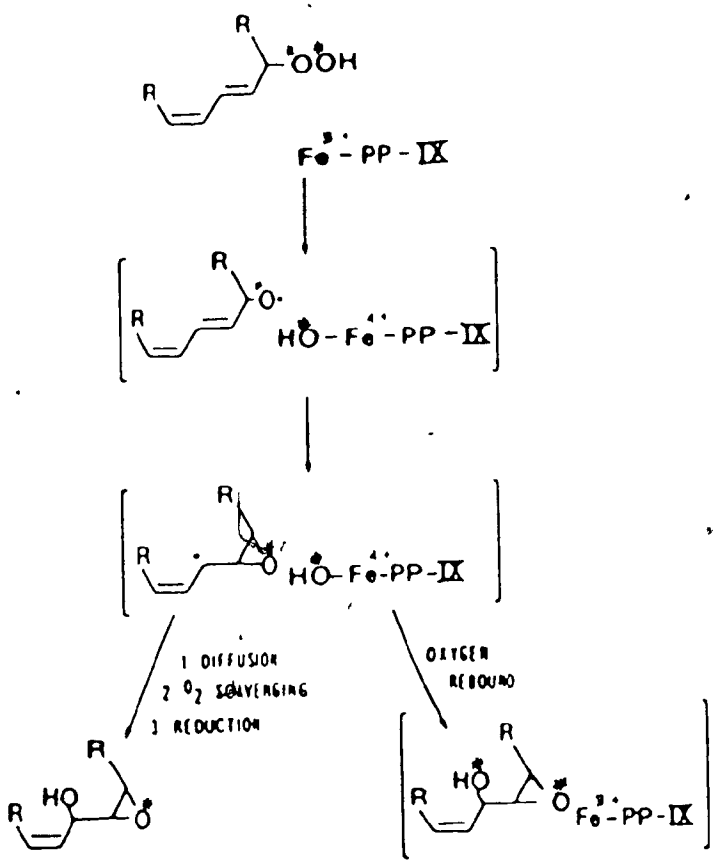

Fig.45. Scheme of the proposed mechanism for the hematin catalyzed conversion of hydroperoxy fatty acids to epoxyhydroxy fatty acids (ref. 283).

from 12hp-20:4 by rat pancreatic islets (288). Hepoxilin A dose dependently enhanced the release of insulin during glucose (10 mM) stimulation of perifused islets (120 ± 51 % at 0.5 x 10^{-6} M and 282 ± 58 % at 2.1 x 10^{-6} M).

4.4. Trihydroxy PUFA Metabolites

9hp-18:2 and 13hp-18:2 can be converted by aorta to trihydroxy products via epoxyhydroxy intermediates (Fig.22). This pathway becomes significant at high substrate concentrations (Fig.28) or in the presence of other polyunsaturated fatty acids, such as 20:4 (section 3.2.7., Fig.30). Hydroperoxides derived from 18:2 and 18:3 are also converted to trihydroxy products by various plant lipoxygenases. Soybean lipoxygenase in the presence of guaiacol has been reported to convert a mixture of 13hp-18:2 and 9hp-18:2 to 9,10,13th-18:1 and 9,12,13th-18:1, along with a number of other products (263) (see Fig.19C). 18:2 and 18:3 are converted by enzymes in wheat flour principally to their 9,12,13-trihydroxy metabolites, via the intermediate 9-hydroperoxides (274) (Fig.19A). Negligible amounts of the 9,10,13-trihydroxy isomers are formed (Table 4). The major products isolated after incubation of a 4:1 mixture of 13hp-18:2 and 9hp-18:2 with ferric chloride/cysteine were 9,12,13th-18:1 and 9,10,13th-18:1 in a ratio of 3:2 (273).

Although, as in wheat flour, 9hp-18:2 is the major initial product of 18:2 metabolism by aorta, the major trihydroxy product is 9,10,13th-18:1, which is accompanied

by a smaller amount of 9,12,13th-18:1. The ratio of 9,10,13th-18:1 to 9,12,13th-18:1 (2.7:1) is similar to that of 9h-18:2 to 13h-18:2 (see Fig.12), raising the possibility that 9hp-18:2 is the precursor of the 9,10,13-trihydroxy isomer, whereas 13hp-18:2 is the precursor of the 9,12,13-trihydroxy isomer.

