STUDIES ON THE PROSTAGLANDIN SYSTEM IN BRAIN

(With Emphasis on the Precursor-Product Relationship)

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ABSTRACT

The arachidonic acid released post-mortem from the complex lipids of rat forebrain labelled with [$^{3}H_{8}$]-arachidonic acid had a specific activity close to that of the total phospholipids and much lower than that of the neutral lipids. The arachidonic acid is probably released from a mixture of phospholipids which excludes phosphatidylserine and phosphatidic acid. In the microsomal fraction, the specific activity of free arachidonic acid was very close to that of phosphatidylinositol. In rat cerebral cortex slices incubated in the presence of [$^{2}H_{8}$]-arachidonic acid, prostaglandin $F_{2\alpha}$ reached maximum labelling before any of the lipids examined except for a pool of rapidly labelled free arachidonic acid located at the damaged surfaces of the tissue. In homogenates the deuterium to protium ratio of the total free arachidonic acid was similar to that of prostaglandin $F_{2\alpha}$. However, the probable occurrence of an isotope enrichment effect suggests that the total free arachidonic acid fraction is not identical with the precursor pool used for prostaglandin $F_{2\alpha}$ synthesis. *In vivo*, the amount of free arachidonic acid and prostaglandin $F_{2\alpha}$ in rat forebrain was increased following convulsions induced by carbachol and metrazol. The level of thromboxane B_2 was not affected. Post-mortem levels of unesterified fatty acids were decreased in forebrains of phenytoin treated rats.

RESUME

Le niveau de radioactivité par microgramme d'acide arachidonique était presque le même pour cet acide gras trouvé dans l'ensemble des phospho-lipides que pour celui libéré post-mortem suite a l'hydrolyse de certains lipides du cerveau de rat auquel on avait injecté une quantité d'acide arachidonique-[$^{3}H_{8}$]. Cet acide gras libre provient probablement d'un groupe de phospho-lipides qui n'inclus pas le phosphatidylsérine et l'acide phosphatidique. L'acide arachidonique libre et celui trouvé dans le phosphatidylinositole ont, pour une quantité donnée, un même niveau de radioactivité lorsqu'on les extrait des microsomes du tissu cérébrale. Dans le cas de tranches de cortex placées dans un milieu contenant l'acide arachidonique-[$^{2}H_{8}$], la valeur maximale d'un rapport de molécules possédant des atomes de deutérium a celles n'en possédant pas fut atteint plus rapidement par les molécules de prostaglandine $F_{2\alpha}$ que par celles d'acide arachidonique trouvées dans les divers lipides du tissu. Cependant les acides gras libres présents aux surfaces endommagées des tranches de cortex firent exception a cette règle. Ce même rapport était presqu'identique pour les molécules de prostaglandine $F_{2\alpha}$ et pour celles d'acide arachidonique libre extraites d'homogénat du cortex auquel avait été ajouté l'acide arachidonique-[$^{2}H_{8}$]. La quantité de prostaglandines $F_{2\alpha}$ ainsi que celle d'acide arachidonique non-estérifié était élevée dans le cerveau du rat après une période de convulsions causées par l'injection de carbachole ou de métrazole. La quantité de thromboxane B_2 n'a pas été modifiée.

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CHAPTER I

INTRODUCTION

A) Landmarks in the History of the Prostaglandin System

In the 1930's, Kurzrok and Lieb (1) reported that human semen could contract or relax the uterus *in vitro*. Goldblatt (2) and von Euler (3) working independantly also observed the smooth muscle stimulating activities of seminal fluid and demonstrated their vasodepressor properties. Von Euler (4) found the active factor to have the properties of an acidic lipid and proposed to name it "Prostaglandin". Subsequently, he determined that "Prostaglandin" represented a mixture of unsaturated hydroxy fatty acids (5). However, the full chemical characterization of six primary prostaglandins (PGF_1, PGE_2, PGE_3, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and $PGF_{3\alpha}$) by Bergström, Samuelsson and co-workers (6-9) was acheived some 30 years later in 1962-63. The following year, Bergström's group in Sweden (10) and Van Dorp's group in the Netherlands (11) demonstrated that prostaglandins were formed by the enzymatic oxygenation of certain polyunsaturated fatty acids. The major pathways of metabolism of prostaglansins were identified in the same year (12). The cyclic endoperoxides, PGG_2 and PGH_2, intermediates in the biosynthesis of prostaglandins were isolated by Nugteren *et al* (13) and Hamberg *et al* (14) almost ten years after their existence had been postulated in 1965 by Samuelsson (15). The total synthesis of prostaglandins was acheived by Corey *et al* (16) in 1968. The accurate measurement of microgram and submicrogram amounts of prostaglandins was acheived by Gréen *et al* (17) who developed a gas chromatographic-mass spectrometric method of quantitation using deuterated prostaglandins as internal standards and carriers.

Knowledge of the pharmacological properties of the prostaglandins also progressed quickly. For instance, prostaglandins of the E series were found to lower blood pressure (6) and stimulate adenyl cyclase in various tissues (18), while prostaglandin $F_{2\alpha}$ was found to be luteolytic in many species except in man where it is only oxytocic (19). Clinical trials of the use of prostaglandin E_2 and $F_{2\alpha}$ to terminate pregnancies and induce labor were started in 1969 (20) and the use of synthetic analogs was implemented a few years later (21). The discovery in 1971 by Vane (22) and by Smith and Willis (23) that aspirin and indomethacin were potent inhibitors of prostaglandin synthesis indicated that prostaglandins probably played a role in fever production and the inflammatory process. Recently, Olley et al (24) demonstrated that E-prostaglandins could maintain the potency of the ductus arteriosus and could be used in the treatment of newborns with certain congenital heart lesions.

In the last few years, two important members were added to the family of products derived from prostaglandin endoperoxides. In 1975, Hamberg et al (25) discovered the conversion of the endoperoxides to thromboxane A_2, a non-prostanoid compound with properties identical to that of the rabbit aorta contracting substance (RCS) reported earlier by Piper and Vane (26). Malmsten et al (27) demonstrated that endoperoxides were potent platelet aggregators but this activity results from their conversion to thromboxane A_2 (25). The following year, Gryglewski et al (28) and Moncada et al (29) found that the prostaglandin endoperoxides could be converted by certain tissues, particularly the vascular endothelium, into a substance that inhibited platelet aggregation. The substance was

originally called prostaglandin X, but Johnson et al (30) who determined its structure and achieved its chemical synthesis proposed the name prostacyclin. The structure of prostacyclin is very similar to that proposed by Pace-Asciak and Wolfe (31) a few years earlier for a novel prostaglandin formed from arachidonic acid by rat stomach. Pace-Asciak (32) also demonstrated that prostacyclin was non-enzymatically hydrolyzed to 6-keto-$PGF_{1\alpha}$. Prostacyclin was found to be 20 to 50 times more potent in increasing cAMP levels in platelets than PGE_2 (33,34) and to be the endogenous metabolite responsible for relaxation of coronary arteries induced by arachidonic acid (35). Chemical synthesis of analogues of prostacyclin were undertaken in the hope of finding an efficient agent against thrombosis (36). A search for selective inhibitors of thromboxane and prostacyclin synthesis was also undertaken. Moncada et al showed that imidazole is a potent inhibitor of thromboxane synthetase and found that 15-hydroperoxy arachidonic acid was a potent inhibitor of prostacyclin synthesis (38).

B) Distribution of Prostaglandins and Thromboxane A_2

The primary prostaglandins ($F_{2\alpha}$, $F_{1\alpha}$, E_2, E_1, D_2) are synthesized by most mammalian tissues as well as some lower vertebrate and invertebrate tissues (39,40). Prostaglandin I_2 (prostacyclin) and thromboxane A_2 are also generated in a large number of animal tissues (41,42) (see Table 1). The exact combination of prostaglandins and related products found is tissue and species specific (40,41,43,44). Prostaglandins are not stored in a preformed state (45-47); rather, their formation is initiated by a variety of stimuli such as hormones,

TABLE 1 .

Principal Prostaglandin Endoperoxide Products Found in Various Tissues

Tissue	Endoperoxide Products
Platelets	TxA_2
Macrophage	TxA_2
Vascular endothelium	PGI_2 , PGE_2
Umbilical artery	TxA_2 , PGE_2
Renal cortex	PGI_2
Renal medulla	PGE_2
Ductus arteriosus	PGI_2
Stomach	PGI_2 , PGE_2
Lung	PGI_2 , PGE_2 , TxA_2
Liver	$PGF_{2\alpha}$, PGE_2
Uterus	$PGF_{2\alpha}$, PGI_2
Bone	PGI_2 , PGE_2
Brain	PGD_2 , PGE_2 , $PGF_{2\alpha}$, TxA_2
Fetal tissues	PGI_2

enzymes, ischemic/hypoxic insult and inflammation (44,48). Minute amounts of prostaglandins are released in many body fluids such as seminal fluid (1-3), menstrual fluid (49), amniotic fluid (50), vitreous humour (51), stomach fluid (52), exudate of skin (53) and CSF (54).

C) Effects of the Prostaglandin System on Cyclic Nucleotides and Calcium Mobilization

The primary prostaglandins have a long list of pharmacological and possible physiological roles (55-57). Thromboxane A_2 is a potent aggregating agent and vasoconstrictor (25,58). Prostaglandin I_2 has properties opposite to those of thromboxane A_2 (28) and also appears to mediate renin release brought about by renal arterial hypotension (59). The involvement of the prostaglandins and related substances in a great variety of physiological processes is to be expected since they can modify the level of cyclic nucleotides and affect the mobilization of calcium in many cell types. In most tissues, including nervous tissue, prostaglandins of the E type increase the intracellular concentration of cAMP (60-65), except in isolated fat cells (19) and toad bladder (66) where they antagonize the hormone stimulated rise in cAMP. In fibroblasts (67) and blood platelets (33,34), PGI_2 caused the largest increase in cAMP. PGD_2 also increased the level of the nucleotide in platelets (68). Prostaglandin endoperoxides and thromboxane A_2 inhibit the PGE_1 and I_2 stimulated cAMP rise in platelets (33,69). On the other hand, prostaglandins of the F type promote cGMP accumulation in the rat uterus (70), in dog and bovine vein (71) and in guinea pig lung (72). The guanylyl nucleotides, GTP and GDP, appear to potentiate the effect of prostaglandins of the E series on the level of cAMP (73,74).

Prostaglandin endoperoxide formation may be involved in the elevation of cGMP levels during platelet aggregation (75). Since cAMP and cGMP have opposite effects on many cellular processes (76), it has been suggested that the selective stimulation of cyclic nucleotides by prostaglandins of different types constitutes a regulating mechanism of cellular activity (77).

The effect of prostaglandins and thromboxanes on many cellular processes may also be mediated by calcium. In stomach muscle, prostaglandin E_1 causes the retention of calcium in a slowly exchangeable fraction (78) while $PGF_{2\alpha}$ promotes influx of the cation in the uterus (79). Other effects of prostaglandins on calcium movement include the release of the cation bound to the lipid phase of turkey erythrocyte membranes (80), the release of calcium accumulated under ATP stimulation in a uterine microsomal fraction (81) and from rat liver mitochondria (82) and the increased binding of calcium to rat liver mitochondrial inner membrane (83). Also, the prostaglandins of the E type liberated during stimulation of the sympathetic innervation of many organs may exert a local negative feedback action on the release of further neurotransmitter through inhibition of calcium uptake by noradrenergic nerve terminals (84). Prostaglandin endoperoxide analogues can produce calcium efflux from rat liver mitochondria (85) and cause influx of calcium into sickle erythrocyte ghosts (86). Finally, the initial aggregatory response of platelets to thromboxane A_2 appears to be mobilization of intracellular calcium (87).

The study of the physiological roles and pharmacological effects of prostaglandins is complicated by the facts that different tissues

produce these compounds in varying proportions (70), catabolize them at different rates (88,89) and respond in opposite directions (90) and with a wide range of sensitivities to a given prostaglandin (87, 91). Furthermore, alterations of prostaglandin synthesis, catabolism and action may occur during development (92,93) and physiological cycles (94,95).

D) Uptake and Binding of Prostaglandins

The fact that endogenous prostaglandins readily go into the extracellular fluid or the incubation medium suggests that they can cross cell membranes or at least can liberate themselves from them (47,96). However, red blood cells are impermeable to exogenous prostaglandins while the tissues of structures such as the lung and kidney can accumulate prostaglandins by a carrier mediated mechanism(97). The prostaglandins of the extracellular fluid of the brain and eye may be removed into the blood circulation by a similar process (97). At high concentration, the non-specific solubilization of prostaglandins in membrane is to be expected because of the lipophilic nature of the compounds.

The binding of prostaglandins to tissues is correlated to their biological activities (98,99), including stimulation of adenyl cyclase activity (100-105). The effect of these compounds on cyclic nucleotide levels suggest that they need not penetrate cells to influence metabolism. Studies on the competitive binding of various prostaglandins and prostaglandin antagonists revealed a high degree of specificity of the binding sites (103,105-110). Prostaglandin receptors are located in the plasma membrane of many cell types (107-109,111). The absence of competition

for binding between prostaglandins and various hormones suggest the existence of prostaglandin specific binding sites on adenyl cyclase (107,104).

E) Structure and Nomenclature

The biochemical analysis of the prostaglandins is made difficult by their instability and the very close chemical similarity among the members of this family of compounds as well as by the low amounts present in tissues.

Prostaglandins have the basic 20 carbon cyclopentane carboxylic acid structure. For the physiologically important primary prostaglandins (E_1, E_2, $F_{2\alpha}$, D_2*) the carboxyhexyl side chain on the cyclopentane ring is in the α configuration and the hydroxyoctyl side chain is in the β configuration while the carbon 15 hydroxyl has the S or L form. The E and F prostaglandins have the characteristic 11 α hydroxy group on the 5 membered ring but the E type has a 9 keto group which is reduced to a hydroxy in the F type. Prostaglandins of the D type have a 9 α hydroxy and an 11-keto group. Dehydration of PGE yields the 10-11 unsaturated ketone PGA which by rearrangement gives the double conjugated ketone PGB.

In prostaglandin I_2, carbon number 9 in the cyclopentane ring is linked by an oxygen to carbon number 6 in the carboxyhexyl side chain to form a 6-9 epoxy structure to the cyclopentane ring. The enol-ether

*The numerical subscripts 1,2,3 written with each type designation indicates the number of carbon-carbon double bonds in the side chains. All the natural prostaglandins and thromboxanes have the Δ^{13} trans double bond. The other double bonds if present would be Δ^5 cis and Δ^{17} cis.

double bond (Δ^5) is in the Z configuration (112). Finally, thromboxane A_2 is a bicyclic compound with an oxygen atom between carbons 11 and 12 forming a heterocyclic non-prostanoate structure and another oxygen forming a 9-11 oxane ring. The side chains on the 6 membered ring are in the same configuration as in the prostaglandins.

F) Mechanisms of Biosynthesis

The enzymic conversion of a number of methylene interrupted cis polyunsaturated long chain fatty acids to the various prostaglandins is accomplished by an enzyme complex termed prostaglandin synthetase. The components of this enzyme system are all tightly bound to the microsomal fraction of all tissues studied (113-115). One exception is the soluble PGD_2 isomerase of rat brain (41), stomach and lung (13). The physiologically important primary prostaglandins are derived from unesterified dihomo-γ-linolenate for PGE_1 and $PGF_{1\alpha}$ and from unesterified arachidonate for PGE_2, PGD_2 and $PGF_{2\alpha}$ (116-119). These fatty acids are members of the ω-6 family derived from dietary linoleate.

The first step in the synthesis of prostaglandin is the removal of a prochiral S hydrogen at carbon 13 of the precursor fatty acid through a lipoxygenase-like reaction. This is followed by the isomeration of the Δ^{11} bond to the Δ^{12} position. Subsequently, there is the insertion of molecular oxygen at C-11 and C-15 with double bond rearrangement to give the Δ^{13} trans double bond. Next comes the formation of the 9,11 endoperoxide with the oxygen at C-9 being derived from the same molecule of oxygen as that at C-11. Concurrent formation of the C-8, C-12 bond by further shifting of double bonds results in a bicyclic endoperoxide

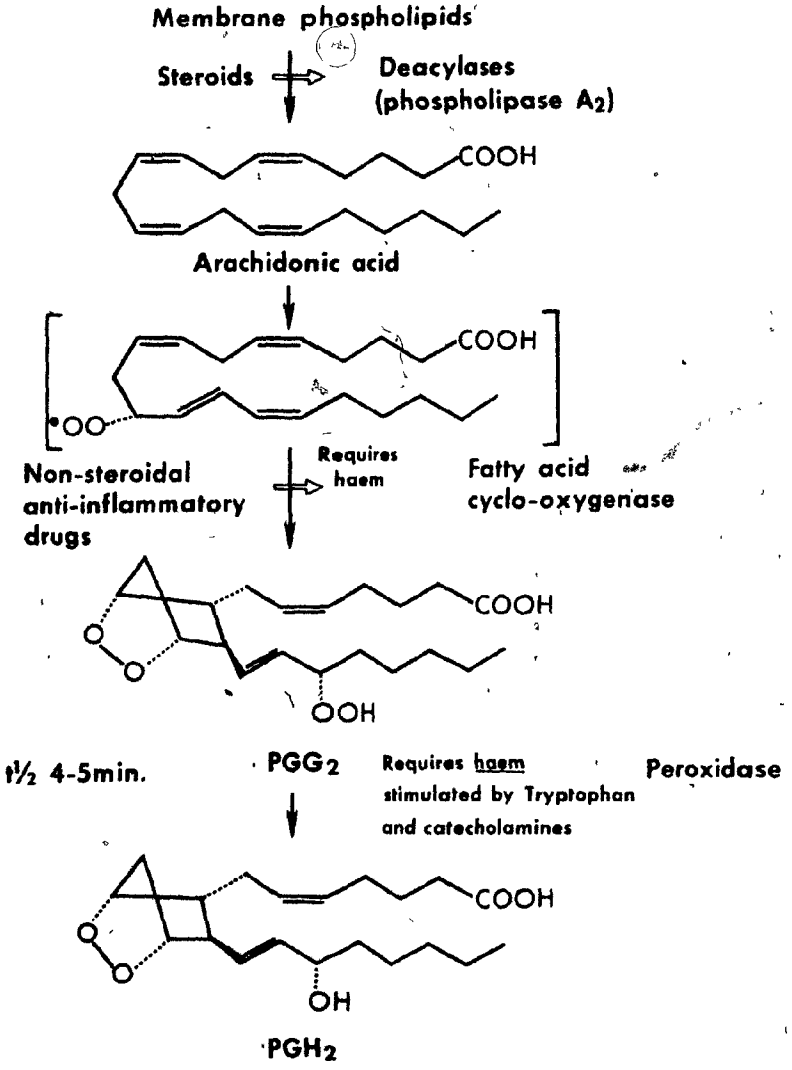
called 15-hydroperoxy-prostaglandin endoperoxide or PGG_2 (14,13,120) (see Figure 1).

A reduced form of PGG_2, 15-hydroxy prostaglandin endoperoxide (PGH_2) has also been isolated (13,121). These two compounds have a half-life of approximately 5 minutes in aqueous media at 37°C (14). The enzymes which catalyze the above reactions have been purified several hundred-fold from sheep (122,123) and bovine vesicular gland (124). The bis-dioxygenase activity requires protoheme whereas the peroxidase activity is stimulated by aromatic compounds such as tryptophan as well as by heme which may be the cytoplasmic activating factors. The ferri-heme dioxygenase is called fatty acid cyclo-oxygenase (14) although this activity has never been separated from the 15-hydroperoxy-peroxidase activity (123).