We isolated 3 stereoisomers of each of 9,10,13th-18:1 and 9,12,13th-18:1 (section 3.2.1.(a) fractions f and g). The 9- and 13-hydroperoxy precursors of these compounds are probably each mixtures of D and L isomers, since Hamberg and Samuelsson (289) found that PG endoperoxide synthase from sheep seminal vesicles converts 18:2 to a 4:1 mixture of the L and D forms, respectively, of 9h-18:2 and 13h-18:2. Addition of oxygen to the hydroperoxides could give a total of four possible diastereoisomers for each epoxyhydroxy product. Hydrolysis of the epoxide could yield additional stereoisomers.

It is not clear whether all the steps in the formation of trihydroxy metabolites from polyunsaturated fatty acids in aorta are enzymatically catalyzed. The mixture of stereo and positional isomers observed for the trihydroxy metabolites of 18:2 could be interpreted as an indication of at least some non-enzymatic steps. However, as discussed above, it must be kept in mind that the first step in the metabolism of 18:2, which is catalyzed by PG endoperoxide synthase may result in the formation of four isomeric products (i.e. the D and L forms of 9h-18:2 and 13h-18:2).

In a recent report, isomers of 9,10,13th-18:1 and

9,12,13th-18:1 were isolated from onion bulbs and were found to exhibit prostaglandin-E like activity, detected as vascular smooth muscle relaxing and non-vascular smooth muscle stimulating activity in a cascade superfusion system (291). These products, though, were three orders of magnitude less potent than PGE_2. They did not inhibit platelet aggregation in concentrations up to 10 µg/ml. It may very well be that trihydroxy PUFA products represent decomposition products of their hydroperoxy precursors and they may serve no special cellular function.

Incubation of human platelets with 20:4 (111-113) or 20:3 (290) also has been reported to give rise to epoxyhydroxy and trihydroxy products via 12hp-20:4 and 12hp-20:3, respectively. Rabbit polymorphonuclear leukocytes convert 20:4 to epoxyhydroxy and trihydroxy metabolites of 15hp-20:4, which is formed by a lipoxygenase in these cells (130). Therefore, in addition to its frequent occurence in plants, the conversion of fatty acid hydroperoxides to epoxyhydroxy and trihydroxy products also seems to be a common feature of many mammalian cell types.

4.5. Metabolism of Epoxyhydroxy Products to Trihydroxy Products

The two stereoisomers of 11,12e-10h-17:1, formed from 20:3 by aorta, were shown to be enzymatically converted to 10,11,12th-17:1 (Fig.10). Since there are two stereoisomers of the epoxyhydroxy compound, one would expect at least two stereoisomers of the corresponding trihydroxy product.

Presumably the chromatographic system used in this study was incapable of resolving these isomers, since we only obtained a single broad peak using isocratic conditions. 9,10e-11h-18:1 was converted to 9,10,11th-18:1 by an epoxide hydrolase in a manner similar to 11,12e-10h-17:1.

The rates of metabolism of 11,12e-10h-17:1 and 9,10e-11h-18:1 by fetal calf aorta are relatively slow compared to the rates of metabolism of similar compounds (e.g. 11,12-epoxy-5,8,14-eicosatrienoic acid and 14,15-epoxy-5,8,11-eicosatrienoic acid) by microsomal fractions from rabbit liver and renal cortex (142). This could be due to the proximity of the hydroxyl substituent, which could interfere with the access of the epoxy group to the active site of the enzyme. It is not clear whether the other epoxyhydroxy intermediates, 9,10e-13h-18:1 and 12,13e-9h-18:1 (see Fig.22), are converted enzymatically to the corresponding trihydroxy products. These allylic epoxides may undergo rapid non-enzymatic solvolysis. On the other hand, 12,13e-9h-18:1 was the main product isolated after incubation of 13hp-18:2 with hemoglobin in buffer, while very little 9,12,13th-18:1 was formed (271). This suggests that 12,13e-9h-18:1 is fairly stable chemically, and that our failure to isolate the two allylic epoxide intermediates could be due to their rapid metabolism by an epoxide hydrolase. These substances could be metabolized at a faster rate than 9,10e-11h-18:1, since their hydroxyl groups are further away from the epoxide group, permitting a greater degree of interaction with the enzyme.

The partially purified epoxide hydrolase from rat lung cytosol was able to completely convert both 11,12e-10h-20:3 and 11,12e-8h-20:3 to the corresponding trihydroxy products in 20 min (284). This enzyme may, therefore, be more active or may be found in larger amounts in the lung than in aorta, although direct conclusions cannot be formulated since the extent of purification of the enzyme upon ammonium sulfate precipitation was not given.