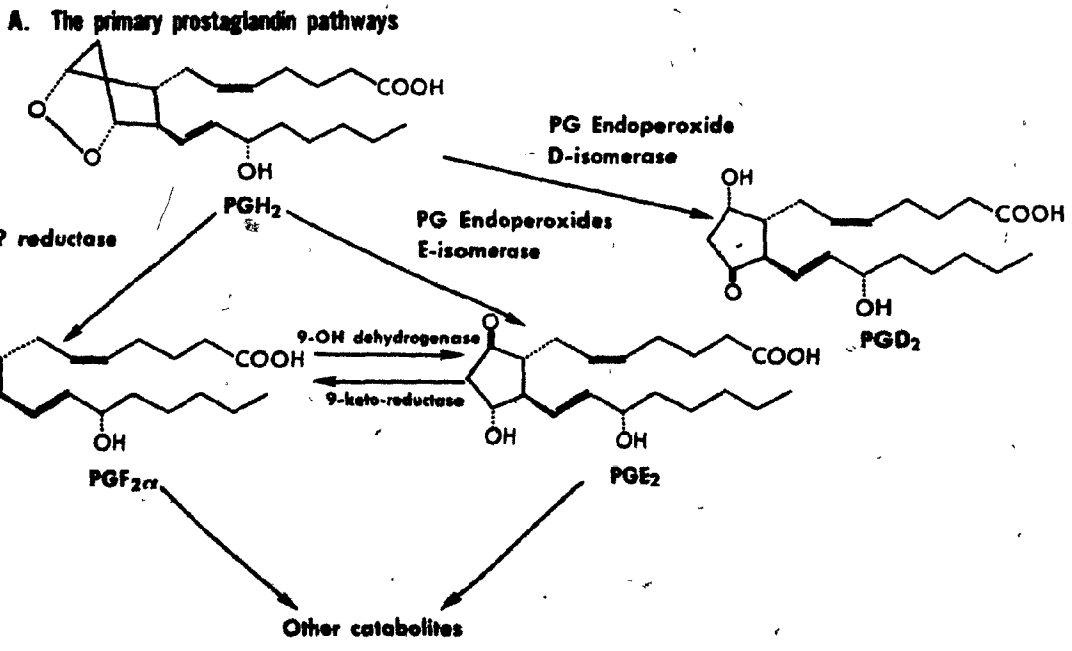
In broken cell preparations (13,126-131) or as the purified enzyme (123,124,132), the fatty acid cyclo-oxygenase shows a time-dependant deactivation resulting from the production of peroxide functions during the reaction. The effect may not require a peroxidase activity (132) as has been suggested (133). The product inhibition appears to be irreversible but the enzyme can be protected by compounds capable of supplying reducing equivalents or of acting as free radical scavengers such as indole, catecholamine, quinone and phenol derivatives (124,134, 135). However, in intact cell preparations the rate of PG production is slower and the cyclo-oxygenase has a much longer life than in homogenates (47,136,137) which suggests that in vivo the hydroperoxy endoperoxides may be produced at a rate slow enough that they are not able to accumulate to the point of inhibiting the enzyme. Interestingly, the

FIGURE 1

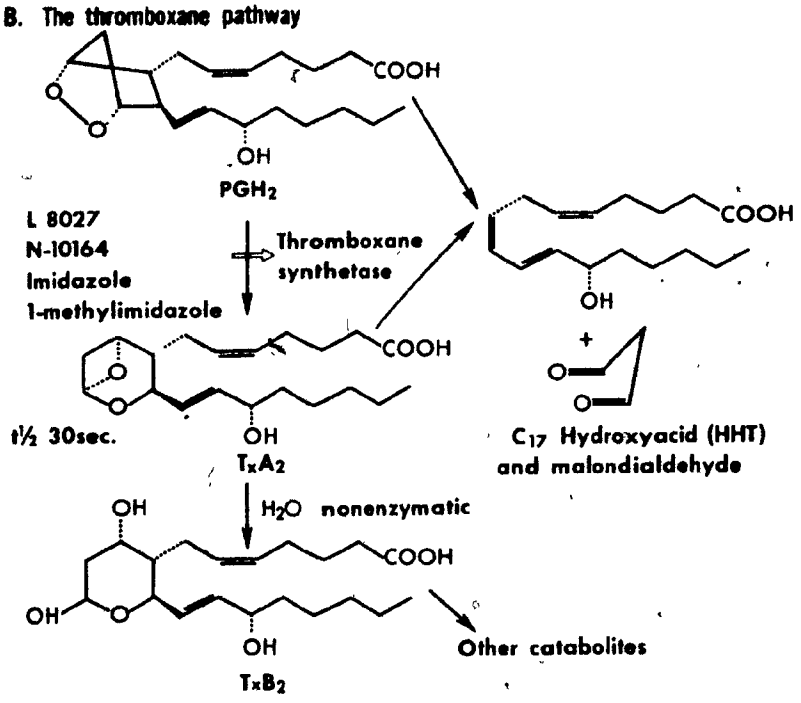
Biosynthesis of Prostaglandin Endoperoxides



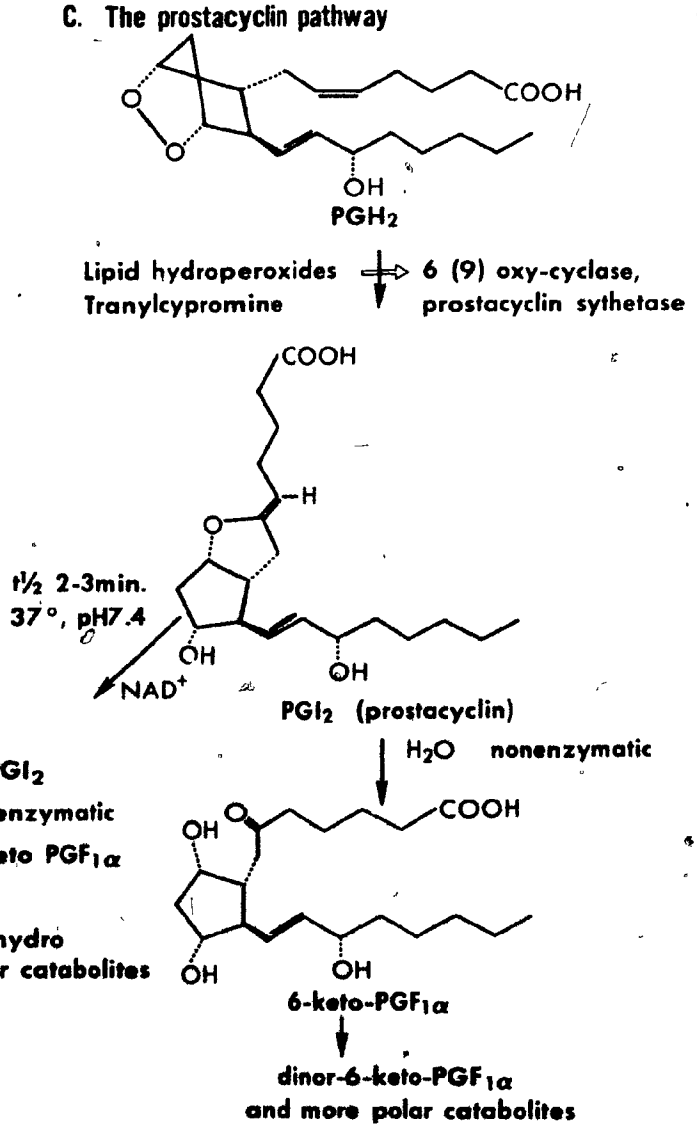
Metabolites of Prostaglandin Endoperoxides FIGURE 2



B. The thromboxane pathway



C. The prostacyclin pathway



cyclo-oxygenase shows an initial activation by PGG_2 as well as the subsequent deactivation (132).

The prostaglandin endoperoxides undergo many different metabolic transformations depending on the tissue where they are produced. PGH_2 rather than PGG_2 is converted to the prostaglandin of the E type by an endoperoxide isomerase from bovine seminal gland, a glutathione requiring enzyme (138). In sheep vesicular gland, the production of PGE_2 may involve first an endoperoxide isomerase and secondly a peroxidase activity (139). Prostaglandins of the F type are formed possibly through the action of an endoperoxide reductase (115) or by chemical reduction in the presence of agents such as metal complexes of organic compounds (135,140). In some tissues, there appears to be a 9-keto reductase which converts prostaglandins of the E type to the F type (141,144) (see Figure 2a).

Prostaglandins of the D type are also formed through the action of a specific isomerase but like all other prostaglandins, they may result from the slower chemical decomposition of endoperoxides (13,14,121,129). Formation of prostaglandins of the A type by a PGE dehydrase may occur; however much if not all arises from the chemical modification of prostaglandins of the E type during extraction under acidic conditions (145).

In blood platelets, brain, lung and many other tissues, the prostaglandin endoperoxides formed from arachidonic acid can be converted to a non-prostanoic acid structure, thromboxane A_2 by a microsomal enzyme (41,146). This is acheived by the insertion of one of the endoperoxide oxygens in the cyclopentane ring to form a six membered heterocyclic structure. The other oxygen forms the 9-11 oxane ring. The resulting bicyclic compound has a half-life of 30 seconds in aqueous media at 37°C

and is non-enzymatically hydrolyzed to a cyclic hemi-acetal pyranose called thromboxane B_2, a stable compound with little biological activity (25). The compounds, 12L-hydroxyheptadecatrienoic acid and malondialdehyde were thought to be breakdown products of thromboxane A_2 but recent reports indicate that they are formed from the endoperoxides through a different but related pathway (147) (see Figure 2b). The fomration of thromboxane A_1 from dihomo-Y-linolenate by platelets has been reported (148).

Prostaglandin I_2 can be produced from PGG_2 or PGH_2 by microsomes of vascular tissues, stomach fundus and many other organs (28,38,41). This compound was first found in rat stomach (32). The prostaglandin I_2 probably arises from the reaction of one of the endoperoxide oxygen with the cis olefin bond at C-5 of the carboxyhexyl side chain to form the 6-9 epoxy linkage. The Δ^5 unsaturation is reformed by subsequent loss of a proton from C-6. In aqueous media, this compound degrades rapidly to the 6-keto $PGF_{1\alpha}$ which is in equilibrium with its lactol form (32) (see Figure 2c).

The relative amounts of the endogenous substrate fatty acids, dihomo-Y-linolenate or arachidonate, will regulate the proportion of prostaglandins differing in degree of unsaturation. Most tissues have a paucity of dihomo-Y-linolenate in their phospholipids (149).

Synthesis of the various products of the prostaglandin synthetase complex may be selectively inhibited by compounds which affect differentially the various enzymes of the complex. For example, in sheep vesicle gland preparations, bicycloheptenes (150) and phenylbutazone (151)

inhibit production of prostaglandins of the E type while gold salts lower prostaglandin $F_{2\alpha}$ production (151). Phenylbutazone also inhibits the production of prostaglandins of the F and E types but not of the D series in rabbit kidney microsomes (152). Imidazole, 1-methyl-imidazole (37), N-0164, a phenylphosphonate (153) and L8027, a non-acidic anti-inflammatory indole derivative (154) preferentially inhibit the thromboxane synthetase of platelets. Tranylcypromine and 15-hydroperoxy-arachidonate specifically inhibit prostaglandin I_2 synthetase in vascular tissues (28).

Inhibition of the cyclo-oxygenase by non-steroidal antiinflammatory drugs blocks formation of the endoperoxides and related products in vitro (22,155-157) and in vivo (158,159). The microsomal cyclo-oxygenase prepared from different tissues may show different susceptibilities to these drugs (160,161). Non-substrate unsaturated fatty acids (127, 162-164), complexing agents and antioxidants (130), also inhibit cyclo-oxygenase activity in vitro. Steroid anti-inflammatory agents inhibit prostaglandin synthesis by blocking the release of arachidonic acid from complex lipids (165-168). A wide variety of other compounds have been shown to inhibit prostaglandin synthesis (169).

G) Biodegradation

Prostaglandins are biodegraded near their site of origin or in specialized tissues such as lung, kidneys and liver after entering the circulation. The complex sequence of enzymatic degradation has been extensively reviewed (88,170-173). Enzymatic dehydration of the 15-hydroxy group as a first step and subsequent reduction of the Δ^{13} trans double bond occur in the cytosol of most cells while beta and omega

oxidation occur in the specialized tissues. The dehydrogenase is present in two forms: a NAD^+ - dependent type and a $NADP^+$ - requiring type (174). These metabolic transformations may occur to various extent in different tissues from the same species or in the same tissue at various developmental stages (92,93).

Prostaglandins of the A type are not removed effectively from the circulation by the lungs (175) and are not good substrates for its prostaglandin dehydrogenase (176). These prostaglandins are metabolized by liver microsomal enzymes to ω-1 and ω-2 hydroxylated compounds and dioic acids (169).

Prostaglandin I_2 is also not metabolized in transit through the lung (177-179) but it is metabolized rapidly by the kidney (180). In the latter organ, PGI_2 undergoes oxidation of its C-15 hydroxy group, reduction of its C-13,14 double bond and β-oxidation to yield 7,9 dihydroxy-4,13-diketo dinor prostanoic acid (181). The non-enzymatic hydrolysis product of PGI_2, 6 keto-$PGF_{1\alpha}$ may also undergo β-oxidation to yield dinor-6 keto $PGF_{1\alpha}$. The major urinary metabolites of 6-keto-$PGF_{1\alpha}$ injected intravenously in rat were β and ω oxidation products (182) while PGI_2 gave 15-keto metabolites (183). Blood vessels can metabolize PGI_2 via a 15-hydroxydehydrogenase (183). The PGI_2 produced by lung can enter directly into the circulation and thus is the only PG that could be regarded as a true circulating hormone.

The rapid formation of thromboxane B_2 from thromboxane A_2 described above (see page 13) indicates that thromboxane cannot act as a circulating hormone. In monkey and man, 2,3 dinor thromboxane B_2 was the major urinary metabolite of TxB_2 (184,185). Dehydrogenation of the hemiacetal alcohol group at C-11 may also be a pathway for the metabolism of TxB_2 (186).

H) Role of Acylhydrolases in Prostaglandin and Thromboxane Biosynthesis

The biosynthesis of prostaglandins occurs strictly from certain unesterified polyunsaturated fatty acids (116,117). However, in many tissues, the in vivo levels of the free fatty acid precursors are extremely low (118, 129,187-190). On the other hand, large amounts of arachidonic acid and smaller amounts of dihomo-γ-linolenate are esterified to complex lipids especially at position 2 of phospholipids (191-198). In some tissues or their homogenates, addition of arachidonic acid increases prostaglandin synthesis as does treatment with phospholipase A_2 (116,117,189,199-203). This enzyme appears to play a major role in the stimulation of prostaglandin synthesis. Arachidonic acid is released from specific endogenous phospholipids following the application of stimuli which initiate prostaglandin and/or thromboxane synthesis in blood platelets (204,205) and transformed mouse fibroblasts (206). Certain inhibitors of prostaglandin synthesis such as tetracaine (207) meparine (208) and steroid anti-inflammatory agents (165-168,209,211) exert their effect by inhibiting phospholipase activities. Furthermore, the release of arachidonic acid, prostaglandins and related products is stimulated by the ionophore A23187 in blood platelets (212-214). The ionophore also activated the production of prostaglandins and thromboxane in many other tissues (215). Conversely, the complexing agent, EDTA, inhibited release of the precursor fatty acid from phospholipids in homogenates of platelets (216), stomach (188) and thyroid gland (217). This is in keeping with the fact that most phospholipase A preparations require calcium for their activity (218).

Interestingly, in the renal medulla, the arachidonic acid released by exogenous triglyceride lipase is available for prostaglandin synthesis (131). The triglycerides found in the lipid droplets of the interstitial

cells of this tissue have an unusually high arachidonate content (219,220) which increases in hydronephrotic and indomethacin treated rabbits (221, 222). The production of prostaglandins in culture of reno-medullary interstitial cells can be inhibited by mepacrine (223). This antimalarial drug probably inhibits triglyceride lipase (224) as well as phospholipases (225).

Triglycerides may also supply some arachidonate for prostaglandin synthesis in the thyroid gland through the action of a lipase sensitive to the increase in cAMP induced by TSH (226). In the uterus and ovaries, a cholesterol esterase may be similarily activated by luteinizing hormone (70). The released cholesterol could be used for hormone synthesis while the arachidonate which makes up a large part of cholesterol ester fatty acids would be transformed to prostaglandins. The cholesterol esters are a major source of arachidonic acid for prostaglandin synthesis by adrenal cortex in the presence of ACTH (227). Phospholipids need not be the only source of arachidonic acid used for prostaglandin synthesis. However, the rate controlling step in endogenous biosynthesis of prostaglandins *in vivo* appears to be an acylhydrolase reaction releasing long chain fatty acids from one or more complex lipids following stimulation.

I) Occurence of Prostaglandins and Thromboxane in Brain

(1) Synthesis and Catabolism

Several reports demonstrate the presence of prostaglandins in brain of many animal species (51,228-233). The *in vivo* levels of prostaglandins in brain are exceedingly small but quickly rise after death of the

animal (47). All brain regions examined in rat, cat and human could synthesize prostaglandins although with different capacities and proportion of E and F types of prostaglandins (47,234,235). In rats killed by microwave irradiation, the median eminence contained a much higher level of prostaglandins of the E series when compared to the medial basal hypothalamus and the anterior pituitary gland (236). In quick frozen brain from rats killed by decapitation, the median eminence had the highest $PGF_{2\alpha}$ and E_2 levels of 24 brain areas (237).

Thromboxane B_2 was synthesized in equal or greater amounts than prostaglandins by the cerebral cortex of rat and guinea pig (238). However, prostaglandin D_2 has been identified as the major prostaglandin endoperoxide product in homogenates of rat brain (239). Prostaglandin I_2 was found in fetal sheep brain (240) but adult rat brain shows little capacity to synthesize this compound (28,38,41).

In vivo, prostaglandins are spontaneously released into superfusates of various brain regions (230,241-247). The levels are increased several fold following the application of neuroleptics (248) and neurotransmitters (241,244) and during electrical stimulation of various brain structures and peripheral nerves (241,249). On the other hand, barbiturates decrease the basal release of prostaglandins in encéphale isolé preparations (248). Prostaglandin content of the CSF is increased in pathological conditions such as pyrogen fever, surgical trauma, meningitis, epilepsy, cerebrovascular accidents (54) and during drug induced convulsions (250). Tissue levels also rise quickly during electroconvulsive shock (251) and convulsions induced by metrazol (252).

The types of prostaglandins spontaneously released from nervous tissue often differ from that of prostaglandins released following stimulation (241,245,249). The differential activation of prostaglandin endoperoxide isomerase and reductase, alterations in co-factor levels and involvement of different cell types could explain the change in the type of prostaglandin released under these two conditions. The non-enzymatic reduction of the prostaglandin endoperoxide to prostaglandin $F_{2\alpha}$ (253) and the greater water solubility of the trihydroxy prostaglandin may also be factors in the preferential release of this prostaglandin. The activation of a 9-keto reductase might account for the change in the type of prostaglandin seen during stimulation. This activity has been reported in monkey, pigeon and rat brain (142,143) and frog spinal cord (144). However assays were generally done with prostaglandin E_2 concentrations far exceeding those found in brain even after prolonged incubation. The 9-keto reductase activity in brain may result from a non-specific reductase activity uncovered when high concentration of PGE are used (47).

Cerebral cortex homogenates have a more rapid initial rate of synthesis than that of slices but stop forming prostaglandins sooner even though the precursor is still plentiful (47). The prostaglandin synthetase in the homogenate may be deactivated by one of the intermediates in prostaglandin endoperoxide synthesis accumulating too rapidly as seems to occur in the homogenate of many tissues (see page 10). Biogenic amines extend the life of the prostaglandin synthetase of brain as well as stimulate the production of prostaglandins (47). Biogenic amines have the same effect on prostaglandin synthesis in stomach fundus

homogenates (129) and seminal vesicles (254). Chemical reduction of the hydroxyperoxide or of the endoperoxide function of the intermediates in prostaglandin formation is probably the mechanism by which these compounds affect the production of prostaglandins (133,253,255). In rat brain homogenates, the biosynthesis of prostaglandin E_1 and E_2 from radioactive precursors is stimulated by ADP and cAMP respectively (256). Electrical pulses applied to the homogenate stimulate prostaglandin E_1 synthesis in the presence of ADP (256). However, in these experiments the prostaglandins were assayed simply by measuring the radioactivity which co-chromatographed with prostaglandin standards after ether extraction. Exogenous free arachidonic acid does not stimulate significantly $PGF_{2\alpha}$ and TxB_2 synthesis by brain tissue in vitro (229,235).

The catabolism of prostaglandins by neonate sheep brain (92) and mature rat and dog brain is negligible (47,257). However, the fetal brain of sheep but not of rat possess very high 15-hydroxy prostaglandin dehydrogenase activity (93). A prostaglandin dehydrogenase activity which uses $NADP^+$ as co-factor was partially purified from the high speed supernatant of mature monkey brain and fetal human brain (174). Very little Δ^{13} reductase activity was detected in monkey brain (174). Low levels of prostaglandin dehydrogenase and somewhat higher levels of Δ^{13} reductase have been reported in swine brain (88). The former activities are also present in frog spinal cord (258).

In brain tissue, a small population of cells may be enriched in prostaglandin degrading enzymes as suggested by the work of Siggin et al who found by histochemical techniques that prostaglandin dehydrogenase activity was concentrated to the Purkinje cells and some molecular layer elements of rat cerebral cortex (259). However, it has been suggested that in vivo most of the prostaglandins are removed into

circulation and metabolized by extra-neural tissues (260).

(2) Cellular and Subcellular Localization of Prostaglandin Synthesis in Brain

The increased release of prostaglandins during stimulation in brain and other nervous tissues especially the frequency dependent evoked release suggests a neural rather than glial site of synthesis. This is supported by the fact that the cerebral cortex grey matter contains more prostaglandins than the underlying white matter (229, 232). Furthermore, immunohistochemical methods revealed the presence of cyclo-oxygenase antigenicity in neurons of guinea pig brain (261). However, glial as well as neuronal cell lines produce prostaglandins in vitro and synthesis in both cell types is increased by dibutyryl cAMP (262,263).

As in other tissues, the prostaglandin synthetase activity of brain is found predominantly in the microsomal fraction (229,264-266). Synthesis of prostaglandins in whole brain is stimulated by norepinephrine but that of synaptosomes is unaffected (266). Although the major fraction synthesizing prostaglandins is not the synaptosomal fraction, the latter was thought to store prostaglandins in relatively large amounts (265). This contention has been disproven in subsequent experiments where most of the prostaglandins were found in the high speed supernatant of rat brain (267). As in all other tissues studied, prostaglandins are not stored in brain but synthesized de novo in the presence of appropriate stimuli.

J) Free Arachidonic Acid in Brain

(1) Release of Arachidonic Acid

Brain tissue quickly frozen in liquid nitrogen (268-270) or microwave irradiated (246) contains very little free fatty acids. Unesterified arachidonic acid, the precursor for the prostaglandins and thromboxanes produced by brain, is practically undetectable under these conditions (268,269). However, anoxia induced *in vivo* (271) or death of the animal (269,270,272) result in a rapid rise in free fatty acid levels in brain. Arachidonic acid is released post-mortem at a faster rate than palmitate, stearate, oleate and docosahexaenoate (268). The increase in free fatty acids is probably due to hydrolysis of brain lipids and not to *de novo* synthesis since in mouse brain slices the only unesterified fatty acid labelled by 1-^{14}C acetate is palmitate in the short period of time involved (273,274). The cerebellum released proportionally less arachidonic acid than the cerebral cortex post-mortem (234).

In vivo, electroconvulsive shock results in a transient increase in free fatty acids (268). The administration *in vivo* of the convulsant drug metrazol also leads to an increase in free fatty acid levels in brain (275). Intracerebral injections of large doses of carbachol and eserine are reported to increase free fatty acid levels *in vivo* (276) where as dibutyryl cAMP is not effective (275). On the other hand, in nerve endings of guinea pig brain norepinephrine and 5-hydroxytryptamine stimulated fatty acid release from endogenous substrates but acetylcholine and dibutyryl cAMP were without effect (277). The hydrolysis of lipids in subcellular fraction other than the synaptosomal one was

not affected by the above chemicals.

The arachidonic acid released in brain following anoxic/hypoxic insult is not limited to its role as a substrate for prostaglandin synthetase. Its accumulation and that of other unsaturated fatty acids is associated with irreversible loss of mitochondrial energy metabolism in rat liver (278) and brain (279).

(2) Subcellular Localization of Arachidonic Acid Release

The larger portion of the fatty acids released post-mortem in rodent brain remains associated with particulate fractions (269). In rats made anoxic by exposure to a nitrogen atmosphere, free fatty acids increased proportionally more in the brain microsomes than in the crude cytoplasmic extract, the cytosol showing no increase (271). The yield and composition of the free fatty acids are somewhat different for each brain subcellular fraction suggesting that lipolysis occurs independantly in each (269).

A comparison of the fatty acid composition of lipids suggests no obvious link between the phospholipids of whole brain (195) or subcellular fractions (280,281) and the unesterified fatty acids released post-mortem. The free fatty acids are relatively rich in palmitate while all the phospholipids except phosphatidylcholine are poor in this fatty acid. Similarly, arachidonic acid comprises a large percentage of the free fatty acids but only a low percentage of the fatty acid of phospholipids except in the phosphatidylinositides. In whole brain, the diglycerides have a fatty acid composition similar to that of the free fatty acid pool (282). However, it must be remembered that the composition of the free fatty acid pool may reflect not only the

distribution of the fatty acids esterified to brain lipids but also the activities and specificities of lipolytic enzymes present in the subcellular fractions.

(3) Post-Mortem Changes in Other Brain Lipids

Other brain lipids besides free fatty acids are quickly altered post-mortem. Phosphatidylinositol monophosphate and phosphatidylinositol diphosphate (283-285) show a rapid loss whereas diglycerides show a rapid increase (282,286). The newly produced diglycerides are rich in arachidonate and stearate as are the inositolphosphatides (282). The rapid disappearance of the former lipids could suggest that they give rise directly or via diglycerides to the free fatty acids. This may not be since the phosphatidylinositol polyphosphates are concentrated in myelin (287,288) and fatty acid release occurs in grey matter rather than in white matter (289). Furthermore, their rate of degradation post-mortem decreases with age (290-292) whereas that of fatty acid release increases (270). Also, the levels of the above phospholipids are not affected by electroconvulsive shocks (284) which are known to raise free fatty acid levels <u>in vivo</u> in rat brain (268). The concentration of the ethanolamine and choline plasmalogens are reported to decrease in brain during post-decapitative ischemia and hypoxia (293). However, these lipids also predominate in the white matter of nervous tissue (294).

K) Stimulation of Lipolytic Enzymes in Brain

(1) Stimulation and Distribution of Phospholipases of the A Type

In anoxia and during electroconvulsive shocks, the high rate of release of arachidonic acid suggests that a phospholipase A_2 is involved

since the unsaturated fatty acids of brain are esterified mainly at the 2 position of phospholipids (195). Also part of the tetraenoic species of phospholipids, especially that of phosphatidylinositol, is formed by a deacylation-acylation system involving a phospholipase A_2 (295-298). Since the saturated fatty acids account for about half of the fatty acids released (268), a phospholipase A_1 must also be active during anoxia and electroconvulsive shocks. Brain phospholipases of the A type may not be responsive to neurotransmitters and cyclic nucleotides. The acyl moiety of rat brain phospholipids prelabelled with 1-^{14}C acetate is not modified by acetylcholine (299). Carbamylcholine injected *in vivo* in rat brain stimulated the incorporation of arachidonic acid into phosphatidic acid but did so probably by increasing the availability of diglycerides from other phospholipids (301). The report that norepinephrine and cAMP accelerated phospholipase A_2 activity of some subcellular fractions of guinea pig brain has been retracted (302). The failure of 50mM KCl to increase the levels of lysophosphatidylcholine or the incorporation of fatty acids into the phospholipids of rat brain synaptic vesicles also suggests that the phospholipases of the A type of this fraction are unresponsive to neurotransmitters (203). In contrast, the incorporation of oleic acid into brain phospholipids is increased by electrical stimulation applied chronically *in vivo* (304). However, *in vitro*, electrical stimulation does not modify the labelling of the acyl moiety of brain phospholipids (299).

In rat brain the highest phospholipase A_2 activity is found in the mitochondrial fraction and the highest phospholipase A_1 activity is

found in the microsomal fraction at physiological pH (305). Synaptosomes also contain a significant phospholipase A_2 activity. Neurons have 5 to 8 times the phospholipase A activities of glial cells at pH 7.4 (306). Acidic phospholipases are also present in rat, calf and human brain (307,308). The release of fatty acids which occurs upon decapitation in brain probably does not result from the activity of acidic phospholipases since lysosomal enzyme release is a relatively slow process in the anoxic/ischemic brain (309,310).

Brain contains phospholipases of the A type which apparently do not require calcium (308,312) and others which may require divalent cations (311). The stimulation of phospholipase A activity in brain by the application of electroconvulsive shock and by decapitation could result from the rapid fall of the ATP level in the tissue since the nucleotide may chelate the cation. Dramatic decreases of brain ATP levels have been observed under the above conditions (313). Chelation of calcium by ATP has been proposed to explain the inhibition of phospholipase A activity in fresh rat liver mitochondria (278).

The hydrolysis of exogenous phospholipids by partially purified acidic phospholipase A from whole brain is regulated by product inhibition (307). A similar inhibition of brain phospholipases may occur with endogenous substrates at physiological pH since fatty acid release practically stops within 15 minutes post-mortem in rat brain with only a very small percentage of the phospholipids being hydrolyzed (270). Although the lysophosphatidylcholine content of rat cerebral hemispheres increases during incubation in a nitrogen atmosphere (314), the lysophospholipase activity of brain (315,316) probably precludes

a stoichiometric correspondence between the latter lipid and the phosphatidylcholine lost post-mortem in this tissue. Lysophosphatidylinositol has been tentatively identified in ox brain (317). When injected in rat brain, this lipid appears to be degraded very rapidly (195). On the other hand, lysophospholipids are readily acylated in the presence of ATP and coenzyme A by rat brain homogenates or subcellular fraction (196, 318,319). The marked requirement of the acylating system for ATP and coenzyme A suggests that reacylation of fatty acids would be slow in brains left in situ after death of the animal.

(2) Stimulation and Distribution of Neutral Lipid Lipases

Neutral lipid lipases of guinea pig synaptosomal membranes are stimulated by many neurotransmitters and putative neurotransmitters when exogenous substrates are used (320). Activity is found in all subcellular fractions from rat (321) and guinea pig brain (320).

If a neutral lipid is the source of the free fatty acids which appear post-mortem, it would have to turn over completely many times to account for the amount of fatty acids released. However, neutral lipid lipases may be active enough to give rise to the free fatty acids provided new substrate is produced. The brain diglyceride pool does increase rapidly post-mortem (286,282). The diglycerides could be cleaved from the phosphatidylinositols by the action of a phospholipase of the C type (286,296,322). The small brain triglyceride pool (268) contains very little arachidonate (322) but in this lipid, the latter fatty acid has a high turnover rate <u>in vivo</u> (296). However, triglycerides decrease by only 20% in one hour post-mortem in rat brain (268).

3) Stimulation and Distribution of Phospholipases of the C Type

Electrical stimulation, cholinergic and adrenergic agonists increase the turnover of the phosphate portion of phosphatidylinositol and phosphatidic acid and of the inositol moiety of the former lipid in ganglia and brain (323). The metabolism of these phospholipids is stimulated by many neurohormones in glial as well as neuronal enriched fractions (324-326) and cultured cell lines (327). The "phospholipid effect" was first observed in pigeon pancreas by Hokin and Hokin in 1953 (328). Since then, the turnover of phospholipids in many tissues has been shown to respond to various stimuli (323).

The mechanism by which neurotransmitters and electrical stimulation increase the turnover rate of phospholipids in brain and ganglia is not clearly understood. It might be that a specific diglyceride pool is generated by a phosphatidylinositol phosphodiesterase and/or a phosphatidic acid phosphatase sensitive to the above stimuli (297,298,324,330). In secretory cells, these enzymes may be of lysosomal origin (331). The newly produced diglycerides would then be reincorporated into the latter phospholipids. Diglyceride kinase could also be a key enzyme in this "phospholipid effect" but a purified kinase preparation from brain was not affected by acetylcholine (332). The diglycerides which may be intermediates in the stimulated metabolism of the above phospholipids could be substrates for the neutral lipid lipases under certain conditions.

Brain phosphatidylinositol phosphodiesterase activity is found in the soluble and particulate fractions of brain (333,334). The cytoplasmic and bound activities require calcium. There is some correlation between the localization of the membrane bound phosphodiesterase activity and

enzymic activities associated with plasma membranes (333). A molecular aggregation between the enzyme and microtubules of rat brain has also been observed (335). Phosphatidylinositol phosphodiesterase activity of the nerve endings is specifically stimulated by low amounts of acetylcholine (336). Brain phosphatidic acid phosphatase is found in all particulate fractions (326,337) and is more active in grey than white matter (338). Much activity is localized to a plasma membrane fraction derived from synaptosomes (339). The enzyme in all subcellular fractions examined was stimulated by acetylcholine (326).

L) Difficulties in Identifying the Complex Lipid Precursor(s) of Prostaglandins in Brain

A major difficulty in identifying the specific complex lipid precursor(s) of the arachidonate used for prostaglandin synthesis results from the large difference in the amount of this fatty acid in the structural lipids of the tissue relative to the amount of prostaglandins produced. Brain contains milligrams of esterified arachidonate per gram tissue while it produced only a few micrograms of prostaglandins per gram. Since the arachidonate actually transformed to prostaglandins represents an extremely small proportion of that found in the complex lipids, it would be very difficult to follow the change in the level of esterified arachidonate directly related to prostaglandin synthesis.

Another difficulty arises from the fact that nearly every complex lipid in brain contains arachidonic acid (296,280,281) and from the likelihood that endogenous lipids are available to different degrees to the acylhydrolases depending on their distribution in the membranes. Indeed,

subcellular fractions of brain differ in the compositions of their phospholipid classes (280,281,340). Furthermore, the fatty acid composition within a given phospholipid class also differs from fraction to fraction (280,281,340-344). Finally, the phospholipids may be distributed asymmetrically across the plane of the subcellular membranes of brain (345). Thus, the use of exogenous complex lipid substrates to identify the origin of the arachidonic acid used for prostaglandin synthesis would not be very informative since the restrictions imposed by membrane organization on the possible endogenous precursors would not operate.

An additional problem is that very little radioactivity can be found in the prostaglandin produced by brain homogenates or microsomes incubated with radioactive arachidonate in the medium (346). Since trace amounts of prostaglandins may be formed non-enzymatically from arachidonic acid (347), the biosynthesis of these compounds from this radioactive fatty acid cannot be convincingly demonstrated in brain. The low conversion by brain of exogenous arachidonate is probably due for the most part to dilution by the relatively large amount of arachidonate released post-mortem from endogenous lipids. The difference that exists between the amount of free arachidonate present <u>in vitro</u> in the tissue and the amount of prostaglandin produced is such that only a very low percentage of the exogenous precursor fatty acid can be converted to prostaglandins.

Another factor which would limit the conversion of exogenous arachidonate to prostaglandins might be the difficulty of access to the prostaglandin synthetase of exogenous free fatty acids in intact brain tissue.

That this phenomenon exists is suggested by the lack of inhibition of prostaglandin synthesis in cortex slices by eicosatetraynoic acid which is a very effective inhibitor in homogenates of the same tissue (47). Finally, a controlled system of delivery of arachidonate to the prostaglandin synthetase tightly coupled to a specific complex lipid might exclude direct use of exogenous arachidonate.

The origin of the unesterified arachidonic acid released post-mortem in rat brain is of particular interest since the fatty acid is the immediate precursor of the major prostaglandins and thromboxanes produced by the tissue. Some of the experiments described hereafter were designed to identify the complex lipid precursor(s) of the unesterified arachidonic acid by following the specific activity of the arachidonic acid released at different times after decapitation compared to that of the arachidonic acid in individual complex lipids. An inverse relation was also sought between the levels of arachidonic acid and radioactivity in the unesterified fatty acid fraction and those in the complex lipids in rat forebrain. The microsomal fraction from rat forebrain was also studied because most of the prostaglandin synthetase activity is located there.

To study the compartmentation of the arachidonic acid used for prostaglandin synthesis by rat cerebral cortex, the deuterium to protium ratios were measured at various time points in the prostaglandin $F_{2\alpha}$ produced by the tissue slices or homogenates incubated in the presence of octadeuterated arachidonic acid and were compared to the values obtained in the arachidonic acid of different lipid fractions of the

incubated brain tissue. It has been shown that octadeuterated arachidonic acid can be used to measure by gas chromatography-mass spectrometry the specific formation of prostaglandin $F_{2\alpha}$ from exogenous precursors in cerebral cortex slices and homogenates (47).

In vivo experiments were also carried out to establish a correlation between the prostaglandin endoperoxide products which appear during drug induced convulsions and the arachidonic acid released under these conditions in rat forebrain. The effect of anticonvulsants on the post-mortem release of fatty acids was also examined.

CHAPTER 2

MATERIALS AND METHODS

A) Intracerebro-ventricular Injection Procedure

Male Wistar rats (Canadian Breeding Farm, Laprairie, Que.) weighing 250-350 gms were anesthetized with ether and positioned in a stereotaxic head holder. After exposure of the cranium, a 1mm burr hole was made at a point 2mm from the sagittal suture and 1mm from the coronal suture without perforation of the meninges. The opening was sealed with bone wax. A 10µl Hamilton syringe held vertically over the small opening by means of the stereotaxic apparatus, was lowered 4.5cm through the bone wax and meninges down to the lateral ventricle. Injection of methylene blue dye in 3 animals confirmed the placement of the needle into the lateral ventricle. The injection mixture had a volume of 8µl and was delivered in approximately 30 seconds. The needle was held in place 1 minute after injection and withdrawn slowly.

B) Injection of [$^3H_8$]-Arachidonic Acid *In Vivo* and Time Course of Incorporation

Tritiated arachidonic acid (New England Nuclear, Boston, Mass. 80 Ci/millimole) was purified by thin layer chromatography (TLC) on silica gel G with cold arachidonic acid (Nu-Check, Elysian, Minn.) added as carrier. The developing solvent system was isopropylether : n-heptane : acetic acid, 40 : 60 : 3 by volume. The area corresponding to the reference arachidonic acid spot was eluted with chloroform : methanol (C:M) 2 : 1. As a precaution against oxidation all steps including

spotting on TLC were carried out in a nitrogen atmosphere. The purified fatty acid was neutralized with 1mM NaOH and complexed with fatty acid free bovine serum albumin (Sigma, St Louis, Mo) in 5mM phosphate buffer pH 7.4 in a molar ratio of fatty acid to albumin of 5 : 1. The solution for injection contained 4.5μCi of tritium and 2.2μg of carrier arachidonic acid in 8μl of buffer.

The rats were kept in a cage with free access to water for two hours 15 minutes after the intracerebral injections of label. At this time they were killed by decapitation. Complete heads were rapidly frozen in liquid nitrogen. The frozen forebrain regions were chipped out of the skull and the pieces kept in liquid N_2 until weighing and homogenizing in C:M, 2 : 1 by volume. In other cases, heads were kept at room temperature and the tissue homogenized in C:M, 2 : 1 after 2, 15 and 30 minutes post-mortem. After 2 hours and 15 minutes, very little of the label remained as unesterified fatty acid since [$^3H_8$]-arachidonic acid injected intracerebrally has a half life of 5 minutes in the free fatty acid fraction of mouse brain (348).

c) Administration of Drugs

Carbamylcholine (Sigma, St. Louis, Mo.) in normal saline was injected into a cerebral lateral ventricle of adult male Wistar rats (100μl/brain). In some cases, the animals were kept under ether for 30 minutes after injection. The animals were decapitated 30 minutes after injection and the heads were rapidly frozen in liquid nitrogen.

Rats fasted overnight were given intraperitoneally two doses (40 mg/Kg and 80mg/Kg), 10 minutes apart, of pentylenetetrazol (Metrazol,

Bilhuber-Knoll Corp., Orange, NJ.). The animals were decapitated 15 and 75 seconds after the onset of generalized convulsions which occurred approximately 2 minutes after the second injection. The heads were rapidly frozen in liquid nitrogen.

Sodium phenobarbital (Brinkman and Co., Montreal, Que.) 150mg/Kg, phenytoin sodium (Dilantin, Parke-Davis, Mi.) 0.33g/Kg and diazepam (Valium, Roche) 8.5mg/Kg, were administered intraperitoneally. The drugs were given 30 minutes before decapitation. In these experiments, the severed heads were kept at room temperature and the forebrains homogenized in C:M, 2 : 1 by volume at 2 minutes post-mortem. In all other experiments the frozen forebrains were chipped out of the skull and the pieces kept in liquid nitrogen until weighing and homogenization in C:M, 2 : 1 by volume for the extraction of neutral lipids and of phospholipids. Homogenization was done in ethanol when prostaglandins and thromboxanes were to be measured. The symptomatic effect of the drugs were recorded and only those animals which had the expected reactions were used.

D) Extraction, Isolation and Saponification of Lipids

After homogenization of the brain tissues in 20 volumes of C:M, 2 : 1 the filtrate was concentrated and the polar and neutral lipids were separated on short columns of silicic acid (Bio-Rad Laboratories, Richmond, Ca.) and celite (John-Manville, Co.) (2 : 1, w/v). The free fatty acids and neutral lipids were eluted with chloroform and the polar lipids with methanol.

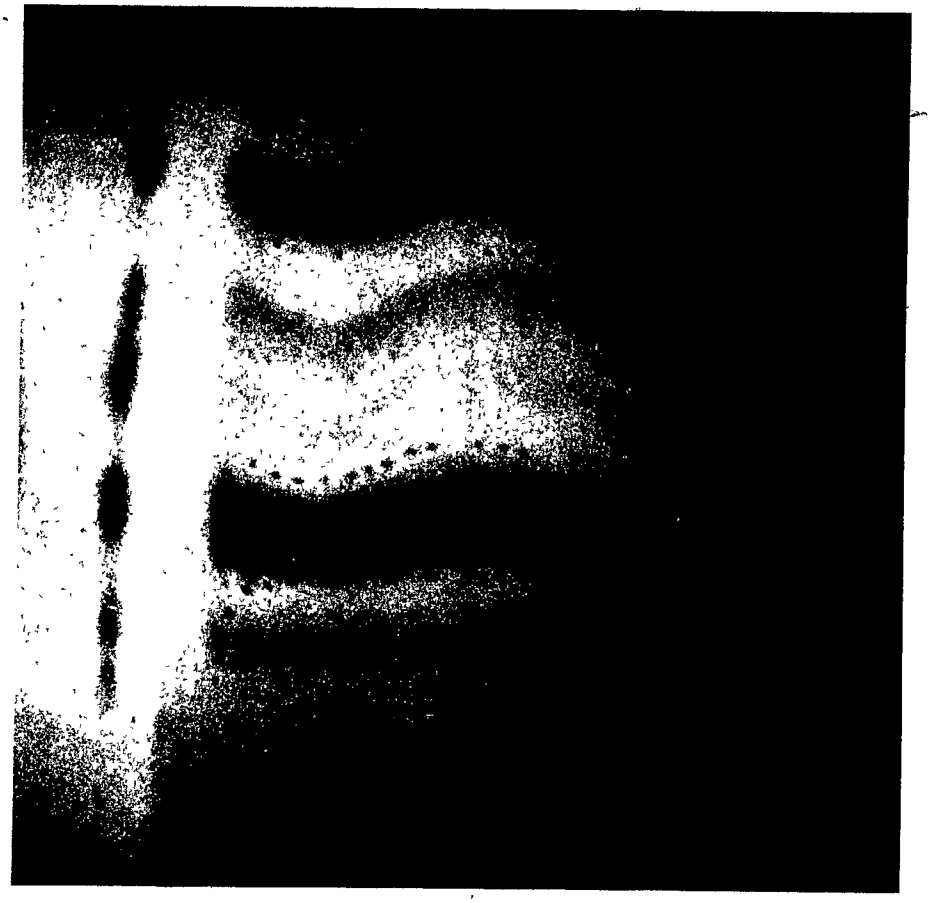
The chloroform eluted lipids were separated by TLC on silica gel G (0.5mm) developed with isopropylether : n-heptane : acetic acid, 40 :

60 : 3. The diglycerides are not well resolved from the cholesterol by the above solvent system. The above two lipids were separated by TLC using chloroform : acetone : methanol, 96 : 4 : 1 as developing solvent mixture (296). The polar lipids were chromatographed on TLC plates of silica gel HR60 (0.5mm) prepared in 1mM Na_2CO_3 according to the method of Skipski et al. (349). These plates were run in chloroform : methanol : acetic acid : water, 75 : 45 : 12 : 6 by volume. The lipids were located with 2,7 dichlorofluorescein dye. The lipids which co-chromatographed with lipid standards (Nu-Check, Elysian, Minn.) were scraped off and eluted with 100ml of C:M 2 : 1 if non-polar or 100ml of methanol if polar. Saponification of lipids and extraction of free fatty acids was done by methods outlined by Christie (350). The efficiency of the hydrolysis and work-up procedure was better than 80% based on the recovery of radioactivity from the lipids extracted from rat brain labelled with tritiated arachidonic acid. It is unusually difficult to separate all the phospholipids in one chromatographic step; however, all but one were clearly resolved by the method of Skipski as seen on the plates (Figure 3). In this system, phosphatidic acid probably contains cardiolipin (349).

E) Subcellular Fractionation

Brain tissue was homogenized after 30 minutes post-mortem in 0.32M sucrose to give a 10% homogenate, w/v. Diisopropylfluoracetate (DFP), a brain phospholipase A inhibitor (308) was added to the sucrose solution at a concentration of 10^{-3}M before homogenization. The previous and subsequent steps were carried out at 0°C. The pellet obtained after centrifugation of the homogenate at 1100g for 11 minutes was discarded while the supernatant was centrifuged under 17,400g for 45 minutes. This

Figure 3: Thin layer chromatograph of rat brain phospholipids prepared according to the method of Skipski (see text). The lipids were located with 2,7 dichlorofluorescein dye. Lipid standards are from bottom: lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and phosphatidic acid.