Current data suggest that epoxide hydrolases in nuclear and microsomal liver fractions are similar (292). Mitochondrial and cytosolic liver epoxide hydrolase activities differ from that found in microsomal fractions with respect to pH optima and substrate specificity (see 293). However, both the microsomal and cytosolic epoxide hydrolase enzymes exhibit a fairly broad substrate specificity (293,294). It is quite likely, then, that the aortic cytosol and microsomal epoxide hydrolase activities display a wide range of substrate specificity and represent different forms of the enzyme.

Epoxide hydrolases seem to operate by a common mechanism (295). They generally display low enantioselectivity and high regiospecificity. Their action involves specific activation of water, probably by means of general base catalysis, followed by nucleophilic attack of hydroxide at the less hindered epoxide carbon, giving rise to the threo 1,2-diol. Elucidation of the mechanism of action of the aortic epoxide hydrolase enzyme was not possible since

we did not investigate the stereochemistry of the epoxyhydroxy and trihydroxy products, or study the incorporation of labeled oxygen into the epoxyhydroxy metabolites.

Liver microsomal epoxide hydrolases are thought to serve a protective function by converting chemically reactive epoxides formed in situ by cytochrome P-450 monooxygenases to less toxic 1,2-diols (296). Without this conversion these epoxides are capable of irreversibly binding to nucleophilic sites present in tissue macromolecules and may initiate toxic or carcinogenic reactions. It is possible that the aortic enzyme(s) may serve a similar function on blood-borne epoxides or epoxides formed locally within the aorta.

4.6. Effects of 18:2 on Prostacyclin Production.

It was originally speculated that 18:2 or its hydroperoxy metabolites might interfere with the synthesis of PGI_2 by aorta (cf. ref. 178,179) but this was not supported by competition experiments in the present study (Fig.30). It is possible that if the metabolism of 9hp-18:2 and 13hp-18:2 to monohydroxy and epoxyhydroxy products is diminished in some circumstances these intermediates could accumulate, resulting in inhibition of PG endoperoxide synthase or prostacyclin synthase. However, the rate of oxygenation of 18:2 is much slower than that of 20:4 (Fig.21), so that hydroperoxy intermediates such as PGG_2 and 15-hydroperoxyPGI_2 (254), derived from 20:4 itself, might be more important in regulating PGI_2 synthesis by vascular

tissue. The inhibitory effect of 18:2 on PGI_2 formation observed in cultured cells may not be inconsistent with our data carried out with particulate fractions. An intact cellular structure may be required to observe this inhibition. 18:2 could alter the lipid micro-environment of membrane-bound cyclooxygenase and/or prostacyclin synthase, thus affecting their activities.

4.7. Oxygenated PUFA Metabolites Synthesized by Vascular Slices

Once the major pathways of PUFA metabolism by aorta and ductus arteriosus had been elucidated, the next step was to quantitate the major PUFA metabolites synthesized from endogenous substrate by vascular tissue. A method was therefore developed to measure the amounts of these products synthesized by slices of blood vessels incubated in physiological medium. After preliminary investigations to screen for the feasibility of quantitating various monohydroxy and trihydroxy PUFA products and prostaglandins a GC-MS assay with selected ion monitoring was established to measure some of the major PUFA products.

Routinely, 9 products were quantitated. Three were derived from 18:2 (9h-18:2, 13h-18:2 and th-18:1) and six were derived from 20:4 (12h-17:3, 11h-20:4, 12h-20:4, 15h-20:4, PGE_2 and 6-oxo$PGF_{1\alpha}$). The production of these 18:2 and 20:4 metabolites by aorta was investigated in three different species: rat, rabbit and bovine. Differences in the production of these products in fetal and adult bovine aorta were also investigated.

4.7.1. 20:4 Metabolites

Although there were considerable quantitative differences between species, the major oxygenated PUFA metabolite released by aorta was, in all cases, 6-oxo$PGF_{1\alpha}$. Rat aorta produced very large amounts of this product, whereas rabbit aorta released only about one-tenth as much. Adult bovine aorta produced an intermediate amount of 6-oxo$PGF_{1\alpha}$. Much smaller amounts of PGE_2 and 12h-17:3 were detected in all cases.

Fetal calf aorta was much more active than adult bovine aorta in producing 6-oxo$PGF_{1\alpha}$ and other cyclooxygenase products. This cannot be explained by a higher ratio of endothelial cells to tissue weight for fetal aorta, since the outer layer (outer media + adventitia) of fetal aorta also had much higher cyclooxygenase activity than the outer layer of adult aorta. It is possible that the increased production of PGI_2 could serve some special function in the fetus, for example, decreasing pulmonary vascular resistance.