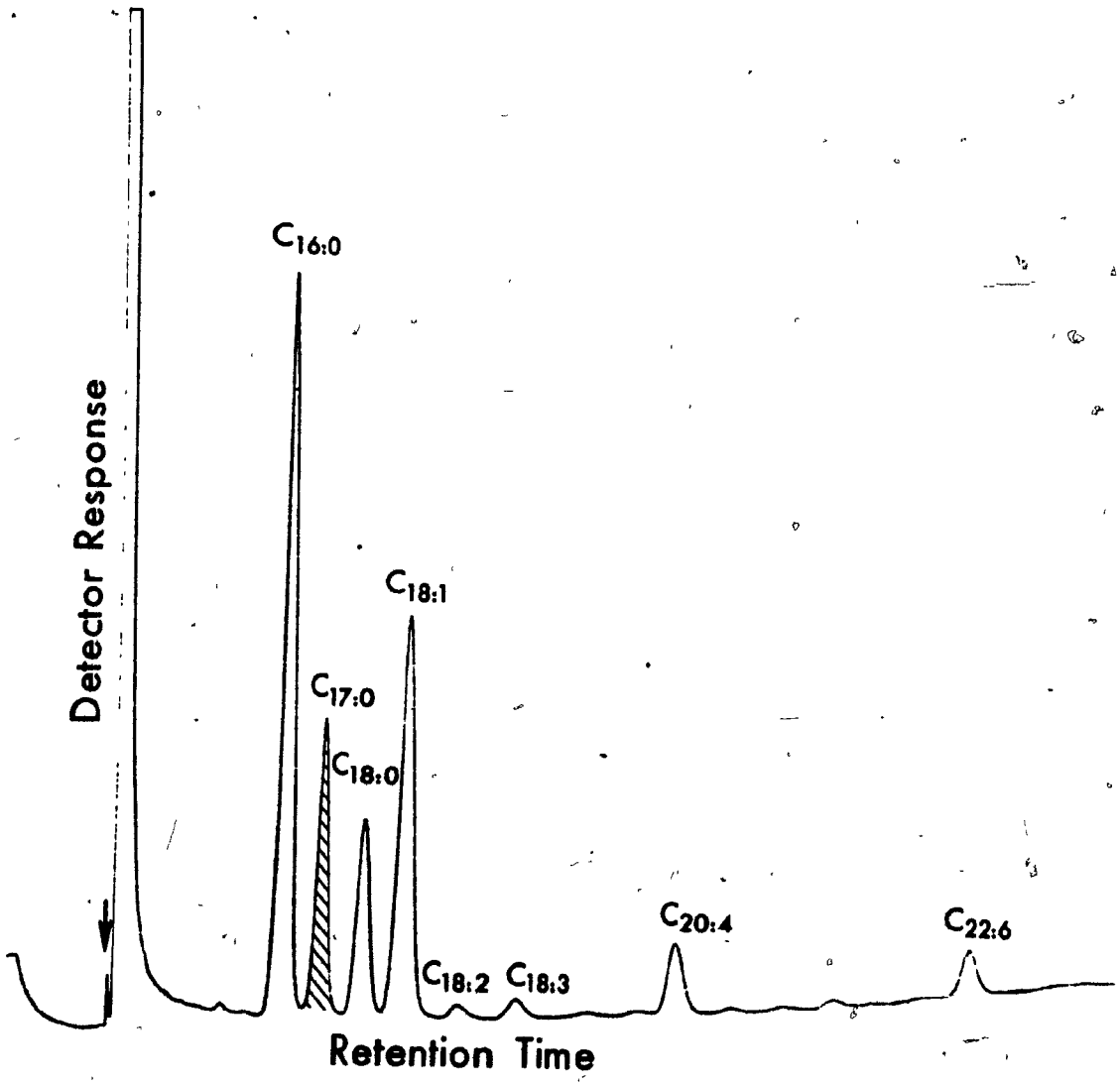
step gave a crude mitochondrial fraction. Further centrifugation of the supernatant at 105,000g for 1 hour yielded a microsomal pellet. Centrifugations were carried out in a Sorval RC2B and a Spinco ultra-centrifuge. Protein determinations were done by the method of Lowry (351).

F) Quantitation of Fatty Acids and Measurement of Radioactivity

Fatty acids were methylated with a ethereal diazomethane in the presence of 10% methanol. These in turn were separated and quantitated by gas liquid chromatography (GLC) on a 10% Silar 10C 6 foot column (Applied Science Lab, State College, Pa.) with a temperature program from 170°C to 230°C increasing at a rate of 3°C/minute. Identification of the fatty acid peaks was done by comparison of their retention times with those of fatty acid standards run under identical conditions (Figure 4). In some instances, the identity of the fatty acids was checked on an LKB-9000 gas chromatograph-mass spectrometer run in the full scan mode using a Silar 10C column and the same temperature program. Source temperature was 250°C, ion current was 60μA and electron energy 20eV. Correction was made for background GLC peaks due to solvents, silicic acid and visualizing spray.

For quantitation of the free fatty acid fraction the internal standard heptadecanoic acid was added to the brain tissues after homogenization in C:M, 2 : 1. When the specific activities of the arachidonic acid from various brain lipids fractions were to be determined, heptadecanoic acid was added after the isolation of the fatty acids. The radioactivity in the fatty acid fractions was assumed to be entirely associated with arachidonic acid since 85% of the radioactivity in rat brain phospholipids

Figure 4: Photograph of a typical gas liquid chromatograph of the fatty acids of rat brain phosphatidylcholine. Sample was run on a 10% silar 10 C 6 ft. column with a temperature program from 170°C to 230°C increasing at a rate of 3°C/min. Heptadecanoic acid ($C_{17:0}$) served as internal standard. The fatty acids are in order of retention times: palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), arachidonic acid ($C_{20:4}$) and docosahexaenoic acid ($C_{22:6}$).


Detector Response
C16:0
C17:0
C18:0
C18:1
C18:2
C18:3
C20:4
C22:6
Retention Time

remained as [$^{3}H_{8}$]-arachidonic acid 24 hours after its administration (352). Radioactivity of all lipids was determined in an Intertechnique liquid scintillation spectrometer using the channel-ratio method with automatic external standardization.

G) Synthesis of Octadeuterated Arachidonic Acid

[5,6,8,9,11,12,14,15-$^{2}H_{8}$ all cis]-eicosatetraenoic acid was synthesized by the method of Hamberg et al (353). The procedure consists of reducing the acetylenic bonds of the corresponding eicosatetraynoic acid (supplied by Dr. D. Garmais, Abbott Laboratories, Montreal, Que.) with deuterium gas to the olefin stage. The reaction is done in the presence of Lindlar's catalyst which gave cis additions to carbon-carbon triple bonds. The deuterium gas was obtained from Merck-Sharp and Dohme, Montreal. The reactions was carried out in methanol at room temperature and at atmospheric pressure. Lindlar's catalyst was synthesized by the method of Lindlar and Dupuis (354) and the amount of quinoline added to further poison it was equal to the weight of the catalyst used.

The hydrogenation reaction was monitored with the manometer of a standard hydrogenation apparatus. The reaction was stopped when an abrupt change in the slope of the hydrogenation curve occured. The catalyst was filtered out, the filtrate diluted with 10 volumes of acidified water (pH 3) saturated with NaCl. The aqueous phase was extracted twice with a double volume of hexane. The organic phase was evaporated and the residue spotted directly on silica gel HR60 (0.5mm) TLC plates containing 6% $AgNO_3$. The plates were activated at 100°C for 2 hours before use. The developing mixture was 2,2,4 trimethylpentane, ethyl acetate and acetic acid (10 : 10 : 0.1). The combined first two

solvents were saturated with water before addition of glacial acetic acid. The band corresponding to the major reaction product (which co-chromatographed with arachidonic acid) was cut out and eluted with C:M, 2 : 1.

The identity and the isotopic purity of the major product was determined by mass spectrometry on a LKB-9000 mass spectrometer interfaced with a Varian SS100MS data machine. The gas chromatography was done on a 6 foot glass column packed with 10% Silar 10C on chromasorb Q. The operating conditions were: oven temperature 200°C, electron energy 20eV, ion source temperature 250°C and trap current 60μA. The compound was analyzed as the methyl ester.

The determination of the chemical purity and the quantitation of the isolated [$^2H_8$]-arachidonic acid was done by gas chromatography using a Silar 10C column. This packing separates geometrical and positional isomers of polyunsaturated fatty acid (355).

H) Synthesis of [$^2H_8$]-Prostaglandin $F_{2\alpha}$

Sheep seminal vesicles (25% w/v) were homogenized in 0.15M phosphate buffer pH 7.4 containing 1mM EDTA and 0.1mM norepinephrine. The supernatant from the first 8000 g x 10 minutes centrifugation was centrifuged at 105,00 g for 60 minutes. The pellet was re-suspended in buffer and centrifuged again to give a microsomal fraction according to Samuelsson et al (356).

Microsomes from 2g of tissue were suspended in 4ml of a 0.15M phosphate buffer pH 7.4 containing 1mM EDTA, 1mM norepinephrine and 1mg of octadeutero-arachidonic acid. The mixture was incubated at 37°C for

25 minutes in a 95% oxygen atmosphere. Ethanol (6 volumes) was added to stop the reaction, the mixture stirred for 20 minutes and then filtered. Tritiated $PGF_{2\alpha}$ (2×10^5 cpm, 1ng) was added to the ethanol extract to monitor recoveries from columns and TLC plates (47). The filtrate was evaporated in vacuo, the residue dissolved in 3ml of distilled water, acidified to pH 3.5 with 1N HCL and introduced into washed Amberlite XAD-2 columns (1.7 x 10cm) which were eluted first with water and then with 100 ml of ethanol. All radioactivity was found in the ethanol fraction. The ethanol fraction was evaporated to dryness, the residue dissolved in 100 µl of C:M (2 : 1 by volume) and applied as a central band to 0.5mm thick Silica Gel G plates and developed with chloroform : methanol : acetic acid : water (90 : 9 : 0.65 by volume). Standards of $PGF_{2\alpha}$ and PGE_2 were run on each side of the plate and detected by spraying with 10% phosphomolybdic acid in ethanol with local heating after covering the central band with a glass plate. The zone of silica gel corresponding to $PGF_{2\alpha}$ was scraped off, placed in glass columns fitted with sintered disks and eluted with 90% methanol. The eluate was evaporated to dryness, 2ml methanol was added, then 30ml water, 80ml diethyl ether and the lower phase was acidified to pH 3.0 with 1N HCL. The lower phase was extracted again with 80ml of diethyl ether and the ether phase was washed with distilled water until neutral. The combined organic phases were evaporated to dryness and redissolved in 6% methanol in chloroform. Samples were then introduced onto Pasteur pipette silicic acid - HA columns (0.6 x 5.3cm) and eluted with 2% methanol in chloroform (5ml) then with 20% methanol in chloroform ($PGF_{2\alpha}$ fraction). The compound was analyzed as the methyl ester, TMS derivative. An aliquot of the isolated material

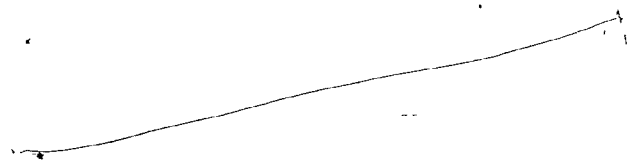
was evaporated to dryness and methylated by treating the sample with 0.5ml freshly distilled ethereal diazomethane (9 : 1 by volume) for 60 minutes at room temperature. Samples were then transferred to small capillary tubes then fitted with rubber septa. TMS ether derivatives of the $PGF_{2\alpha}$ fractions were prepared by adding 10µl of Tri-Sil Z (Pierce Chemical Co., Rockford, Ill.) and heating at 60°C for 5 minutes. The identity and the isotopic purity of the extracted $PGF_{2\alpha}$ were determined by mass spectrometry. The gas chromatography was carried out on a 6 foot glass column packed with 1% OV-101 on chromasorb W HP. The operating conditions were: oven temperature 220°C, electron energy 20eV, ion source 290°C and trap current 60µA. The $[^2H_8]$-$PGF_{2\alpha}$ was quantitated by G.C. using a glass column packed with 1% SE 30 with an oven temperature of 220°C and 20-ethyl $PGF_{2\alpha}$ was used as internal standard (Figure 5). The C-value of these compounds was determined and the G.C. response of the internal standard compared to that of authentic $PGF_{2\alpha}$ (gift from Dr. John E. Pike, Upjohn Co., Kalamazoo, Mich.).

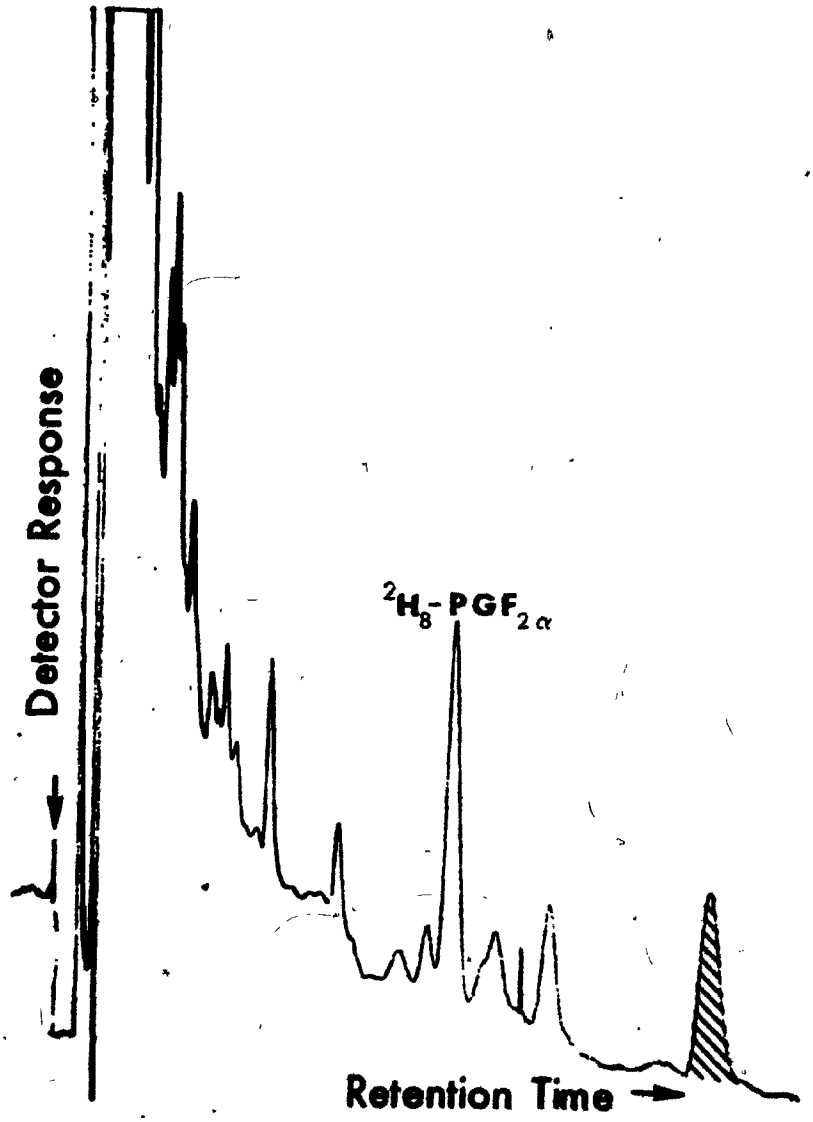
The amount of protium form in $[^2H_8]$-$PGF_{2\alpha}$ was quantitated by multiple ion analysis using $[3,3,4,4,-^2H_4]$-$PGF_{2\alpha}$ (supplied by Dr. John E. Pike, Upjohn Co.) as internal standard (47). The ions monitored were m/e 423 for the protium form and 427 for the tetradeuterated form.

I) Synthesis of $[^2H_8]$-Thromboxane B_2

$[5,6,8,9,11,12,14,15-^2H_8]$, thromboxane B_2 was prepared by incubation of freshly collected human platelets for 5 minutes at 37°C withh 800µg of octadeutero-arachidonic acid. Also added were 7µCi of $[1-^{14}C]$-arachidonic acid (Amersham, England, 58mCi/mmole).

Figure 5. Photograph of a gas liquid chromatograph of the prostaglandin $F_{2\alpha}$ produced by sheep seminal vesicle microsomes incubated in the presence of [$^2H_8$] arachidonic acid. The prostaglandin $F_{2\alpha}$ isolated from the reaction mixture was run on a 1% SE 30 6 ft. glass column with an oven temperature of 220°C. The internal standard was 20-ethyl $PGF_{2\alpha}$. The C-value for $PGF_{2\alpha}$ was 24.1 and for the internal standard, 25.7.




Detector Response
$^2H_8$-PGF$_{2\alpha}$
Retention Time

The platelets were collected with 7.5% (v/v) of 77mM EDTA and centrifuged at 200 x g for 15 minutes in siliconized tubes at room temperature. The plasma was transferred with siliconized pipettes to other tubes and centrifuged at 650 g for 15 minutes. The pellets from the plasma suspended in 0.15M NaCL - 0.15M Tris HCl buffer pH 7.4 - 77mM sodium EDTA (90 : 8 : 2 by volume) and centrifuged again at 650 g for 15 minutes. This last pellet was suspended in Krebs-Hanseleit medium without calcium for the incubation (14). The following purification was done by the method of Hamberg et al (156). The ethanol extract (20 volumes) was evaporated to dryness in vacuo, the residue dissolved in 20ml of acidified water and partitioned with ether as described in the preceeding section for the isolation of $PGF_{2}\alpha$. The organic phases were combined, evaporated to dryness and the residue redissolved in ether : petroleum ether (25 : 75) for transfer to silica gel columns (3g SilHA, Bio-Rad Laboratories, Ca.). The columns were eluted with 10ml of the following solvents: ether : petroleum ether (25 : 75) and (40 : 60) and ethyl acetate. The last fraction was evaporated and the residue methylated as described for $PGF_{2}\alpha$. The methylated material was then chromatographed on silica gel G plates using as solvent system the organic upper phase of ethyl acetate: 2,2,4 trimethylpentane : water, 25 : 50 : 100. Thromboxane B_2 standard (Upjohn Co.) was spotted on the sides of the plate and was later visualized by spraying with phosphomolybdic acid and local heating. The band corresponding to the standard TxB_2 was eluted with ethyl acetate : methanol (3 : 2). The eluting solvent was then evaporated and the residue spotted on a second TLC plate of silica gel G and developed in ehtyl acetate saturated with water.

The TMS ether, methyl ester derivatives of the compound which co-chromatographed with a standard TxB_2 was analyzed by GC-MS. A 6 foot glass column packed with 1% OV-101 and an oven temperature of 220°C were used. Ion source temperature was 290°C, electron energy 70eV and trap current 60μA. The mass spectrometer was operated in the full scan mode to obtain the complete mass-spectrum of the compound for identification and estimation of isotopic purity. Multiple ion analysis using the alternating voltage acceleration was also used to quantitate $[^2H_8]$-TxB_2 against standard TxB_2. The fragment ions monitored were the base peaks of the above compounds with m/e of 260 and 256 respectively. The multiple ion analysis was performed at an electron energy of 20eV. The GC-MS response of labelled against unlabelled TxB_2 is linear and the standard curve has a slope of 1 according to Hamberg et al (358). The C-value of the derivatized $[^2H_8]$-TxB_2 was obtained on the same column as above. The % protium in the $[^2H_8]$-TxB_2 could be easily obtained from mass fragmentograms since the intensities of the ions monitored for these two isotopic forms of the compound would be equal for equal amount of material

J) Preparation and Incubation of Cerebral Cortex Tissues for Production of PG Endoperoxide Derivatives

Unanaesthetized male Wistar rats were decapitated and after quick removal of the brain, two to three slices of cerebral cortex, 0.5mm thick were cut out with a Stadie-Riggs blade in a humid chamber. To measure the production in vitro of $PGF_{2\alpha}$ from endogenous and exogenous precursors, the slices were incubated immediately for 5 and 60 minutes in 3ml of

Ringer-Bicarbonate glucose (RBG) medium, pH 7.4 containing 25μg of [$^2H_8$]-arachidonic acid per 100mg tissue with or without 1mM norepinephrine at 37°C in a 95% O_2 - 5% CO_2 atmosphere. The time elapsed between decapitation and beginning of incubation was 5 minutes. Homogenates were prepared from slices suspended in the incubation medium in a glass homogenizing tube with a teflon pestle rotating at 2000 rpm for 6 thrusts.

K) Extraction of PG Endoperoxide Products and of Other Lipids

At the end of the incubation period the slices were homogenized in organic solvents separately from the medium or combined with it. Ethanol (20 volumes) was used to extract the prostaglandins and thromboxane B_2 and C:M (2 : 1) to extract the neutral and polar lipids. In the latter case, the separated slices were washed for 3 minutes in buffer containing fatty acid-free bovine serum albumin in a molar ratio of 5 : 1 of tissue fatty acids to bovine serum albumin before homogenization (359).

The organic solvent extracts were worked-up according to the procedure already described in preceeding section on the isolation of octadeutero-prostaglandins $F_{2\alpha}$ produced by sheep seminal vesicle microsomes and of octadeuterothromboxanes B_2 from blood platelets or on the isolation of individual lipids from rat cerebral hemispheres (see pages 36,45,49).

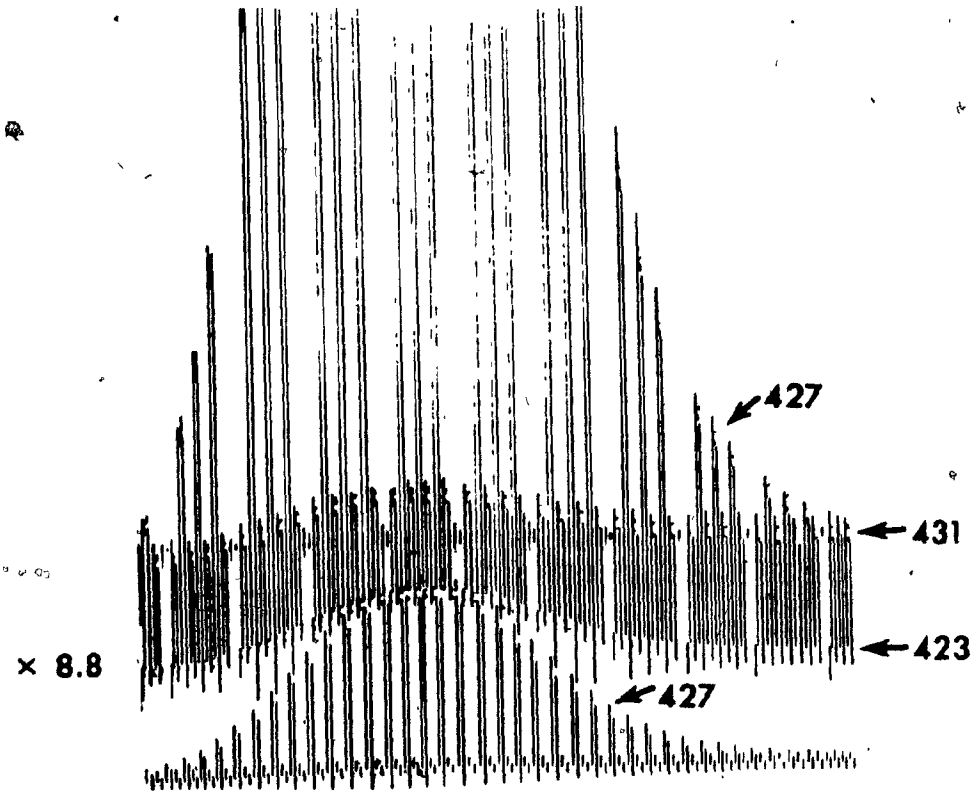
L) Quantitation of $PGF_{2\alpha}$ Produced by Cerebral Tissue

[3,3,4,4,$^2H_4$]-$PGF_{2\alpha}$ served as internal standard to quantitate by gas chromatography-mass fragmentography, the $PGF_{2\alpha}$ and the octadeutero-$PGF_{2\alpha}$ produced by cerebral tissue. The [$^2H_4$]-$PGF_{2\alpha}$ (one microgram) was added

to the ethanol extract at the start of the purification procedure. Analysis of the samples were carried out immediately after derivatization on a LKB-9000 instrument using the multiple ion detector unit to monitor intensities alternatively of the protium and deuterated prostaglandins in the same injection sample. The ions monitored for the TMS methyl esters of $PGF_{2\alpha}$, $[^2H_4]$-$PGF_{2\alpha}$ and $[^2H_8]$-$PGF_{2\alpha}$ were m/e 423, 427 and 431 respectively. Gas chromatography was carried out on a 6 foot glass column packed with 1% OV-101 Chromasorb W-HP. The operating conditions were: oven temperature 220°C, electron energy 20eV, ion source 290°C and trap current 60μA (Figure 6).

Standard mixtures of $PGF_{2\alpha}$ and $[^2H_4]$-$PGF_{2\alpha}$ were run with every group of four to six samples (5, 10 and 20ng $PGF_{2\alpha}$/μg $[^2H_4]$-$PGF_{2\alpha}$). The amount of protium in the deuterated standard was subtracted from sample measurements. A standard line was prepared from mixtures of 20 to 80ng of $[^2H_8]$-$PGF_{2\alpha}$ added to 1μg of the internal standard $[^2H_4]$-$PGF_{2\alpha}$ and was plotted as ng $[^2H_8]$-$PGF_{2\alpha}$ measured versus ng $[^2H_8]$-$PGF_{2\alpha}$ per μg $[^2H_4]$-$PGF_{2\alpha}$. The contribution of the internal standard to the intensity of the ion at m/e 431 was subtracted from that of $[^2H_8]$-$PGF_{2\alpha}$. The deuterium to protium ratio in the $PGF_{2\alpha}$ produced by rat cerebral cortex incubated for 5 to 60 minutes in the presence of $[^2H_8]$-arachidonic acid was obtained by taking the ratio of the amount of $[^2H_8]$-$PGF_{2\alpha}$ to the amount of protium form produced during incubation. The amount of $PGF_{2\alpha}$ synthesized during the time required to prepare the tissue for incubation as measured by Wolfe et al (47) was subtracted from the $PGF_{2\alpha}$ recovered from the incubated slice. In establishing a precursor-product relationship between the arachidonic acid in a lipid

Figure 6: Photograph of a mass fragmentogram of a prepared mixture consisting of 1µg of $[^2H_4]$-$PGF_{2\alpha}$ and 20 ng of $PGF_{2\alpha}$ and $[^2H_8]$-$PGF_{2\alpha}$. Analysis was carried out on an LKB 9000 gas chromatograph-mass spectrometer using the accelerating voltage alternator device for multiple ion detection. The ions monitored for the trimethylsilylether methyl esters of $PGF_{2\alpha}$, $[^2H_4]$-$PGF_{2\alpha}$ and $[^2H_8]$-$PGF_{2\alpha}$ were m/e 423, 427 and 431 respectively. The tracings produced by the two galvanometers are attenuated in the ratio of 1 to 8.8. Retention time for $PGF_{2\alpha}$ was approximately 4 minutes on a 1% OV 101 6 foot column at 220°C. The difference in the level of the base lines for the different fragment ions is due to column bleed.


427
431
423
× 8.8
427

fraction and the $PGF_{2}\alpha$, a correction for the enrichment in deuterium of the product over the precursor should be considered. The estimation of the percentage enrichment is described in the appendix (see page 117).

M) Quantitation of the TxB_2 Produced by Cerebral Tissues

[5,6,8,9,11,12,14,15-$^{2}H_{8}$]-TxB_2 prepared previously served as internal standard to quantitate the TxB_2 produced by cerebral tissues. To the ethanol extract 1.5 to 2μg of [$^{2}H_{8}$]-TxB_2 containing [1-^{14}C]-TxB_2 (4×10^{5} cpm) was added. The radioactive TxB_2 allows monitoring of recoveries from columns and thin layer plates.

TxB_2 was analyzed as the methyl ester-TMS ether derivatives prepared as described previously for [$^{2}H_{8}$]-$PGF_{2}\alpha$. Analysis was done by multiple ion monitoring with an LKB-9000 instrument. The ions monitored were m/e 256 for TxB_2 and m/e 260 for [$^{2}H_{8}$]-TxB_2. A 6 foot glass column packed with 1% OV-101 was used with an oven temperature of 220°C. The operating conditions were the same as used in the GC-MS of the [$^{2}H_{8}$]-TxB_2 prepared previously from blood platelets (see page 50). The amount of protium in the deuterated standard was subtracted from sample measurements and standard mixtures were prepared.

N) Measurement of D/H in the Arachidonic Acid from Lipids of Cerebral Cortex Lablled <u>In Vitro</u>

The methyl ester of the arachidonic acid from the free fatty acid fractions or from the isolated complex lipids were also analyzed using the multiple ion detection unit of the LKB-9000 mass spectrometer. The

ions monitored alternatively were the molecular ion of the protium and the octadeutero form of arachidonic acid (m/e 318 and 326 respectively). The operating conditions were the same as used for the analysis of chemically prepared $[^2H_8]$-arachidonic acid (see page 56). Standard mixtures of $[^2H_8]$ and $[^1H_8]$-arachidonic acid were prepared and the line relating the amount of deuterated arachidonic acid in these mixtures to the amount measured by mass-fragmentography was obtained. Undeuterated arachidonic acid served as internal standard.

O) Isolation and Quantitation of Prostaglandin Endoperoxides, Prostaglandin $F_{2\alpha}$ and Thromboxane B_2 Produced by Rat Brain In Vivo

As described in section C, the brains of rats previously injected intracerebroventricularly with various drugs or with saline were frozen in liquid nitrogen. The frozen tissues were homogenized in 10 volumes of ethanol and then filtered. The $PGF_{2\alpha}$ and the TxB_2 were extracted and quantitated as described in sections H and I. The amount of prostaglandin endoperoxide present in the frozen tissue was determined as the increase in $PGF_{2\alpha}$ when stannous chloride (20mM) was added to the ethanol used for extraction (14).

CHAPTER 3

RESULTS

A) Fatty Acid Composition of Phospholipids Isolated From Rat Forebrain

The fatty acid composition of the phospholipids of rat forebrain given in Table 2 is very similar to that of whole rat brain (149, 195, 360). The fatty acid composition of the phosphatidic acid plus cardiolipin fraction is also close to that found for phosphatidic acid of rat whole brain. However, the percentage of docosahexaenoic acid is much higher in the above lipid mixture than in the purified phosphatidic acid. Since brain cardiolipin contains only traces of docosahexaenoic acid (149), a third component rich in the latter fatty acid must be present in the lipid mixture. Phosphatidylethanolamine which runs close to phosphatidic acid and cardiolipin on the thin layer chromatogram may be the contaminating lipid.

The fatty acid composition of phosphatidylserine has not been reported for rat whole brain. However, the fatty acid composition of this phospholipid in rat forebrain is very similar to that reported for rabbit cerebral cortex (280). In this case also, docosahexaenoic acid is present in higher proportion in the rat forebrain lipid but the same high percentage of the latter fatty acid has been found in the phosphatidylserine of human grey matter (149). Myristate, palmitoleate, linoleate, linolenate and docosatetraenate each accounted for at most four percent of the fatty acids of individual phospholipids.

TABLE 2

PERCENTAGE COMPOSITION OF THE MAJOR FATTY ACIDS FROM PHOSPHOLIPIDS OF RAT FOREBRAIN FROZEN IN LIQUID NITROGEN UPON DECAPITATION

Total fatty acids from phosphatidylethanolamine contained 5-6% of docosatetraenoic acid (22 : 4). Each value is the mean of three determinations given with the standard deviation (± S.D.)

Lipid	Palmitate 16 : 0	Stearate 18 : 0	Oleate 18 : 1	Arachidonate 20 : 4	Docosahexaenoate 22 : 6
Phosphatidylinositol	10.2 ± 0.16	33.8 ± 2.3	16.7 ± 1.4	32.7 ± 1.5	6.6 ± 1.3
Phosphatidylcholine	44.5 ± 4.8	14.1 ± 1.0	31.4 ± 2.1	7.1 ± 0.7	4.6 ± 0.01
Phosphatidylethanolamine + Plasmalogens	10.2 ± 1.5	27.5 ± 4.5	24.1 ± 4.2	14.7 ± 1.6	22.6 ± 0.7
Phosphatidylserine	4.8 ± 2.0	42.4 ± 3.7	19.1 ± 0.5	3.6 ± 0.5	30.1 ± 1.6
Phosphatidic acid + Cardiolipin	21.1 ± 3.5	15.7 ± 3.1	38.6 ± 4.8	13.7 ± 1.6	11.0 ± 4.6

B) Analysis of $[^2H_8]$-Arachidonic Acid Prepared from Eicosatetraynoic Acid

The chemical purity of the arachidonic acid prepared from eicosatetraynoic acid was better than 98% as evaluated by gas chromatography. The mass spectra of this product taken at the descending slope of the gas-chromatograph peak maximum showed a molecular ion of m/e 326 and no ions of m/e 318-325 which indicates the presence of only octadeuterated arachidonic acid (Figure 7). The line relating the amount of $[^2H_8]$-arachidonic acid in the standard mixture to the amount measured had a slope very close to the theoretical 45° (figure 8a) indicating that equal amounts of deuterated and undeuterated arachidonic acid would give rise to molecular ions of nearly equal intensities.

C) Mass-Spectrometric Determination of the Identity and Purity of $[^2H_8]$-Prostaglandin $F_{2\alpha}$

The mass-spectrometric analysis of the $PGF_{2\alpha}$ produced by sheep seminal vesicle microsomes incubated in the presence of $[^2H_8]$-arachidonic acid indicated that this product contained mainly $[^2H_8]$-$PGF_{2\alpha}$. However, the mass spectrum contained an ion at m/e 423 derived from $[^1H_8]$-$PGF_{2\alpha}$. Quantitation of this ion by multiple ion analysis using $[^2H_4]$-$PGF_{2\alpha}$ as internal standard showed that $[^1H_8]$-$PGF_{2\alpha}$ represented 18% of the total $PGF_{2\alpha}$ quantitated by gas chromatography. The mass spectrum of the $[^2H_8]$-$PGF_{2\alpha}$ as the methyl ester, trimethylsilyl ether derivative had a M+ of m/e 592 and all the prominent fragment ions already reported (361). (Table 3). The line relating the standard amounts of $[^2H_8]$-$PGF_{2\alpha}$ added

Figure 7. Mass spectrum of $[^2H_8]$-arachidonic acid methyl ester. The spectrum was taken on an LKB 9000 GC-MS instrument. Operating conditions are described in the text. The background was automatically subtracted from the spectrum by a SS100 Varian computer interfaced to the GC-MS. The molecular ion has a m/e value of 326.

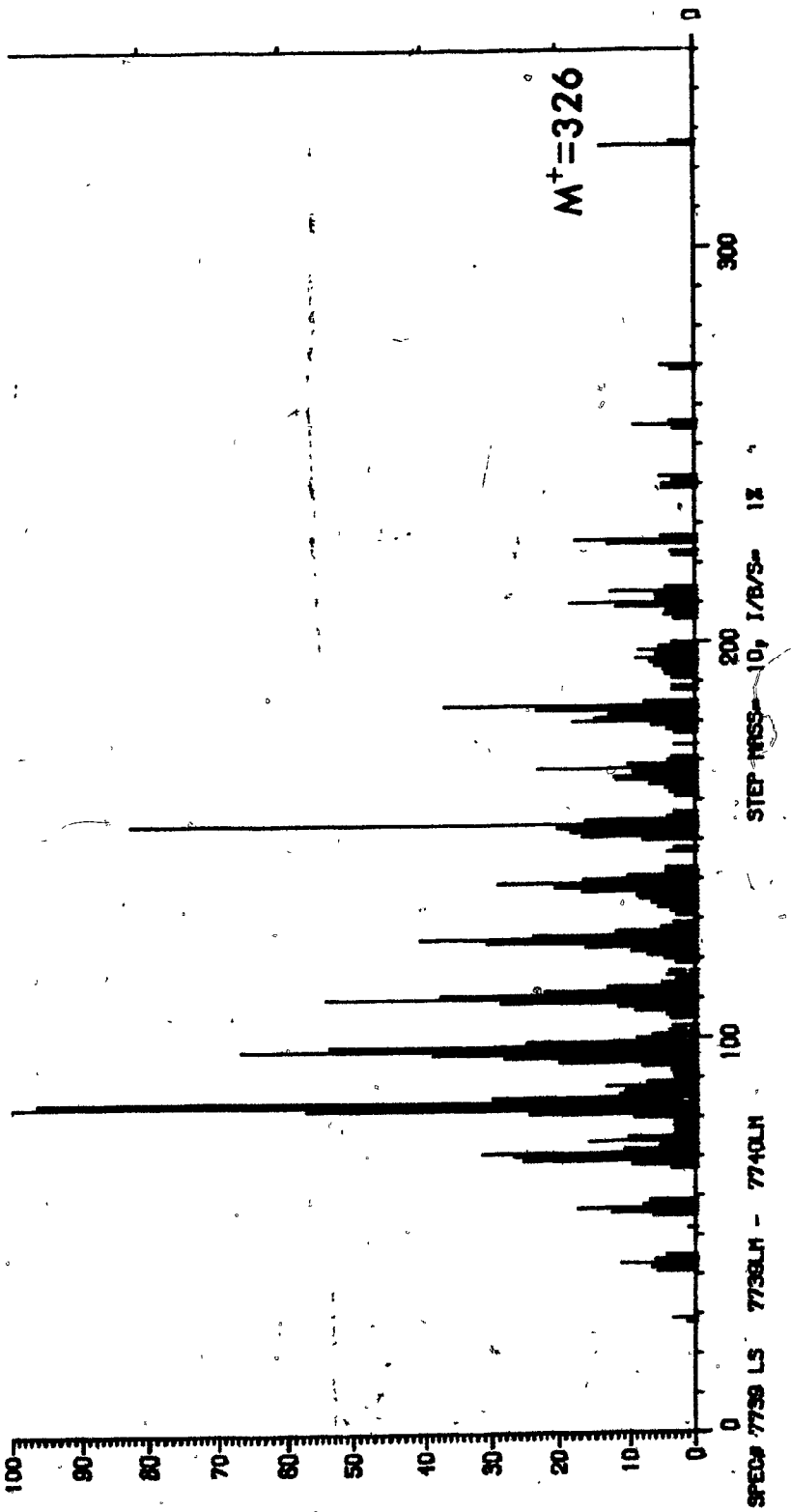

M+=326
SPEC# 7739 LS 7738LH - 7740LH
STEP MASS= 10, I/B/S= 1%
0
100
200
300
100
90
80
70
60
50
40
30
20
10
0

Figure 2.

a. Standard line relating the amount of [$^{2}H_{8}$]arachidonic acid added to 1 µg of [$^{1}H_{8}$]arachidonic acid and the amount of [$^{2}H_{8}$]arachidonic acid measured by mass fragmentography. The undeuterated arachidonic acid served as internal standard. The multiple ion detector unit of the LKB 9000 GC-MS was used to monitor alternatively ion intensities of the deuterated and undeuterated arachidonic acid. Operating conditions are described in the test. Fragment ions corresponding to the molecular ions of the [$^{2}H_{8}$] and [$^{1}H_{8}$]arachidonic acid were monitored (i.e. m/e values of 326 and 318, respectively). The slope of the line is 0.93.

b. Standard line relating the amount of [$^{2}H_{8}$]$PGF_{2\alpha}$ added to 1 µg of [$^{2}H_{4}$]arachidonic acid and the amount of [$^{2}H_{8}$]$PGF_{2\alpha}$ measured by mass fragmentography. The tetradeuterated $PGF_{2\alpha}$ served as internal standard and carrier. Operating conditions are described in the text. The fragment ions monitored for the TMS methyl ester derivatives of [$^{2}H_{8}$] and [$^{2}H_{4}$]$PGF_{2\alpha}$ were m/e 431 and 427, respectively. The slope of the standard line is 0.49.

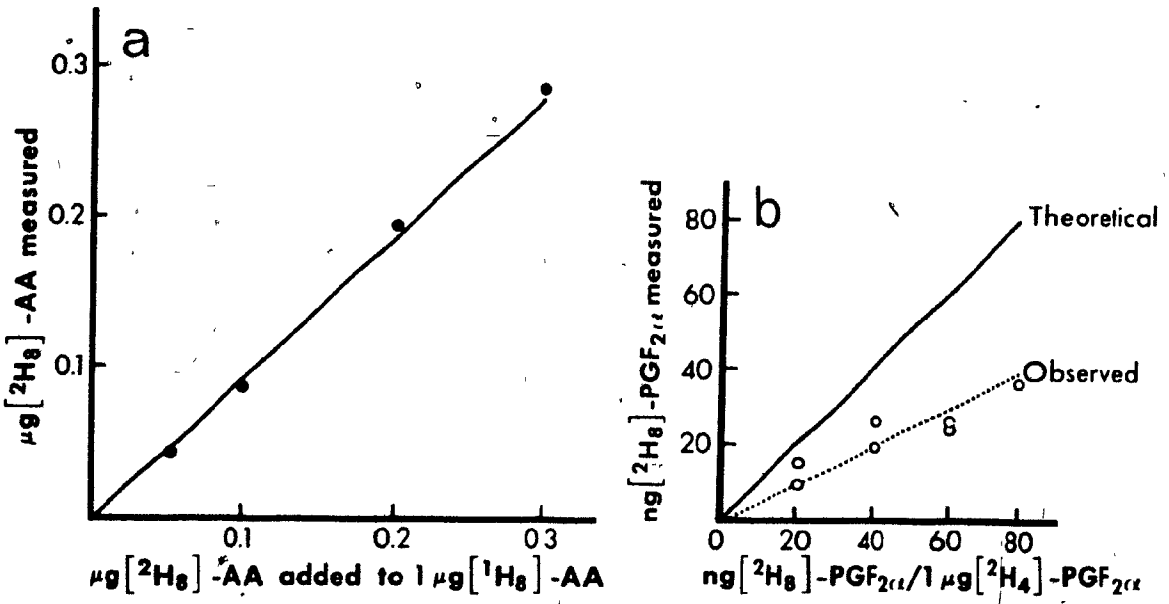

a
0.3
0.2
0.1
μg[²H₈]-AA measured
0.1
0.2
0.3
μg[²H₈]-AA added to 1 μg[¹H₈]-AA
b
80
60
40
20
ng[²H₈]-PGF₂α measured
Theoretical
Observed
0
20
40
60
80
ng[²H₈]-PGF₂α/1 μg[²H₄]-PGF₂α

TABLE 3

Prominent Mass Spectral Fragments of $[^2H_8]$-PGF$_{2\alpha}$

Fragment Ions	m/e value
Molecular ion	592
$M^+ - CH_3$	577
$M^+ - C_5H_{11}$	521
$M^+ - {}^1$HOTMSi	502
$M^+ - {}^2$HOTMSi	501
$M^+ - (C_5H_{11} + {}^1$HOTMSi)	431
$M^+ - (C_5H_{11} + {}^2$HOTMSi)	430
$M^+ - ({}^1$HOTMSi + ^{2}HOTMSi)	412
$M^+ - (2 \times {}^2$HOTMSi)	411
$M^+ - (C_5H_{11} + {}^1$HOTMSi + ^{2}HOTMSi)	340
$M^+ - (C_5H_{11} + 2 \times {}^2$HOTMSi)	339
$TMSiO^+ = C^2H$-OTMSi	192
$TMSiO^+ = C^2H$-C_5H_{11}	174

to 1μg of $[^2H_4]$-$PGF_{2\alpha}$ and the amount of $[^2H_8]$-$PGF_{2\alpha}$ measured by multiple ion analysis had a slope much less than 45° (Figure 8b). This deviation is due to two similar fragmentations of $[^2H_8]$-$PGF_{2\alpha}$ to yield ions with m/e values of 430 and 431 of nearly equal intensities. The latter ion, used for quantitation, arises from the loss of C_5H_{11} + TMSO^{1}H fragments and the former from the loss of C_5H_{11} + TMSO^{2}H fragments. The slope of the standard line (0.49) was used for calculating the amount of $[^2H_8]$-$PGF_{2\alpha}$ produced by cerebral cortex tissues incubated with $[^2H_8]$-arachidonic acid since $[^2H_4]$-$PGF_{2\alpha}$ also served as internal standard in this case.*

D) Mass-Spectrometric Determination of the Identity and Purity of $[^2H_8]$-Thromboxane B_2

$[^2H_8]$-Thromboxane B_2 trimethylsilylether, methyl ester should have a molecular ion at m/e 608 (362). The ion was not found in the mass-spectrum of the compound synthesized by human blood platelets incubated in the presence of $[^2H_8]$-arachidonic acid (Figure 9). However, the fragment ion giving rise to the base peak at m/e 260 probably corresponds to the one giving rise to the base peak of non-deuterated thromboxane B_2 at m/e 256. This fragment ions contains carbons 5,6,8 and 9 which, in the labelled thromboxane B_2, would carry a deuterium atom each. The relationship between fragment ions reported in Table 4 and those of thromboxane B_2 (362) suggest that the compound isolated from human blood platelets is

*The values which we reported in reference 9 for the amount of $[^2H_8]$-$PGF_{2\alpha}$ synthesized by rat cerebral cortex in vitro are low since standard line for the measurement of this product had not been made at that time.