There was very little difference between the amounts of oxygenated 18:2 and 20:4 metabolites formed by fetal calf aorta and the ductus arteriosus. The ductus arteriosus produced about 4200 ng 6-oxo$PGF_{1\alpha}$/g tissue, compared to only about 100 ng PGE_2/g tissue. This relatively small amount of PGE_2 could be quite important, however, since PGE_2 has very potent vasodilatory effects on the ductus, and appears to be necessary for maintaining its patency during fetal life (234). PGI_2, on the other hand, is a much weaker

vasodilator with respect to the ductus, and is over 1000 times less potent than PGE_2, at least in the sheep (235).

The major monohydroxy 20:4 products released by slices of aorta and ductus arteriosus were 11h-20:4 and 15h-20:4, which were previously identified, but not quantitated, after incubation of slices of fetal calf aorta in physiological medium in the absence of exogenous substrate (254). These products have also been identified after incubation of cultured smooth muscle cells from rat (297) and rabbit (298) aorta with exogenous arachidonic acid. Incubation of rabbit aorta smooth muscle cells with 20:4 also led to the formation of 5, 8, 9, and 12 monohydroxy metabolites (298). We were able to detect little or none of the former three products after incubation of rabbit aorta in physiological medium. In the three species we investigated, 12-lipoxygenase activity, although it was always detectable, was very low. This could still be important, however, since 12h-20:4 has been reported to be an extremely potent chemokinetic agent for vascular smooth muscle cells (299).

4.7.2. 20:3 Metabolites

Aorta formed only small amounts of 20:3 metabolites. The major product formed from exogenous 20:3 by particulate fractions from adult and fetal bovine aorta was 12h-17:2 (256). Epoxyhydroxy and trihydroxy metabolites derived from 12hp-17:2 were also formed, but only small amounts of PGE_1 and $PGF_{1\alpha}$ were detected (256). In preliminary experiments only small amounts (2-3 ng/g tissue) of 8,11,12th-17:1 from

fetal calf aorta could be detected. Larger amounts of 12h-17:2 (ca. 150 ng/g tissue for fetal calf aorta) were formed, but in subsequent experiments, the combined amounts of this product and 12h-17:3 were measured.

4.7.3. 18:2 Metabolites

(a) Released Products

Substantial amounts of the 18:2 metabolites (9h-18:2, 13h-18:2 and th-18:1) were found to be released from aorta, especially from rat and rabbit aortae, which contain large amounts of 18:2. In contrast to particulate fractions, PG endoperoxide synthase was not required for the formation of these products, since their formation was not inhibited by indomethacin. It is possible that they could have been formed by a lipoxygenase from free 18:2. However, lipoxygenases are quite specific in their formation of a particular isomer and in these studies approximately equal amounts of both the 9- and 13- isomers were produced. Alternatively, the 18:2 metabolites could have been released by the hydrolysis of lipids containing oxidized forms.

The 12-hydroxystearate detected in the hydrogenated free and esterified fractions from aorta could have arisen from 12-hydroxy-9,13-octadecadienoic acid (12h-18:2), which is a singlet oxygen derived metabolite of 18:2 (300). Alternatively, 12-hydroxystearate itself could have been present in aorta. Various hydroxystearate isomers, synthesized by microoganisms in the gut, have been detected in human fecal lipids (301). Another possible source is

ricinoleic acid (12-hydroxy-9-octadecenoic acid), which is the major fatty acid in castor bean seeds, and has been shown to be incorporated into triglycerides from rat fat pads after administration of castor oil (302). A product which gives rise to 12-hydroxystearate after hydrogenation has also been reported to be released from rabbit peritoneal tissue (281). Although this product was assumed to have been derived from 12h-18:2, this was not supported by any experimental evidence.

(b) Esterified Products

The major proportion of oxygenated 18:2 metabolites were present in the esterified form in lipids. These oxygenated products did not appear to be synthesized to a large extent during the 20 min incubations, but were present in the tissue prior to incubation, as shown in Table 7. Although the absolute amounts of 9h-18:2, 13h-18:2, and th-18:1 varied considerably from one species to another, they represented about the same proportion (ca. 0.2%) of total 18:2 in aorta from adults of all three species. 20:4 metabolites (11h-20:4, 12h-20:4, and 15h-20:4) were also present in aortic lipids, but in much smaller quantities, which, in adult aortae, amounted to only about 0.02-0.03% of total aortic 20:4. This could possibly be due to different distributions of 18:2 and 20:4 in different lipid fractions. We have not as yet determined the type of lipid which is the source of the oxygenated PUFA metabolites. Cholesteryl hydroxylinoleate has previously been identified in samples

of atherosclerotic human aorta (303). It was not detected in normal aorta, however, but the method used was not very sensitive. Esterified hydroxy-PUFA have also been detected by HPLC in liver phospholipids after treatment of mice with carbon tetrachloride (304).

There are several possible mechanisms for the formation of the esterified oxygenated PUFA. One possibility could be that free 18:2 and 20:4 were oxygenated and then incorporated into lipids in a manner similar to the incorporation of 5h-20:4 into lipids in leukocytes (277). Free 13h-18:2 and 9h-18:1 were not well incorporated into either vascular endothelial or smooth muscle cells, however, so it seems much more likely that it was the esterified fatty acids which were oxygenated. These oxygenated lipids are unlikely to have arisen by the action of cyclooxygenase, since they are not substrates for this enzyme (305). Moreover, indomethacin did not affect their formation. The oxygenated products could have accumulated in lipids either due to autoxidation in vivo, or to the action of an enzyme similar to the lipoxygenase in reticulocytes (306) which has been reported to convert esterified 18:2 and 20:4 in rat liver mitoplasts to monohydroxy and trihydroxy metabolites (307).

The precursors of the monohydroxy and trihydroxy lipids in aorta are presumably hydroperoxy lipids, which may be enzymatically or non-enzymatically reduced to the corresponding hydroxy lipids. If they are not reduced

rapidly enough, they could have toxic effects, and could inhibit PGI_2 formation, as do hydroperoxy fatty acids and their methyl esters (174). As well as being reduced to hydroxy lipids, the lipid hydroperoxides could be converted nonenzymatically to epoxyhydroxy products. In situations where lipid hydroperoxides may accumulate, as they may do in atherosclerosis (228), there could be an increase in the relative amounts of the trihydroxy metabolites of these epoxyhydroxy compounds.

4.8. Reduction of Hydroperoxides

The cell has developed a host of factors to defend against harmful lipid hydroperoxides. As was discussed earlier, selenium-dependent glutathione peroxidase catalyzes the reduction of hydroperoxides to their corresponding hydroxy derivatives. This enzyme resides in the cytosolic fraction and acts upon a wide variety of free organic hydroperoxides, but is not reactive towards phospholipid hydroperoxides (308). Another selenium-dependent glutathione peroxidase, recently isolated from pig liver (309), heart (310) and brain (311), acts upon these latter products, and has been termed "peroxidation inhibiting protein" (PIP) or "phospholipid hydroperoxide" glutathione peroxidase (312). The amount of phospholipid hydroperoxide reduced by PIP is stoichiometrically equivalent to the amount of oxidized glutathione that is formed (309). Non-selenium dependent glutathione peroxidase is present in rat liver cytosolic fractions (313). This activity has been

identified as two glutathione transferases (B and AA) (314). Non-selenium dependent glutathione peroxidase activity has also been demonstrated in rabbit liver (315) and kidneys from rat (313) and rabbit (315). In contrast to the selenium-dependent form, non-selenium dependent glutathione peroxidase only reduces organic hydroperoxides and shows little activity towards hydrogen peroxide (316).

The peroxidase component of PG endoperoxide synthase, localized in the endoplasmic reticulum, catalyzes the reduction of PGG_2 to PGH_2. This enzyme could also be very important in reducing other PUFA hydroperoxides.

Other important cellular agents, such as vitamin E, ascorbic acid and glutathione, by way of their antioxidant action, influence hydroperoxide levels. Superoxide dismutase and catalase will have a role to play, as well.

4.9. Effect of Selenium Deficiency on the Production of Oxygenated PUFA Metabolites by Aorta Slices

Fatty acid hydroperoxides formed during the metabolism of PUFA by aorta, if not rapidly metabolized, inhibit PGI_2 production. A reduction in the capability of vascular tissue to reduce hydroperoxy PUFA could result in decreased synthesis of PGI_2, and increased metabolism of hydroperoxides via the epoxyhydroxy-trihydroxy pathway. As mentioned above, one mechanism of reduction of cellular hydroperoxy PUFA is by selenium-dependent glutathione peroxidase. We attempted to determine whether a deficiency in this enzyme would result in diminished vascular synthesis of PGI_2.

Aortic glutathione peroxidase activity was virtually completely inhibited in rats after 5-7 weeks on a selenium-deficient diet (Table 9). This degree of inhibition is in agreement with studies from other groups (112,317), who found marked inhibition of glutathione peroxidase activity in platelets (112), liver (317) and erythrocytes (317). On the other hand, selenium deficiency only reduced rabbit aortic glutathione peroxidase activity to 56 % of control values after 7 weeks of feeding. After having started this experiment a few reports were found in the literature indicating that rabbits are unresponsive to a diet deficient in selenium and have a lower selenium requirement than other species (315,318). This is consistent with the smaller effect of selenium deficiency on peroxidase activity in rabbit compared to rat.