Figure 9: Mass spectrum of the trimethylsilylether methyl ester of the $[^2H_8]$-thromboxane B_2 synthesized by human blood platelets incubated in the presence of $[^2H_8]$-arachidonic acid. The spectrum was taken on an LKB-9000 gas chromatography-mass spectrometer. The base peak at m/e 260 arises from a tetra-deuterated fragment ion. The molecular ion is not seen however, the ion at m/e 593 corresponds to M^+-CH_3. Other prominent fragment ions of $[^2H_8]$-thromboxane B_2 can be related to those of thromboxane B_2 (see text). The retention time for $[^2H_8]$-thromboxane B_2 was approximately 4 minutes on 6 foot column OV101 1% at 220°C.

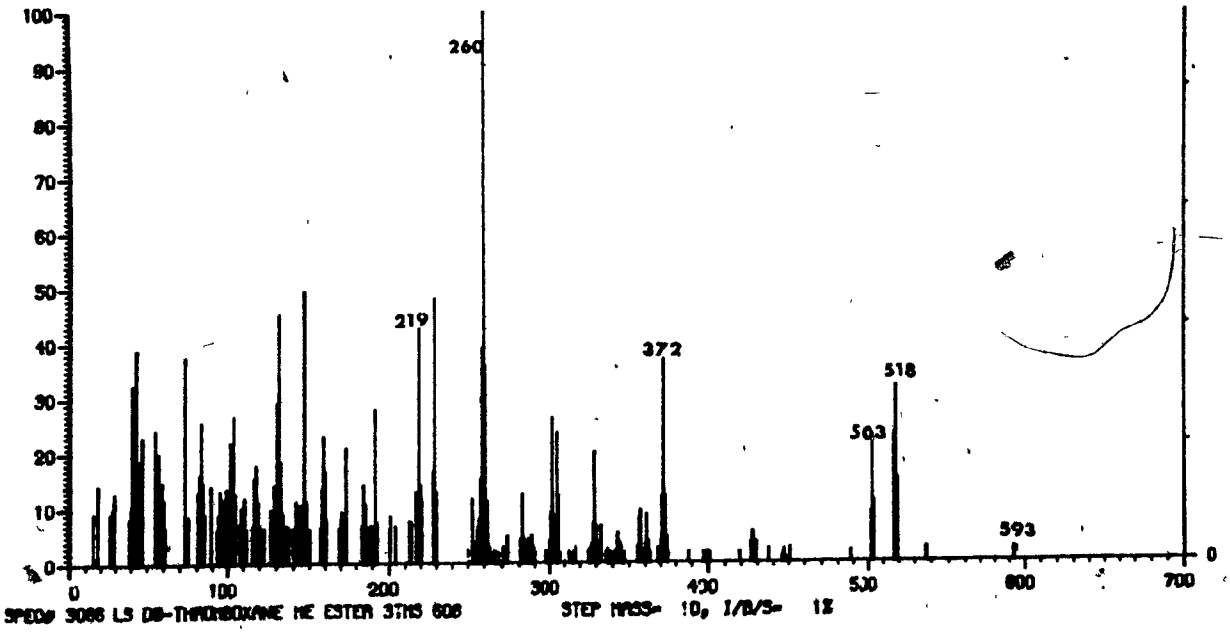

100
90
80
70
60
50
40
30
20
10
0
260
219
372
518
503
593
0
100
200
300
400
500
600
700
SPECH 3086 LS DB-THROMBOXANE ME ESTER 3TMS 60E
STEP MASS= 10, I/B/S= 1%

TABLE 4

Prominent Mass Spectral Fragment of $[^2H_8]$-Thromboxane B$_2$

Fragment Ions	m/e value
$M^+ - CH_3$	593
$M^+ - C_5H_{11}$	537
$M^+ - HOTMSi$	518
$M^+ - {}^2HOTMSi$	517
$M^+ - (HOTMSi + CH_3)$	503
$M^+ - ({}^2HOTMSi + CH_3)$	502
Trimethylsilyl ether, methyl ester $[^2H_6]$-HHT	372
$TMSiO\text{-}C^2H = C^2H\text{-}CH_2\text{-}C^2H = C^2H\text{-}(CH_2)_3\text{-}COOCH_3$	260
$TMSio = C^2H\text{-}CH = C^2H\text{-}OTMSi$	219
$TMSiO = C^2H\text{-}C_5H_{11}$	174

$[^2H_8]$-thromboxane B_2. Furthermore, the C value of the latter compound was 24.4, nearly the same as that reported for thromboxane B_2. A fragment ion with a m/e of 256 was present in the mass-spectrum of $[^2H_8]$-thromboxane B_2 but represented only 1% of the one at 260 as measured by multiple ion analysis indicating that very little protium form is present in the octadeuterated thromboxane B_2.

E) Origin of the Arachidonic Acid Released Post-Mortem in Rat Forebrain

1) Release of Arachidonic Acid from Rat Brain Lipids Post-Mortem

The level of all free fatty acids in rat forebrain quickly removed from heads frozen in liquid nitrogen upon decapitation was very low (Table 5). The total amount of the free fatty acids (40µg/g tissue) was the same as that reported for the brain of rats killed by immersion of the whole animal in liquid nitrogen (270). A rapid increase in the level of all the major free fatty acids and the preferential release of arachidonic acid was also observed in this tissue shortly after decapitation in agreement with previous reports (268,270,272). Arachidonic acid was released at a much faster rate than all the other fatty acids since between zero and 2 minutes post-mortem there was a large disproportionate increase in this free fatty acid. However, between 2 and 15 minutes post-mortem the release of arachidonic acid was less specific since its percentage in the free fatty acid did not increase.

2) Post-Mortem Changes in the Specific Activity of Various Lipids From Rat Brain Injected with $[^3H_8]$-Arachidonic Acid

TABLE 5

MAJOR FATTY ACIDS RELEASED POST-MORTEM IN RAT FOREBRAIN

Zero times values obtained from forebrain of heads quickly frozen in liquid nitrogen upon decapitation. Values at 2 and 15 minutes post-mortem obtained from forebrain of severed heads kept at room temperature. Rats had received an intracerebroventricular injection of [$^3H_8$]arachidonic acid 2 hours and 15 minutes before decapitation. Results expressed as mean ± S.D. of three determinations.

	μg fatty acid/g tissue			Percentage composition		
	Time post-mortem			Time post-mortem		
Fatty acid	0 min	2 min	15 min	0 min	2 min	15 min
Palmitate	14.4 ± 1.2	20.4 ± 2.3	59.1 ± 9.4	36.5 ± 3.0	18.9 ± 1.9	18.1 ± 1.6
Stearate	12.4 ± 1.2	38.6 ± 3.1	83.3 ± 11.8	31.5 ± 3.7	35.8 ± 2.9	25.9 ± 1.1
Oleate	5.7 ± 2.2	12.9 ± 2.1	62.4 ± 17.0	14.5 ± 3.3	11.8 ± 0.4	19.2 ± 2.8
Arachidonate	5.5 ± 0.9	35.5 ± 7.4	106.1 ± 7.2	13.9 ± 1.8	32.1 ± 2.0	33.2 ± 2.6
Docosahexaenoate	1.4 ± 1.2	2.5 ± 1.5	11.1 ± 2.3	3.5 ± 2.7	2.3 ± 1.1	3.4 ± 0.6

The specific activity of the free arachidonic acid at zero time in rat forebrain was more than 5 times that found at 2 minutes post-mortem (1054 and 198 cpm/μg respectively, Table 6). The large initial specific activity of the free arachidonic acid is probably due to residual radioactivity which had not been incorporated in the lipids during the 2 hours and 15 minutes after the intraventricular injection of [$^3H_8$]-arachidonic acid. The amount of radioactivity found at zero time in the free arachidonic acid represented 0.2% of the injected dose. The drop in specific activity is due to the release of arachidonic acid from the complex lipids which occurs following decapitation. After the initial decrease, the specific activity of the free arachidonic acid increased slightly with post-mortem time (Table 6). Changes in the specific activity of the complex lipids between 0 and 30 minutes post-mortem were small. The specific activity of the free arachidonic acid determined for the rat forebrain at 30 minutes after decapitation (288cpm/μg, Table 6) was closer to that of phosphatidylinositol than to that of the other lipids. However, the calculated specific activity of the arachidonic acid released between 0 and 30 minutes post-mortem (232cpm/μg arachidonic acid) was not very different from that of the total phospholipids (207cpm/μg, Table 6). The former value was calculated by subtracting the radioactivity and the amount of arachidonic acid in the free fatty acid fraction at the earlier time from that of the later time.

3) Amount of Free Arachidonic Acid and Specific Activity of Lipids in Rat Brain Subcellular Fractions

In the microsomal fraction, the specific activity of the lipids

was determined only after 30 minutes post-mortem. The ratio of the specific activity of the free arachidonic acid to that of the total phospholipids in this fraction was similar to the same ratio in the whole tissue 30 minutes after decapitation although the specific activity of the microsomal total phospholipid fractions was 1.7 times larger than that of the whole tissue (Table 6). In this subcellular fraction, as in the whole tissue, phosphatidylinositol had a specific activity closest to the uncorrected value for the free arachidonic acid. The amount and specific activity of free arachidonic acid as well as the percentage composition of the free fatty acids of the microsomes differed markedly from those of the crude mitochondrial fraction (Table 7). The supernatant contained relatively little unesterified arachidonic acid.

The specific activity of the free arachidonic acid in subcellular fractions was probably not greatly modified during the time required for their preparation since fatty acid release is completed by 30 minutes post-mortem in rat brain (268) and the fractionation was done at 0°C in the presence of a phospholipase A inhibitor. Tissue homogenized 2 minutes after decapitation and left for 60 minutes at 0°C in sucrose containing 10^{-3}M DFP had 35.5 ± 3.1 (3)µg arachidonic acid per gram tissue. Tissue homogenized in chloroform : methanol, 2 : 1 at 2 minutes post-mortem had 32.9 ± 7.9 (3)µg arachidonic acid per gram tissue

4) Post-Mortem Changes in the Amount of Arachidonic Acid and Radioactivity in Various Lipids of Rat Brain

TABLE 6

Specific Activity of Arachidonic Acid in Lipid Classes of Rat Forebrain*

Zero time values obtained from forebrain of heads frozen in liquid nitrogen upon decapitation. Values at 30 minutes post-mortem for whole tissues and microsomes were obtained from forebrains kept at room temperature. The specific activity of the free arachidonic acid in the whole tissue at 2 and 15 minutes post-mortem was 198 ± 34 and 236 ± 43 cpm/μg respectively. The specific activity of the released arachidonic acid between 0 and 30 minutes post-mortem was 232 cpm/μg. All values are means ± S.D. where appropriate with the number of determinations in brackets.

Lipid	Specific activity of arachidonic acid cpm/μg		
	Whole tissue (3)		Microsomes (2)
	Time post-mortem		
	0 min	30 min	30 min
Free arachidonic acid	1054 ± 517	288 ± 47	423
Triglycerides	3741 ± 181	2898 ± 112	-
Diglycerides	865 ± 246	608 ± 151	1060
Phosphatidylinositol	329 ± 29	298 ± 33	416
Phosphatidylcholine	439 ± 42	385 ± 23	758
Phosphatidylethanolamine + Plasmalogens	99 ± 22	101 ± 18	175
Phosphatidylserine	237 ± 22	193 ± 16	388
Phosphatidic acid + Cardiolipin	67 ± 13	60 ± 21	-
Total phospholipids	224 ± 48	207 ± 38	350

*The specific activities at each time point were normalized as follows:

$$\overline{\text{S.A.}} \text{ of total PL. } \times 1/N \sum_{i=1}^{i=3} \text{S.A.}_i \text{ of each lipid/S.A.}_i \text{ of total PL.}$$

where N= total number of determinations at each time point.

TABLE 7

Free Fatty Acids in Subcellular Fractions of Rat Forebrain at 30 minutes Post-Mortem*

	µgFAA	cpm	% composition of Free Fatty Acids				
	mg protein	µgFAA	16:0	18:0	18:1	20:4	22:6
Homogenate	0.85	235	24.3	26.8	12.6	31.8	4.5
Crude Mitochondria (P_2)	1.85	195	19.2	30.1	16.4	28.3	6.1
Microsomes (P_3)	0.30	423	21.6	36.4	18.7	17.9	5.2
Supernatant (S_3)[1]			22.1	21.4	21.6	20.1	14.7

Each value is the mean of two determinations

*injected intraventricularly with [$^3H_8$]-arachidonic acid 2 hours and 15 minutes before decapitation. Tissue homogenized in sucrose 0.32 + 10^{-3}M DFP (0°) 30 minutes after death.

[1]about 3% of the forebrain free arachidonic acid was recovered from the supernatant by solvent partition extraction.

In forebrain, phosphatidylcholine was the only lipid that showed a significant decrease in the level of radioactivity when expressed as a percent of total lipid radioactivity in the 30 minutes post-mortem period (Table 8). This decrease, amounting to 8%, can account for 63% of the increase in the proportion of radioactivity associated with the free fatty acid fraction. None of the lipids showed a significant decrease in the amount of arachidonic acid (μg/g tissue) between zero and 30 minutes after decapitation (Table 9). The amount of arachidonic acid recovered from phosphatidylcholine decreased by 7% but this difference was not significant. The 20% increase in the amount of arachidonic acid in the diglycerides is probably due to the release of diglycerides rich in arachidonic acid which occurs post-mortem in rat brain (282,286).

F) Relationship Between Arachidonic Acid Release and Prostaglandin $F_{2\alpha}$ Synthesis in Rat Cerebral Cortex Slices

1) Release of Fatty Acids Post-Mortem in Rat Cerebral Cortex Slices

The relationship between the release of individual free fatty acids in rat cerebral cortex slices with incubation time (Figure 10) shows that after 15 minutes, the rates of release decrease greatly. The level of free arachidonic acid increased at the fastest rate as occurs in whole brain post-mortem (268,272). A rapid hydrolysis of cerebral cortex lipids must also take place in the period necessary to prepare the cortex slice (5 minutes) since cerebral cortex from rat brain frozen in liquid

TABLE 8

RADIOACTIVITY IN EACH LIPID OF RAT FOREBRAIN EXPRESSED AS PERCENT OF TOTAL LIPID RADIOACTIVITY

Values obtained from forebrains treated as described in Table 3. Total lipid radioactivity at zero time was $504 \pm 73 \times 10^3$ cpm/g tissue and at 30 minutes $533 \pm 89 \times 10^3$ cpm/g tissue fresh weight. Results are means ± S.D. Number of determinations in brackets.

	Percent of total lipid radioactivity	
	Time post-mortem	
Lipid	0 min (3)	30 min (3)
Free arachidonic acid	1.85 ± 0.90	7.98 ± 0.95**
Triglycerides	3.61 ± 0.86	2.97 ± 0.42
Diglycerides	2.31 ± 0.24	2.32 ± 0.62
Phosphatidylinositol	21.1 ± 4.50	20.00 ± 1.30
Phosphatidylcholine	46.35 ± 2.04	42.49 ± 1.04*
Phosphatidylethanolamine + Plasmalogens	18.80 ± 2.68	19.26 ± 1.41
Phosphatidylserine	3.76 ± 0.55	3.25 ± 0.77
Phosphatidic acid + Cardiolipin	2.23 ± 0.59	2.30 ± 0.88

**The difference between the means is significant at the level of $P<0.01$

*The difference between the means is significant at the level of $P<0.05$

TABLE 9

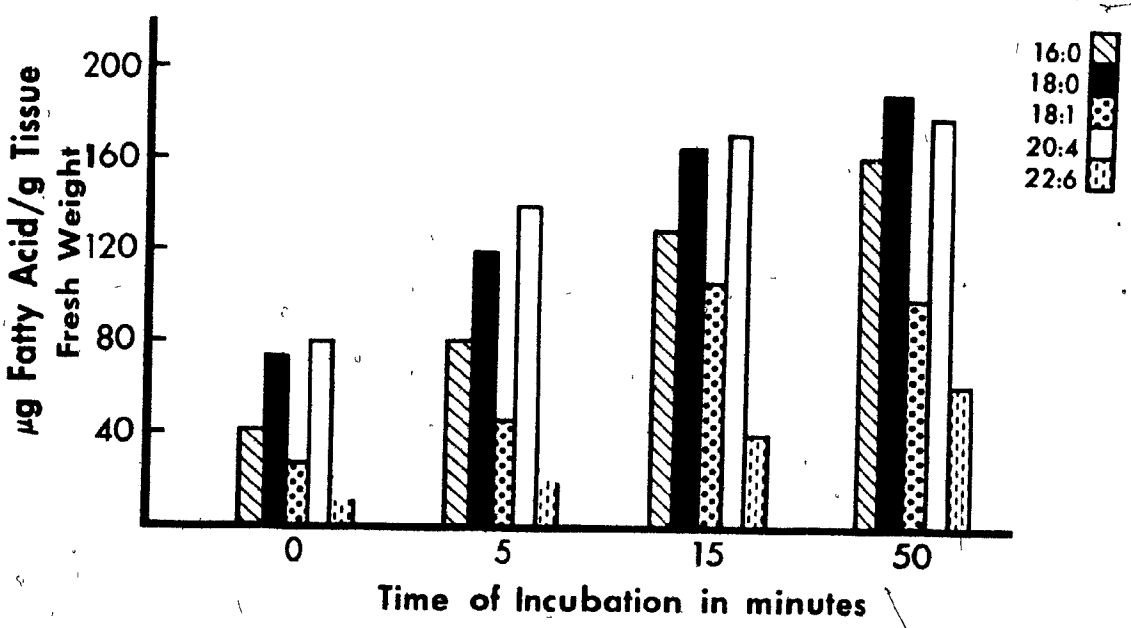
ARACHIDONIC ACID IN LIPID CLASSES OF RAT FOREBRAIN EXPRESSED AS PERCENT OF TOTAL LIPID ARACHIDONIC ACID

Values obtained from forebrain treated as described in Table 3. Total lipid arachidonic acid at zero time was 2053 ± 350 μg/g tissue and at 30 minutes 2345 ± 247 μg/g fresh tissue weight. Values are means ± S.D. Number of determinations in brackets.

Lipid	Percent of total arachidonic acid Time post-mortem	
	0 min (3)	30 min (3)
Free arachidonic acid	0.28 ± 0.03	6.46 ± 1.37
Triglycerides	0.26 ± 0.05	0.23 ± 0.04
Diglycerides	0.70 ± 0.08	0.85 ± 0.10
Phosphatidylinositol	16.23 ± 1.87	15.81 ± 0.57
Phosphatidylcholine	27.51 ± 1.91	25.58 ± 1.63
Phosphatidylethanolamine + Plasmalogens	44.71 ± 1.83	42.53 ± 2.40
Phosphatidylserine	3.72 ± 0.70	3.82 ± 0.33
Phosphatidic acid + Cardiolipin	6.58 ± 2.18	4.75 ± 1.32

Figure 3.

Free fatty acids released post-mortem in rat cerebral cortex slices. Preparation and weighing of the slices required five minutes. The slices were incubated in Ringer-bicarbonate-glucose buffer, pH 7.4, at 37°C in an atmosphere of 95% O_2 and 5% CO_2. The fatty acids were quantitated by gas chromatography using heptadecanoic acid as internal standard. The fatty acids measured were: palmitate (16 : 1), stearate (18 : 0), oleate (18 : 1), arachidonate (20 : 4) and docosahexaenoate (22 : 6). The values at 0 and 50 minutes of incubation are the means of 4 determinations each. The values at 5 and 15 minutes of incubation are the average of 2 determinations each.


µg Fatty Acid/g Tissue
Fresh Weight
200
160
120
80
40
0
5
15
50
Time of Incubation in minutes
16:0
18:0
18:1
20:4
22:6

nitrogen upon decapitation contains only small amounts of free fatty acids (270). After removal of the slice and low speed centrifugation of the incubation medium, less than 10% of each fatty acid was found in the supernatant.

2) Incorporation of [$^2H_8$]-Arachidonic Acid into the Free and Esterified Arachidonic Acid from Incubated Rat Cerebral Cortex Tissue

The deuterium to protium ratios of the free arachidonic acid in cortex slices approached maximum values by 15 minutes of incubation in the presence of [$^2H_8$]-arachidonic acid (Table 10). The maximal ratio observed was 0.22 after 60 minutes of incubation although enough [$^2H_8$]-arachidonic acid was added to the medium to give a deuterium to protium ratio of nearly 1.5 if there was complete mixing of exogenous and endogenous arachidonate. The ratios were measured after washing the slice for 3 minutes in buffer containing fatty acid free bovine serum albumin to remove the exogenous arachidonate which simply adhered to the cell surfaces or which was in the extracellular fluid of the tissue (359). The remaining [$^2H_8$]-arachidonate was presumably not readily available to the albumin. Saturation of the serum albumin was unlikely since it was added in a molar ratio of protein to total tissue free fatty acid of 1 : 5. Furthermore, endogenous free arachidonic acid was not removed from the slice by bovine serum albumin. Only 1 to 1.5μg of free arachidonic acid were recovered with the serum protein per 100mg tissue. This fraction had a deuterium to protium ratio 30 times that of the free arachidonic acid remaining in the slice after 5 minutes of incubation in

TABLE 10

INCORPORATION OF [$^2H_8$]ARACHIDONIC ACID INTO LIPIDS OF INCUBATED RAT CEREBRAL CORTEX TISSUE

Cerebral cortex tissue was incubated in Ringer-bicarbonate-glucose buffer containing 25 μg of [$^2H_8$]arachidonic acid per 100 mg tissue fresh weight. Slices were washed with buffer containing bovine serum albumin (BSA) after incubations as described in the text. The deuterium to protium ratio in the free arachidonic acid of the slice at 15 minutes of incubation was 0.18 ± 0.02 (n = 4). Values are means of 2 determinations unless indicated otherwise. The standard deviation (± S.D.) is given where appropriate.