The production of PGI_2 (measured as 6-oxo$PGF_{1\alpha}$) and th-18:1 were not significantly altered by selenium deficiency, in both the rat and rabbit (Figs. 43 and 44). The object of this study as stated above was designed only to test the effect of decreased selenium-dependent glutathione peroxidase activity on the production of oxygenated PUFA metabolites. The lack of difference in production of PUFA metabolites between control and selenium-deficient groups would seem to indicate that this enzyme is not very important for the reduction of hydroperoxy PUFA to their corresponding hydroxy compounds. This is probably accomplished by other mechanisms as discussed above.

The results reported here tend to differ from similar studies carried out in other laboratories. Masukawi et al. (319) found that aortic rings taken from a selenium-deficient group of Wistar rats released significantly less PGI_2-like bioactivity than a control group. Their results were expressed as the percent inhibition of ADP-induced platelet aggregation (selenium-deficient group: 16.45 ± 3.28, n=8 ; control group: 31.93 ± 6.21, n=8). They also found that the malondialdehyde level of the aorta (an index of lipid peroxide levels) was significantly higher in selenium-deficient rats (1.77 ± 0.15 nmol MDA/mg protein, n=7) than selenium-supplemented rats (1.34 ± 0.11 nmol MDA/mg protein).

Doni et al (320) investigated the influence of selenium administration on PGI_2 production. Adult male Wistar rats were divided into two groups. One group had sodium selenite added to its drinking water for 80 days, corresponding to a daily intake of 0.3 ppm/rat while the other group's drinking water had no supplement. The rats were fed a standard diet, presumably containing selenium. PGI_2-like bioactivity was then assessed, by an aggregometric assay (ADP-induced platelet aggregation), from slices of aorta, vena cava, lower lung lobe and heart apex. They found a 90 % increase of glutathione peroxidase activity in erythrocytes of the selenium-supplemented rats. Aorta from this group released 44 % more ($p < 0.005$, n=12) PGI_2-like activity. However, selenium supplementation had no effect on the production of PGI_2-like activity by other tissues. They also reported

that incubation of aortae from selenium-supplemented rats with a glutathione peroxidase inhibitor (3-amino-1,2,4-triazole) resulted in a 90 % reduction of PGI_2-like synthesis.

In another study (321), it was observed that selenium, when added to culture medium of confluent pig aortic endothelial cells, enhanced the ability of these cells to produce PGI_2 (measured by radioimmunoassay of 6-oxo$PGF_{1\alpha}$) when stimulated with 20:4 or thrombin. Concomitant with this increase in PGI_2 production was a time-dependent increase in glutathione peroxidase activity.

The three studies mentioned above tend to support a role for selenium and glutathione peroxidase in controlling aortic synthesis of PGI_2. The data suggest that glutathione peroxidase is capable of reducing the levels of cellular hydroperoxides, thus protecting prostacyclin synthase from their inhibitory effect. The reason for the apparent discrepancy between their data and that presented here is unknown. It is possible that the method of assay could be an important factor. The use of GC-MS with selected ion monitoring is a very specific assay, whereas measurement of PGI_2-like bioactivity by platelet aggregometry is lacking in this respect. Various differences in the experimental procedure (e.g. incubation conditions, stimulation of tissue) could account for the divergence in results. Alternatively, the strain of rat (Sprague-Dawley vs Wistar) might be a factor.

In addition to the studies performed with aorta, several studies with platelets have been performed. Bryant and Bailey (112,322) found a decreased glutathione peroxidase activity and an altered profile of lipoxygenase products (incubation with [^{14}C]20:4) in platelets from selenium-deficient rats. 12hp-20:4 was increased sevenfold, and its trihydroxy degradation products (8,9,12th-20:3 and 8,11,12th-20:3), 3 to 4 fold. 12h-20:4 synthesis was marginally reduced (8 %) in one of their studies (322) and reduced by 20-30 % in the other (112). These reports support a role for platelet selenium-dependent glutathione peroxidase in the enzymatic reduction of platelet-produced 12hp-20:4. In a similar investigation (323), it was found that selenium-deficient platelets produced larger amounts of thromboxane B_2 compared to control platelets. However, in contrast to the decreased finding of Bryant and Bailey, there was no difference in the production of trihydroxy metabolites of 20:4 between selenium-deficient and control groups. These results suggest that the activity of the cyclooxygenase pathway is increased (increased TXB_2 formation) in selenium deficiency, possibly due to increased hydroperoxide activator levels.