	Lipid	Arachidonic Acid [$^2H_8$]/[$^1H_8$]	
		5 minute incubation	60 minute incubation
BSA washings from incubated slice	Free AA	2.23	1.73 ± 0.27 (3)
Slice	Free AA	0.07	0.22 ± 0.01 (3)
	Diglycerides Phosphatidylinositol Phosphatidylcholine	<0.01	<0.03
	Triglycerides	0.03	0.28 ± 0.05 (3)
Incubated homogenate	Free AA	2.20 ± 0.13 (3)	1.37
	Triglycerides	0.02	0.03 ± 0.003 (3)

AA - Arachidonic acid

the presence of octadeuterated arachidonic acid (Table 10). The isotopic purity of the $[^2H_8]$-arachidonic acid added to the incubation medium decreased from 98% to 91% after 60 minutes of incubation. A loss of 13% of the free arachidonic acid from the slice could account for the decrease in the isotopic purity of the labelled precursor in the medium.

The free arachidonic acid of the incubated homogenates had deuterium to protium ratios much higher than those found in the incubated slices. Most of the deuterated arachidonic acid became bound to tissue elements since 96% of the total (endogenous and exogenous) free arachidonic acid was found in the pellet after centrifugation of homogenates incubated for 60 minutes. The decreased ratios in the free arachidonic acid of the homogenate with incubation time was due to the continued release of the free fatty acid from brain lipids. An average of 68µg of arachidonic per 100mg of tissue fresh weight was liberated between 5 and 60 minutes of incubation. In the slice, the deuterium to protium ratio in the free arachidonic acid increased rather than decreased since penetration of the labelled fatty acid into the tissue was gradual and the release of arachidonic acid was reduced after 5 minutes of incubation (Figure 3 and Table 10).

The triglycerides from the slice reached a deuterium to protium ratio slightly higher than that found in the free arachidonic acid fraction. However, the triglycerides from the homogenate incorporated very little of the labelled fatty acid although present in the homogenate at 60 minutes of incubation at a similar level to that found in the slice before homogenization (i.e. 16,1µg and 13.5µg triglyceride per 100mg tissue fresh weight, respectively). The diglycerides, phosphatidylinositol and

phosphatidylcholine from the cortex slices were also poorly labelled.

3) Transformation of $[^2H_8]$-Arachidonic Acid into PGF$_{2\alpha}$ by Cerebral Cortex *in vitro*

The deuterium to protium ratios in the PGF$_{2\alpha}$ produced by cerebral cortex slices after 60 minutes of incubation was not significantly higher than that found in the PGF$_{2\alpha}$ formed during 5 minute incubations (Table 11). However, at 60 minutes the deuterium to protium ratio in the PGF$_{2\alpha}$ recovered in the medium was approximately twice that found in the PGF$_{2\alpha}$ of the slice. The homogenate when incubated with the same amount of $[^2H_8]$-arachidonate acid produced PGF$_{2\alpha}$ with a deuterium to protium ratio 10 times that of the PGF$_{2\alpha}$ found in the incubated slice.

The amount of PGF$_{2\alpha}$ produced by cortex slices during the time required for their preparation was 8.9ng/100mg fresh weight. Also, cortex slices can bind irreversibly 3.8ng of PGF$_{2\alpha}$ per 100mg tissue (47). We assumed, to calculate the "corrected" $[^2H_8]$: $[^1H_8]$-PGF$_{2\alpha}$ ratios of columns 2 and 4 of Table 10 that 3.8ng/100mg tissue of the PGF$_{2\alpha}$ initially present in the slice remained with it and that the difference, 4.8ng/100mg tissue, was recovered in the medium.

4) Effect of Norepinephrine on the Incorporation of $[^2H_8]$-Arachidonic Acid into Triglycerides and Transformation into the PGF$_{2\alpha}$ of the Incubated Cortex Slices

The addition of norepinephrine to the incubation medium has been shown to increase the production of PGF$_{2\alpha}$ by rat cerebral cortex slices (47).

TABLE 11

INCORPORATION OF [$^2H_8$]ARACHIDONIC ACID INTO PROSTAGLANDIN $F_{2\alpha}$ BY RAT CEREBRAL CORTEX TISSUE IN VITRO

Cerebral cortex tissue was incubated in Ringer-bicarbonate-glucose buffer containing 25 µg of [$^2H_8$]-arachidonic acid per 100 mg tissue. Slices were washed with fresh buffer without added bovine serum albumin. The deuterium to protium ratio in the $PGF_{2\alpha}$ were corrected for the amount of endogenous [$^1H_8$]-$PGF_{2\alpha}$ present in the tissue before incubation as described in the text. Where appropriate, values are means ± S.D. The number of determinations is given in parentheses.

	5 minutes incubations		60 minutes incubations	
	$PGF_{2\alpha}$ [$^2H_8$]/[$^1H_8$]	Ratios corrected for [$^1H_8$]$PGF_{2\alpha}$ at zero time	$PGF_{2\alpha}$ [$^2H_8$]/[$^1H_8$]	Ratios corrected for [$^1H_8$]$PGF_{2\alpha}$ at zero time
Homogenate	-	-	1.06 ± 0.30 (4)	1.25
Slice	0.08, 0.12	0.14	0.10 ± 0.02 (5)	0.15
Medium	-	-	0.23 ± 0.07 (5)	0.30
Slice + Medium	0.18 ± 0.06 (3)	0.21	0.22 ± 0.05 (5)	0.24

In a similar situation, the neurohormone doubled the $[^2H_8]$: $[^1H_8]$ ratios in the $PGF_{2\alpha}$ produced by rat cortex slices incubated for 60 minutes in the presence of $[^2H_8]$-arachidonic acid (Table 12). Norepinephrine also caused a significant although smaller increase in the labelling of the free arachidonic acid but not of the triglycerides. The amount of endogenous free arachidonic acid in the slice (and medium) was not increased by the drug after 60 minutes of incubation.

G) Drug Induced Convulsions and the Production of Prostaglandin Endoperoxide Derivatives in Rat Brain

1) Symptomatic Effects of the Drugs

The animals had generalized convulsions two to three minutes after the intraventricular injection of carbachol and began to recover approximately 30 minutes later. When the rats were kept anesthetized with ether for 30 minutes after injection, their peripheral symptoms were greatly attenuated or absent. The first intraperitoneal dose of metrazol usually caused a very short generalized seizure within 10 minutes of injection. The second dose of metrazol caused a generalized convulsion which lasted approximately 2 minutes.

2) Effect of Drugs of the PG Endoperoxide, $PGF_{2\alpha}$ and Thromboxane B_2 Levels in Rat Forebrain <u>in vivo</u>

Prostaglandin $F_{2\alpha}$ levels in rat cerebral hemispheres were greatly increased following convulsions induced by carbachol and metrazol (Table 13). The convulsion period of 75 seconds with metrazol gave the largest increase

TABLE 12

EFFECT OF NOREPINEPHRINE ON THE INCORPORATION OF [$^2H_8$]ARACHIDONIC ACID INTO PROSTAGLANDIN $F_{2\alpha}$, THE UNESTERIFIED ARACHIDONIC ACID AND THE TRIGLYCERIDES OF RAT CEREBRAL CORTEX SLICES INCUBATED FOR 60 MINUTES.

Cerebral cortex slices were incubated in Ringer-bicarbonate-glucose buffer containing 25 μg of [$^2H_8$]arachidonic acid per 100 mg tissue fresh weight and 1 mM norepinephrine hydrochloride. For the determination of the deuterium to protium ratios in the free arachidonic acid and triglycerides, slices were washed with buffer containing bovine serum albumin. The ratios in the $PGF_{2\alpha}$ were obtained from slices plus medium. Where appropriate, values are means ± S.D. The number of determinations is given in parentheses.

	Deuterium to Protium Ratios		
	-NE	+NE	% increased
Free arachidonic acid	0.22 ± 0.01 (3)	0.30, 0.33	37%
Triglycerides	0.28 ± 0.05 (3)	0.29, 0.32	9%
Prostaglandin $F_{2\alpha}$	0.22 ± 0.05 (5)	0.43 ± 0.10 (4)	95.5%

Table 13. Effects of Drugs on the Production of PG Endoperoxides, $PGF_{2\alpha}$ and Thromboxane B_2 by Rat Cerebral Hemispheres in vivo

Conditions	PG Endoperoxides[a]	$PGF_{2\alpha}$	TxB_2
		ng/g tissue fresh weight	
Control[b]	n.d.	15.9 ± 11(4)	1.4, 3.4
Carbachol (100 μg)	n.d.	83.8 ± 14.4(4)	0.04 ± 0.06(3)
Carbachol (ether anesthesia)	-	78.4, 96.9	-
Control[c]	-	1.2 ± 0.8(8)	-
Metrazol[d]			
15 sec. convulsion	22.1 ± 6.9(3)	9.9 ± 5.4(4)	0.8 ± 1.3(3)
75 sec. convulsion	n.d.	182.7 ± 32.7(3)	3.2
Ischemia[e]			
75 seconds	-	2.28, 0	-

Results expressed as means with S.D. when applicable; n.d. is not detectable. Dash means not determined.

[a] Determined as the increase in $PGF_{2\alpha}$ caused by stannous chloride added to the ethanol used for extraction of the frozen brain tissue.

[b] Injected intraventricularly with 8 μl 0.9 percent saline, head frozen in liquid N_2 30 minutes later.

[c] Injected intraperitoneally with 0.9 percent saline, head frozen in liquid N_2 2 minutes later.

[d] Metrazol seizure induced with two doses of the drug, 10 minutes apart (40 mg/kg and 80 mg/kg, respectively).

[e] Severed heads kept at room temperature for 75 seconds before freezing in liquid nitrogen.

in $PGF_{2\alpha}$. Neither drug modified significantly the level of thromboxane B_2 in rat brain. Prostaglandin endoperoxide could be "trapped" with stannous chloride only at 15 seconds of convulsion induced by metrazol. A period of 75 seconds of anoxia and ischemia following decapitation did not increase the level of $PGF_{2\alpha}$ over that of its control. The value obtained for $PGF_{2\alpha}$ in the controls for the carbachol experiments were higher than that found in the controls for the metrazol experiments. This is probably due to the trauma caused to the brain tissue by the intraventricular injection procedure used in the former experiments.

3) Effect of Drugs on Fatty Acid Levels in Rat Forebrain in vivo and Post-Mortem

Carbamylcholine (carbachol) and pentylenetetrazol (Metrazol) in convulsant doses increased the amounts in vivo of free fatty acids in rat cerebral hemispheres (Table 14). The level of stearic and arachidonic acid was selectively increased following 30 minutes of convulsion induced by carbachol or following 1' seconds of convulsion induced by metrazol. The amount of stearate and arachidonate was increased some two to three fold respectively with both drugs. A longer convulsion period with metrazol (75 seconds) resulted in a general rise in free fatty acid levels in rat brain. The period of anoxia and ischemia following decapitation also caused a general increase in free fatty acids but much more fatty acids were released than in the case of the metrazol convulsions of equal duration. The level of arachidonic acid in the triglycerides and diglycerides after saline injections was 7.5 and 15.7μg/g tissue respectively while after carbachol injections it was 10.1 and 16.7μg/g tissue respect-

Table 14. Effect of Drugs on the Amounts *in vivo* of Free Fatty Acids in Rat Brain Cerebral Hemispheres

μg FA/g tissue fresh wt.

Fatty Acid	Control[a]	Carbachol (100 μg)	Control[b]	Metrazol[c] convulsion period		Decapitation[d]	
				15 sec	75 sec		
	n=6	n=5	n=4	n=6	n=6		
Palmitic (16:0)	14.6 ± 3.5	12.7 ± 2.4	11.5 ± 2.6	16.2 ± 5	28.8 ± 9.1	41.2,	38.7
Stearic (18:0)	10.2 ± 2.1	17.5 ± 3.9	12.4 ± 5	24.2 ± 4.1	27.2 ± 5.1	41.6,	50.3
Oleic(18:1)	5.7 ± 1.5	7.2 ± 1.9	10.1 ± 1.4	12.6 ± 4.1	17.6 ± 4.8	23.1,	17.5
Arachidonic (20:4)	4.1 ± 1.8	13.5 ± 3.5	6.5 ± 2.8	19.9 ± 3.6	18.1 ± 4.6	43.2,	39.2
Docosahexenoic (22:6)	1.4 ± 1.1	3.4 ± 1.5	3.[illegible] ± 1.1	2.9 ± 1.5	3.2 ± 1.5	4.6,	4.5

[a] Injected intraventricularly with saline, brain frozen in liquid nitrogen 30 min later.

[b] Injected intraperitoneally with saline, brain frozen in liquid nitrogen 2 min later.

[c] Metrazol seizures induced with two doses of the drug, 10 min apart (40 mg/kg and 80 mg/kg, respectively).

[d] Severed heads kept at room temperature for 75 sec before freezing in liquid nitrogen.

ively (each value is the average of two determinations).

In contrast, the amount of free stearic acid, oleic acid and arachidonic acid found in brain tissue at 2 minutes post-mortem was decreased by approximately 40% in rats treated with the anticonvulsant drug phenytoin (Table 15). Sodium phenobarbital decreased the amount of arachidonic acid recovered in rat cerebral hemispheres two minutes after decapitation. Diazepam lowered the level of oleic acid in the same conditions.

TABLE 15

Effect of Drugs on the Amount of Free Fatty Acids Found in Rat Cerebral Hemispheres Following Decapitation

μg FA/g tissue fresh wt.

Fatty Acid	Control[a] n=13	Sodium Phenobarbital[b] n=4	Phenytoin[b] n=8	Diazepam[b] n=4
16:0	20.6 ± 6.1	20.6 ± 8.6	17.2 ± 2.3	22.2 ± 8.8
18:0	38.2 ± 8.1	36.6 ± 8.8	21.5 ± 7.6**	33.2 ± 4.1
18:1	10.2 ± 2.5	13.3 ± 5.1	6.7 ± 3.7*	5.9 ± 3.3**
20.4	39.2 ± 8.9	29.1 ± 5.5*	24.9 ± 9.1**	39.5 ± 3.5
22:6	2.8 ± 1.7	3.7 ± 1.3	2.1 ± 1.2	2.1 ± 0.7

[a]0.5 ml 0.9 percent saline injected intraperitoneally, animals killed 30 minutes later by decapitation and cerebral hemispheres homogenized in chloroform-methanol (2:1 by vol.) 2 minutes post-mortem.

[b]Sodium phenobarbital, 150 mg/kg; Phenytoin, 0.33 g/kg; Diazepam, 8.5 mg/kg.

*$P = 0.05$ **$P < 0.01$

CHAPTER 4

DISCUSSION

A) Origin of the Arachidonic Acid Released Post-Mortem in Rat Forebrain

The arachidonic acid released post-mortem in rat forebrain probably originates from a mixture of phospholipids. The specific activity of the arachidonic acid released after decapitation is close to that of the total phospholipids and no individual lipid accounts for all the arachidonic acid and the radioactivity appearing in the free fatty acid fraction Approximately 63% of the radioactivity of the arachidonic acid released post-mortem seems to arise from phosphatidylcholine. However, the specific activity of this phospholipid was 1.7 times that of the arachidonic acid released during the 30 minutes post-decapitation period. Therefore only 37% of the free arachidonic acid could come from phosphatidylcholine. The complementary sources of the released arachidonic acid may be phosphatidylethanolamine and phosphatidylinositol. Although these phospholipids may make significant contributions to the free arachidonic acid fraction, decreases in their content of radioactivity and amount of fatty acid would be difficult to detect since they contain much more arachidonic acid than that released post-mortem. Some contribution from a complex lipid of lower specific activity such as phosphatidylethanolamine may be required since the specific activity of phosphatidylinositol was slightly above that of the released arachidonic acid and that of phosphatidylcholine had an even larger value. The gradual rise following the initial rapid drop in the relative specific activity of the free arachidonic acid recovered at different times after decapitation suggests that phospholipids of higher specific activity make larger

contributions to the free arachidonic acid pool with increasing post-mortem time.

Phosphatidylserine is probably not an important source of the free arachidonic acid. This phospholipid had a specific activity close to that of the released arachidonic acid but it contained about half as much of the fatty acid at zero time than was found in the free fatty acid fraction 30 minutes after decapitation. No decrease in radioactivity and amount of arachidonic acid were observed for this phospholipid. In the phosphatidic acid plus cardiolipin fraction, a decrease in arachidonic acid sufficient to account for 30% of the free arachidonic acid would not be detected because of the large variation in the amount of fatty acid recovered from this complex lipid mixture. However, such a large contribution from these lipids would probably have resulted in a lower specific activity in the arachidonic acid released after decapitation. The arachidonic acid in the phosphatidic acid plus cardiolipin mixture had a specific activity nearly 4 times lower than that of the released fatty acid. The neutral lipids probably do not make a significant contribution to the free arachidonic acid recovered post-mortem. These lipids contain much less of the fatty acid that can be liberated after decapitation and their specific activities are much higher than that of the free arachidonic acid. In vivo, the arachidonic acid in the triglycerides of rat brain has a higher turnover rate as seen following intracerebroventricular injections of [$^{3}H_{8}$]-arachidonic acid (296). However, the specific activity of the brain triglycerides was quite stable from decapitation to 30 minutes post-mortem. Also, the level of triglycerides in rat brain has been reported to decrease by only 20% in

60 minutes following decapitation (268). The rapid in vivo metabolism of triglycerides observed after intracerebroventricular injections of [$^3H_8$]-arachidonic acid is localized mainly in the choroid plexus (see appendix A).

It has been suggested that the ethanolamine plasmalogens may be a major source of arachidonic acid for prostaglandin synthesis (363). Although this lipid is abundant in white matter and myelin and possesses significant levels of arachidonic acid (294) much less radioactivity was incorporated into alkenyl-phosphatidylethanolamine relative to the diacyl class in rat brain injected with [$^3H_8$]-arachidonic acid (364). In our experiments, the specific activity of the plasmalogens would probably be lower than that of the diacyl PE. and thus much lower than that of the arachidonic acid released during post-decapitation ischemia and anoxia.

In the microsomal fraction, the specific activity of the free arachidonic acid was the same as that of the phosphatidylinositol. This does not necessarily indicate that the fatty acid was released from the latter lipid. The fact that brain frozen in liquid nitrogen would not yield typical microsomal fractions does not allow the specific activity of the free arachidonic acid to be corrected for the radioactivity which had not been incorporated 2 hours and 15 minutes after intracerebroventricular injection. On the other hand, the observations that the specific activity of the free arachidonic acid and the percentage composition of the free fatty acids found in the microsomal and crude mitochondrial fractions differed from each other indicate, in agreement with a previous report (269), that subcellular fractions from rat brain can maintain separate free fatty acid pools. As shown in the result

section (see page 72), the specific activity of the free arachidonic acid in the subcellular fractions from brains left in situ 30 minutes after decapitation is not greatly modified during the time required for their preparation. Much of the arachidonic acid release probably occurs in the mitochondria since they contain a large part of the phospholipase A_2 activity of rat brain. Indeed, the crude mitochondrial fraction was the richest in free arachidonic acid per μg protein or in percentage composition.

The existance of a phospholipase A_2 activity sensitive to anoxia and ischemia accompanying decapitation is suggested by the rapid release of arachidonic acid in rat brain shortly after decapitation. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol probably contributed most of the arachidonic acid released in rat forebrain after decapitation. If indeed the free fatty acids are compartmentalized, only the free arachidonic acid released in certain subcellular membranes could be available to prostaglandin synthetase.

B) Relationship between Arachidonic Acid Release and Prostaglandin $F_{2\alpha}$ Synthesis in Rat Cerebral Cortex Tissue

Although the rate of synthesis of $PGF_{2\alpha}$ by rat cerebral cortex slices is linear for periods up to 60 minutes (47), its D/H did not change significantly from 5 to 60 minutes of incubation in the presence of $[^2H_8]$-arachidonic acid. In the slice, $PGF_{2\alpha}$ reached maximum labelling before any other lipid. A pool of endogenous free arachidonic acid must have mixed very rapidly with the exogenous $[^2H_8]$-arachidonic acid and the most likely location of this pool is at the surface of the slice. Indeed,

free arachidonic acid with a high D/H was removed by bovine serum albumin from incubated slices. The high D/H in the $PGF_{2}\alpha$ found in the medium may be due to the rapid access of the labelled precursor to the prostaglandin synthetase present at the damaged surfaces of the slice. Norepinephrine probably increased the D/H of the $PGF_{2}\alpha$ produced by the slice by stimulating directly the surface prostaglandin synthetase. Indeed, most of the $[^{2}H_{8}]$-$PGF_{2}\alpha$ could have been formed at the surface of the slice since it represented only 18% of the total $PGF_{2}\alpha$ production and the $PGF_{2}\alpha$ recovered with the slice was maximally labelled as quickly as the total $PGF_{2}\alpha$ (slice and medium). Furthermore, the D/H of the $PGF_{2}\alpha$ calculated from the rate of entry of $[^{2}H_{8}]$-arachidonic acid into the cortex slice differs from the observed D/H after 60 minutes of incubation (0.17 versus 0.24, see appendix B).

The triglycerides, the complex lipid with the highest D/H, are probably not intermediates in the delivery of arachidonic acid to the prostaglandin synthetase system of cerebral cortex since, in the slice, norepinephrine doubled the deuterium to protium ratio of the $PGF_{2}\alpha$ but did not affect significantly that of the triglycerides. In the homogenate, the $PGF_{2}\alpha$ had a D/H approximately 50 times that of the triglycerides.

The involvement of phospholipids is also unlikely since the half-life of their fatty acid moiety is measured in days in whole brain and its subcellular fraction (365-367). In our experiments, phosphatidylinositol and phosphatidylcholine were poorly labelled by $[^{2}H_{8}]$-arachidonic acid when compared to $PGF_{2}\alpha$. The diglycerides also did not incorporate much $[^{2}H_{8}]$-arachidonic acid. However, the arachidonic acid of CDP-diglycerides does turn over rapidly in rat liver microsomes (368). This

liponucleotide is present in brain (369-370) but its role as a source of arachidonic acid for prostaglandin synthesis remains to be investigated.