No change in the amounts of esterified oxygenated 18:2 metabolites was seen between the control and selenium-deficient groups in the present study (Figs.43 and 44). Selenium-dependent glutathione peroxidase is not capable of reducing hydroperoxidized phospholipids or lowering lipid peroxidation in rat liver microsomes (324). Only after

treatment of phospholipids or microsomes with phospholipase A_2 could this enzyme reduce hydroperoxides (324). In contrast, the selenium-dependent enzyme, PIP or phospholipid hydroperoxide glutathione peroxidase can reduce phospholipid hydroperoxides. If this enzyme is present in rat aorta and reduced in a state of selenium deficiency (results which have not been reported), a difference in the amounts of esterified oxygenated metabolites might have been expected between the control and selenium-deficient groups. However, factors such as vitamin E may be capable of handling an increased oxidative stress in the absence of this enzyme.

The present study, therefore, has raised a number of questions regarding the control of aortic cellular hydroperoxide levels by peroxidases and other non-enzymatic means, and the regulation of PGI_2 synthesis by these factors. Further investigation into the intricate control mechanisms of aortic PUFA metabolism is definitely required.

SUMMARY AND CONCLUSIONS

The metabolism of polyunsaturated fatty acids by vascular tissue has been studied. Particulate fractions from fetal calf aorta converted 20:3 to two stereoisomers of 11,12-epoxy-10-hydroxy-8-heptadecenoic acid, which are present in relatively large amounts after short incubation periods (5-10 min). Their amounts slowly decreased after 10 min due to metabolism by epoxide hydrolase(s) to 10,11,12-trihydroxy-8-heptadecenoic acid.

Linoleic acid is metabolized by particulate fractions and homogenates from aorta and ductus arteriosus to oxo, monohydroxy, epoxyhydroxy and trihydroxy products. The products identified by GC-MS were: 9-oxo-10,12-octadecadienoic acid, 9-hydroxy-10,12-octadecadienoic acid, 13-hydroxy-9,11-octadecadienoic acid, 9,10-epoxy-11-hydroxy-12-octadecenoic acid, 12,13-epoxy-11-hydroxy-9-octadecenoic acid, 9,10,11-trihydroxy-12-octadecenoic acid, 9,10,13-trihydroxy-11-octadecenoic acid and 9,12,13-trihydroxy-10-octadecenoic acid. The latter two trihydroxy products each consisted of mixtures of at least 3 stereoisomers. The initial oxygenation of 18:2 to 9hp-18:2 and 13hp-18:2 by aorta particulate fractions occurs via prostaglandin endoperoxide synthase, since inhibitors of this enzyme, indomethacin and acetylsalicylic acid, blocked the synthesis of 9h-18:2 and 13h-18:2, the major products formed under the incubation conditions. Subsequent transformation of the hydroperoxy intermediates appears to occur by a mixture of enzymatic and non-enzymatic steps. Metabolism to oxo and

epoxyhydroxy products is likely a non-enzymatic process, whereas reduction to hydroxy products may occur either enzymatically, by the peroxidase component of prostaglandin endoperoxide synthase, or non-enzymatically. Epoxyhydroxyoctadecenoic acids are converted by epoxide hydrolase(s) to the corresponding trihydroxy products.

18:2 is not a very potent inhibitor of the metabolism of 20:4 by fetal calf aorta particulate fractions. In contrast, 20:4 markedly inhibits the formation of monohydroxy metabolites of 18:2. The formation of the trihydroxy 18:1 isomers was inhibited to a much lesser extent, probably due to competition with PGG_2 and/or 15hp-PGI_2 for the peroxidase component of prostaglandin endoperoxide synthase.

Incubation of slices of fetal calf aorta in Krebs-Ringer Tris medium resulted in the synthesis of 9h-18:2, 13h-18:2, 9,10,11th-18:1, 9,10,13th-18:1 and 9,12,13th-18:1 from endogenous substrate. A GC-MS assay using selected ion monitoring was developed to quantitate all of the above products (except for 9,10,11th-18:1), along with the major oxygenated metabolites of 20:4 formed by aorta. 6-oxo$PGF_{1\alpha}$ was the major oxygenated PUFA metabolite produced by rat and bovine aorta and fetal calf ductus arteriosus. Much smaller amounts of other cyclooxygenase products (PGE_2, HHT, 11h-20:4 and 15h-20:4) were formed. Substantial amounts of free and esterified monohydroxy and trihydroxy 18:2-derived metabolites were detected, especially in rat and rabbit

aortae. Indomethacin did not inhibit the formation of these oxygenated 18:2 metabolites. Neither exogenous 13h-18:2 nor th-18:1 was incorporated to a large extent into lipids from vascular endothelial or smooth muscle cells, suggesting that the esterified 18:2 oxygenation products had arisen mainly via direct oxygenation of lipids.

Feeding rats a selenium-deficient diet for 5-7 weeks inhibited aortic glutathione peroxidase activity by 97 %. Selenium deficiency, however, did not significantly alter the formation of 6-oxo$PGF_{1\alpha}$ or any other oxygenated PUFA metabolites by rat aorta. Rabbit aortic glutathione peroxidase activity was inhibited by 44 % after feeding a selenium-deficient diet for the same time period. Only the formation of the minor lipoxygenase product, 12h-20:4, was significantly reduced during incubations of rabbit aorta in Krebs-Ringer Tris medium.

The fact that PUFA are readily oxygenated by vascular tissue to potentially harmful hydroperoxy intermediates reveals the need for mechanisms to control their intracellular levels. In situations where lipid hydroperoxides may accumulate, as in atherosclerosis, there could be an increase in the relative amounts of hydroperoxy metabolites, such as trihydroxyoctadecenoic acids. Esterified monohydroxy and trihydroxy PUFA metabolites found within aortic lipids raise the possibility that esterified hydroperoxy precursors are present which may inhibit PGI_2 synthesis as do free hydroperoxy PUFA. Further study is necessary to define the intricate control of hydroperoxide

levels and the role they play in the regulation of PGI_2 synthesis.

CLAIMS TO ORIGINAL RESEARCH

1) Bovine aorta particulate fractions convert 20:3 to two stereoisomers of 11,12-epoxy-10-hydroxy-8-heptadecenoic acid (11,12e-10h-17:1), which were identified by GC-MS.

2) 11,12e-10h-17:1 is converted to 10,11,12-trihydroxy-8-heptadecenoic acid by epoxide hydrolase(s) present in both cytosolic and particulate fractions of aorta.

3) Linoleic acid is converted by homogenates and particulate fractions of bovine aorta and ductus arteriosus to oxo (9-oxo-10,12-octadecadienoic acid), monohydroxy (9-hydroxy-10,12-octadecadienoic acid and 13-hydroxy-9,11-octadecadienoic acid), epoxyhydroxy (9,10-epoxy-11-hydroxy-12-octadecenoic acid and 12,13-epoxy-11-hydroxy-9-octadecenoic acid) and trihydroxy (9,10,11-trihydroxy-12-octadecenoic acid, 9,10,13-trihydroxy-11-octadecenoic acid and 9,12,13-trihydroxy-10-octadecenoic acid) products.

4) Formation of the oxygenated metabolites of 18:2 by aorta particulate fractions is inhibited by prostaglandin endoperoxide synthase inhibitors (indomethacin and acetylsalicylic acid) in a dose-dependent fashion.

5) 9,10e-11h-18:1 is converted to 9,10,11th-18:1 by epoxide hydrolase(s) in cytosolic and particulate fractions of aorta.

6) 20:4 is a much better substrate for prostaglandin endoperoxide synthase than 18:2 and is a potent inhibitor of oxygenation of the latter compound. In contrast 18:2 is not a very potent inhibitor of 6-oxo$PGF_{1\alpha}$ production from 20:4 by aortic particulate fractions.

7) A GC-MS assay with selected ion monitoring has been developed for the quantitation of at least nine oxygenated PUFA metabolites (6-oxo$PGF_{1\alpha}$, PGE_2, 12h-17:3, 11h-20:4, 12h-20:4, 15h-20:4, 9h-18:2, 13h-18:2 and th-18:1). The amounts of these products formed from endogenous substrate by fetal calf aorta and ductus arteriosus and adult bovine, rat and rabbit aortae were determined.

8) Substantial amounts of free and esterified monohydroxy and trihydroxy metabolites of 18:2 were detected, especially in rabbit and rat aortae. The esterified products were present in the tissue even before incubation of the tissue. These products had not been previously detected in normal mammalian tissues.

9) Selenium deficiency does not alter the production of oxygenated PUFA metabolites from rat aorta. Only the minor lipoxygenase product, 12h-20:4, was significantly reduced by selenium deficiency in the rabbit. This suggests that glutathione peroxidase may not be very important for the reduction of hydroperoxy PUFA to their corresponding hydroxy compounds.

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