In the homogenate, the D/H of the $PGF_{2}\alpha$ was very close to that of the total free arachidonic acid after 60 minutes of incubation. In this preparation, production of $PGF_{2}\alpha$ with a large protium content can be accounted for simply by the dilution of the added $[^{2}H_{8}]$-arachidonic acid by the endogenous arachidonic acid released post-mortem. However, the deuterium to protium ratio in the $PGF_{2}\alpha$ may not be directly comparable to that of the arachidonic acid since an isotope enrichment of the product can occur during its biosynthesis from a mixture of $[^{2}H_{8}]$ and $[^{1}H_{8}]$-arachidonic acid, as observed with sheep seminal vesicle microsomes (115). Theoretical calculations (see appendix C) suggest that the deuterium labelling of the $PGF_{2}\alpha$ formed by rat cerebral cortex homogenates could be enriched by approximately 200%. Therefore, the precursor pool used for $PGF_{2}\alpha$ may not correspond to the total arachidonic acid fraction.

Since prostaglandin synthetase is a membrane bound enzyme in brain and since at least 96% of the exogenous and endogenous free arachidonic acid was tissue bound in the homogenate, compartmentation of the precursor in this preparation could occur at the level of the subcellular membranes. That subcellular fractions from rat brains can maintain separate free fatty acid pools has already been indicated (see page 94).

The experiments where $[^{2}H_{8}]$-arachidonic acid was used as a precursor of $PGF_{2}\alpha$ indicate that the fatty acid enters cortex slices with difficulty resulting in a poor labelling of the $PGF_{2}\alpha$ found within the tissue. On the other hand, the prostaglandin synthetase present at the damaged surfaces of the slice has a ready access to the labelled precursor. No

complex lipid was found to act as intermediates in the transfer of exogenous arachidonic acid to the prostaglandin synthetase system. In the homogenate, the endogenous and exogenous arachidonic acid appear to be equally available to the prostaglandin synthetase. However, compartmentation of the free arachidonic acid used for $PGF_{2}\alpha$ synthesis in the homogenate may have been masked by a significant isotope enrichment effect, in the conversion of a mixture of $[^{2}H_{8}]$ and $[^{1}H_{8}]$arachidonic acid to $PGF_{2}\alpha$. The greater part of the endogenous free arachidonic acid present in rat cerebral cortex tissue during the incubations appeared rapidly after decapitation.

C) Drug Induced Convulsions and the Production of Prostaglandin Endoperoxide Derivatives in Rat Forebrain

The present experiments suggest that a relationship exists in vivo between the appearance of free arachidonic acid and the production of $PGF_{2}\alpha$ but not of thromboxane B_{2} in rat cerebral hemispheres. The very rapid accumulation and subsequent disappearance of PG endoperoxides during metrazol convulsions suggests that these compounds are rapidly converted to $PGF_{2}\alpha$ under these conditions. However, in vitro there is accumulation of thromboxane B_{2} as well as $PGF_{2}\alpha$ in rat cerebral tissue and the production of both compounds is stimulated by norepinephrine (47,238). The dissocation of the production of $PGF_{2}\alpha$ and thromboxane B_{2} during drug induced convulsions raises the possibility that under certain conditions the pathways for the synthesis of these two compounds do not share the same pool of endoperoxide and/or that convulsant drugs stimulate

specifically the prostaglandin isomerase and a reductase. Increased levels of PGE_2 as well as $PGF_{2\alpha}$ following metrazol convulsions have already been reported for rat brain (252).

We observed a large increase in the amount of $PGF_{2\alpha}$ formed between 15 and 75 seconds of metrazol convulsions. A post-decapitation period of 75 seconds did not increase $PGF_{2\alpha}$ levels above the unstimulated *in vivo* levels. On the other hand, the production of $PGF_{2\alpha}$ by cerebral hemispheres of rats given convulsive doses of carbachol was not decreased by ether anesthesia which prevented the respiratory difficulties and consequent hypoxia associated with generalized seizures. Thus, the increased amount of $PGF_{2\alpha}$ in rat brain may be a direct consequence of the action of the convulsant drugs on brain tissue rather than due to a modification of blood circulation and breathing.

The levels of all free fatty acids were much higher at 75 seconds post-decapitation than at 15 and 75 seconds of metrazol convulsions or 30 minutes of carbachol convulsions. Also, arachidonic acid was released with greater selectivity during carbachol convulsions than during post-decapitation ischemia. Similar observations have been made in heart where bradykin induced a smaller, more specific release of arachidonic acid than did ischemia (371). The difference between the amounts of free arachidonic acid released either during carbachol or metrazol convulsions and the increase of $PGF_{2\alpha}$ formed through the action of these drugs is about 100-fold. Therefore, $PGF_{2\alpha}$ synthesis does not affect significantly the arachidonic acid pool size. Furthermore, the reduction of this pool size by phenytoin is insufficient to limit $PGF_{2\alpha}$ synthesis.

The neutral lipids may not be the source of the arachidonic acid released during drug induced convulsions since their content in this fatty acid did not decrease significantly. However, hydrolysis of the

diglycerides may have been masked by the activity of a phospholipase C sensitive to cholinergic agents.

It has been suggested that cAMP levels may regulate the activity of brain phospholipases involved in the release of fatty acids (275). This hypothesis is supported by the fact that the cAMP content of brain is increased after electroconvulsive shock and post-decapitation ischemia (372). Phenobarbital inhibits the increase in cAMP which occurs following decapitation in rat brain by approximately 50% at 2 minutes post- mortem (372). However, the fact that this drug had no effect on the post-mortem release of fatty acids in brain except for a small decrease in free arachidonic levels does not support the above hypothesis. Furthermore, the amount of free fatty acids in rat brain was not increased significantly by intraventricular injections of adenosine (unpublished results) or of convulsive doses of dibutyryl-cAMP (275).

The effectiveness of phenytoin in inhibiting fatty acid release may be related to its ability to bind brain proteins and phospholipids and/or its ability to increase the binding of calcium to phospholipids (373). The increased binding of calcium to phospholipids caused by phenytoin may play a role in the effect of the drug on fatty acid release since the activity of some brain phospholipases of the A type may be dependent on divalent cations (311). Phenytoin does not seem to affect the release of palmitate. This specificity in the effect of the drugs is not readily explained since <u>in vitro</u> phenytoin binds more strongly to dipalmitoyl than to distearoyl lecithins (373).

The administration <u>in vivo</u> of metrazol and carbachol in convulsive doses caused a preferential release of arachidonic acid in rat cerebral

hemispheres. This arachidonate appears to have been directed towards $PGF_{2}\alpha$ (and E_2) synthesis at the expense of thromboxane B_2 production under these conditions. Phenytoin was much more effective than phenobarbital and diazepam in inhibiting the release of fatty acids which occurs upon decapitation in rat brain.

APPENDIX A

The Choroid Plexus: A Major Site of Incorporation of Arachidonic Acid Into Triglycerides of Rat Brain

INTRODUCTION

The triglycerides of mouse and rat brain have a much faster turnover rate and a much higher specific activity than the phosphoglycerides following the intracerebral injection of labelled palmitate, oleate or arachidonate (296,348,374,375). The difference in the apparent metabolic patterns between the triglycerides and phosphogylcerides raises the possibility that in certain brain regions or cell types, the fatty acids of triglycerides undergo a particularly rapid turnover. This idea is supported by the finding that the rabbit choroid plexus has a high capacity to incorporate tritiated palmitate in vivo and in vitro (376).

The capacity for incorporation of arachidonic acid into triglycerides and other lipids by rat whole brain and choroid plexus was studied using intracerebroventricular injections of non-trace amounts of [$^{2}H_{8}$]-arachidonic acid. The labelling of brain lipids with the deuterated fatty acid was measured by gas chromatography-mass spectrometry. Trace amounts of [$^{3}H_{8}$]-arachidonic acid were used to study the rates of incorporation and turnover of triglycerides in the above tissues. The amount and the fatty acid composition of triglycerides in rat forebrain, cerebral cortex and choroid plexus were also determined.

MATERIALS AND METHODS

[$^{3}H_{8}$]-arachidonic acid, 4.5μCi, was administered to male Wistar rats (250-350g) by intracerebroventricular injections as described previously (page 34). The rats were decapitated at 20 seconds, 40 seconds, 1 minute,

20 minutes and 60 minutes following injection. Also, injections were done directly into the cortical tissue. The needle was stereotaxically placed at a depth of 1mm in the parietal cortex and at an angle of 23°. After the injection, the needle was kept in the tissue to prevent back-flow of the solution. Alternatively, [$^2H_8$]-arachidonic acid was given intraventricularly in 5 successive injections of 5µl each at 5 minutes of interval. Each of these injections contained 25µg of the deuterated fatty acid. The rats were decapitated 5 minutes after the last one. Unoperated rats were also decapitated and their brains dissected to determine the amount and the fatty acid composition of the triglycerides in the forebrain, cerebral cortex and choroid plexus. The post-mortem time required for the exision of the whole brain was 2 minutes. A further 3 minutes was required for the dissection and weighing of the choroid plexus or of the cerebral cortex.

Brain lipids were extracted with 20 volumes of chloroform: methanol, 2 : 1 and the free fatty acids, triglycerides, diglycerides and phospholipids were separated by column and thin layer chromatography (page 36). Measurements of the deuterium to protium ratio in the arachidonic acid from the above lipids was done by gas chromatography-mass spectrometry (page 55). The radioactivity in the triglycerides was determined in a scintillation spectrometer (page 40). The fatty acids hydrolyzed from the triglycerides of rat whole brain, choroid plexus and cerebral cortex were separated and quantitated by gas chromatography (page 40). The weight of the triglycerides was derived from that of its total fatty acids by the method outlined by Litchfield (377).

RESULTS AND DISCUSSION

The choroid plexus from the lateral ventricle showed a considerable incorporation of non-trace amounts of deuterated arachidonic acid to the triglyceride fraction (Table 1). Furthermore, the labelling of the triglycerides from whole brain and choroid plexus greatly exceeded that of any of the other complex lipids. The deuterium to protium ratio in the free arachidonic acid of whole brain decreased with post-mortem time probably as a result of the release of endogenous arachidonic acid from complex lipids which occurs upon decapitation.

The graph of radioactivity against time (figure 1) shows that the incorporation and decay of radioactive arachidonic acid in whole brain triglycerides parallels that in the choroid plexus triglycerides. Furthermore, the triglycerides of the choroid plexi from the lateral ventricles contained 1/3 or more of the counts found in whole brain triglycerides 5 and 20 minutes after the label was administered. Thus this lipid from the choroid plexi probably accounts for a major component of the time course curve of incorporation of arachidonic acid in the triglycerides of whole brain following intracerebroventricular injections.

The intracortical injections (Figure 2) show that the triglyceride pool in this brain area can also readily incorporate arachidonic acid. The maximum specific activity of the cortical triglycerides reached with this method of injection is comparable to that reached by the triglycerides of whole brain after the intraventricular injections (8.5 and 3.9 x 10^6 cpm/µmole $C_{20:4}$ respectively). However, the maximum specific activity of the choroid plexus triglycerides was 70 x 10^6 cpm/µmole $C_{20:4}$ after intraventricular injections.

TABLE 1

INCORPORATION OF $[^2H_8]$-ARACHIDONIC ACID INTO RAT BRAIN LIPIDS

	Arachidonic Acid $[^2H_8]/[^1H_8]$	
Lipid	Whole brain	Choroid Plexus
Triglycerides	0.29 (2)	4.0
Diglycerides	0.01	-
Free arachidonic acid[1]	0.62	-
Phospholipids	0.01 (2)	0.10

Each value represents one determination UNLESS INDICATED OTHERWISE

$[^2H_8]/[^1H_8]$ in whole brain lipids was determined at two minutes post-mortem.

$[^2H_8]/[^1H_8]$ in choroid plexus lipids of both lateral ventricles was determined at 5 minutes post-mortem.

The rats received 5 successive intracerebroventricular injections of 25μg $[^2H_8]$-AA in 5μl of buffer at 5 minutes of interval.

[1]The $[^2H_8]/[^1H_8]$ ratio in the FFA's of whole brain was 0.33 when determined at 10 minutes post-mortem.

Figure 1: Time course of Intraventricular Incorporation of [$^3H_8$]-Arachidonic Acid into Rat Brain Triglycerides.

Rats were injected intracerebroventricularly with approx. 5μCi of [$^3H_8$]-arachidonic acid described in the text. The full circles represent the radioactivity determined in the whole brain and the open circles the radioactivity determined in choroid plexi of the lateral ventricles. Each choroid plexus weighed approx. 1mg (wet weight).

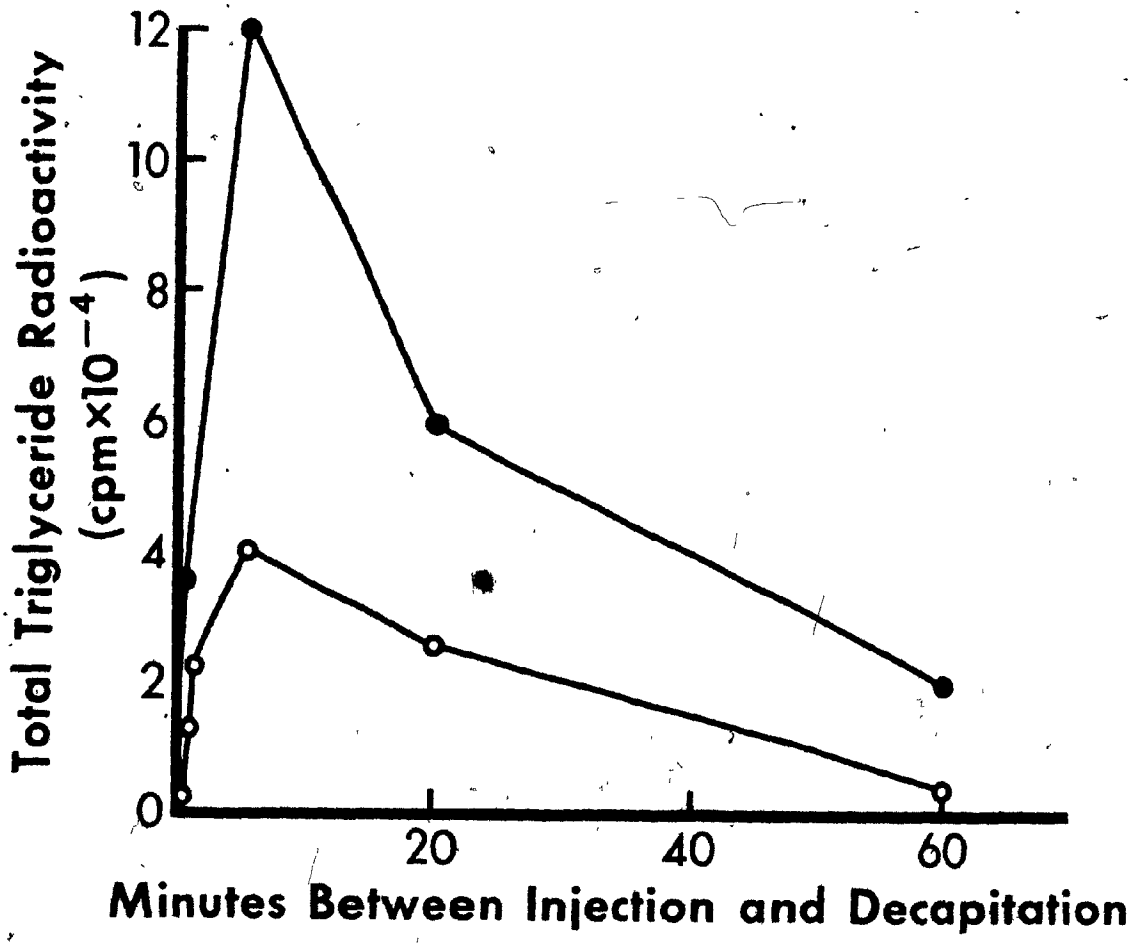
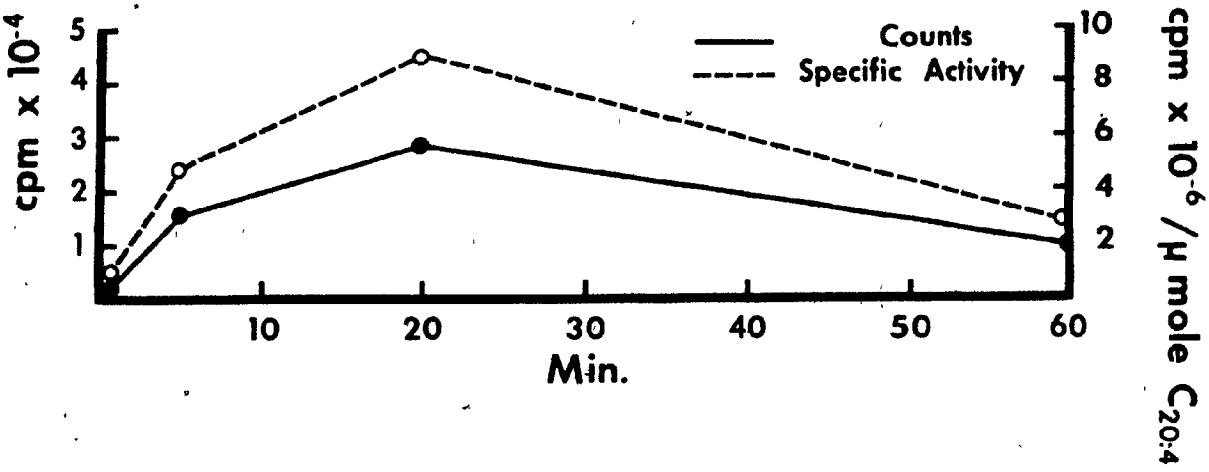

Total Triglyceride Radioactivity
(cpm×10⁻⁴)
12
10
8
6
4
2
0
20
40
60
Minutes Between Injection and Decapitation

Figure 2: Time course of Intracerebral Incorporation of [$^3H_8$]-Arachidonic Acid into Rat Cerebral Cortex Triglycerides.

The Y-axis on the left is used to indicate the radioactivity recovered in the triglycerides from 100mg of parietal cortex on the injected side. The Y-axis on the right is used for the specific activity of the arachidonic acid in the triglycerides. The x-axis represents the minutes elapsed between injection and decapitation. Rats were injected with approx. 5μCi of [$^3H_8$]-arachidonic acid into the parietal cortex as described in the text.

Time Course of Intracerebral Incorporation of $^3H_8C_{20:4}$ into Rat Cerebral Cortex Triglycerides



Interestingly, the peak incorporation of arachidonic acid into the triglycerides occured at 20 minutes after the intracortical injections but at 5 minutes after the intraventricular injections. This could result from the fact that the label, when given intracerebrally, is available for a longer period than when given intraventricularly. In the former case, 85% of the counts found at 20 seconds in the cortex remained after 5 minutes. In the case of the intraventricular injections, less than 60% of the counts found at 20 seconds in whole brain remained after 5 minutes.

The composition for the unsaturated fatty acids is markedly different in forebrain and cerebral cortex triglycerides compared to choroid plexus (Table 2). The first two tissues have a higher percentage of arachidonic acid than eicosatrienoic acid while in the latter, the siutation is reversed. However, standards of docosamonoenoic acid co-chromatographed with w-6 eicosatrienoic acid and a contribution from the former fatty acid to the eicosatrienoic peak cannot be excluded. The choroid plexus triglycerides also contained much less docosahexaenoic acid than that of the forebrain and cerebral cortex. Approximately 30 times more triglycerides per mg tissue was found in the choroid plexus than in the forebrain tissue or the cerebral cortex. The choroid plexus had 4.25μgTG/mg tissue, the forebrain, 0.11μgTG/mg tissue ± 0.02 (3) and the cerebral cortex, 0.13μgTG/mg tissue ± 0.01(4). The percentage of arachidonic acid in the triglycerides of the choroid plexus increased to 7% following repeated injections of the fatty acid (see Table 2).

The present <u>in vivo</u> studies have uncovered a concentrated and highly metabolically active triglyceride compartment in the choroid plexus of rat brain with a rapid turnover rate, a very large capacity to incorporate arachidonic acid and a distinct fatty acid composition. This triglyceride pool makes a major contribution to the time course of incorporation of arachidonic acid into triglycerides of whole brain following intracerebroventricular injection of fatty acid.

TABLE 2

Fatty Acid Composition of the Triglycerides from Different Regions of Rat Brain

	Area %		
Fatty Acid	Forebrain (3)	Cortex (4)	Choroid Plexus*
Myristate (14 : 0)	2.3 ± 0.4	2.2 ± 0.5	5.9
Palmitate (16 : 0)	40.3 ± 2.8	34.2 ± 1.3	36.8
Stearate (18 : 0)	15.2 ± 0.9	14.4 ± 1.1	17.8
Oleate (19 : 1)	22.4 ± 1.6	20.0 ± 1.7	22.0
Linolenate (18 : 2)	5.7 ± 1.3	4.5 ± 0.9	9.8
**Eicosatrienoate (20 : 3)	1.1 ± 0.7	2.0 ± 1.2	4.0
Arachidonate (20 : 4)	4.8 ± 1.0	9.5 ± 0.5	1.8
Docosahexaenoate (22 : 6)	8.2 ± 1.6	12.2 ± 1.2	1.9

*Choroid plexus from lateral ventricles of 6 rats. Forebrain was obtained from heads frozen in liquid N₂ upon decapitation. Choroid plexus and cortex lipids were extracted 5 minutes post-mortem. Arachidonic acid in choroid TG's increased to 7% following the successive intracerebroventricular injections of 5 x 20μgAA in 5μl of buffer each at 5 minutes intervals.

**Retention time on silar 10C columns corresponded to that of the W-6 isomer. Peak may contain docosamonoenoic acid.

APPENDIX B

Theoretical Labelling of $PGF_{2\alpha}$ Calculated from Rate of Entry of $[^2H_8]$-Arachidonic Acid into Cortex Slices

Since $PGF_{2\alpha}$ is produced at a constant rate by brain tissue for incubation periods of up to 60 minutes and is not significantly metabolized (47), its D/H at any given time will be equal to the average D/H of the precursor over the time period of incubation. To obtain the average D/H in a possible precursor pool, we plotted the D/H ratios in the free arachidonic acid of washed cortex slices versus incubation time (Figure 1). The ratios were measured after 0, 5, 15 and 60 minutes of incubation in the presence of $[^2H_8]$-arachidonic acid under the same conditions as described before (Table 10, page 81). The area under the curve divided by the total time of incubation will give the average D/H of the precursor over the 60 minute time period.

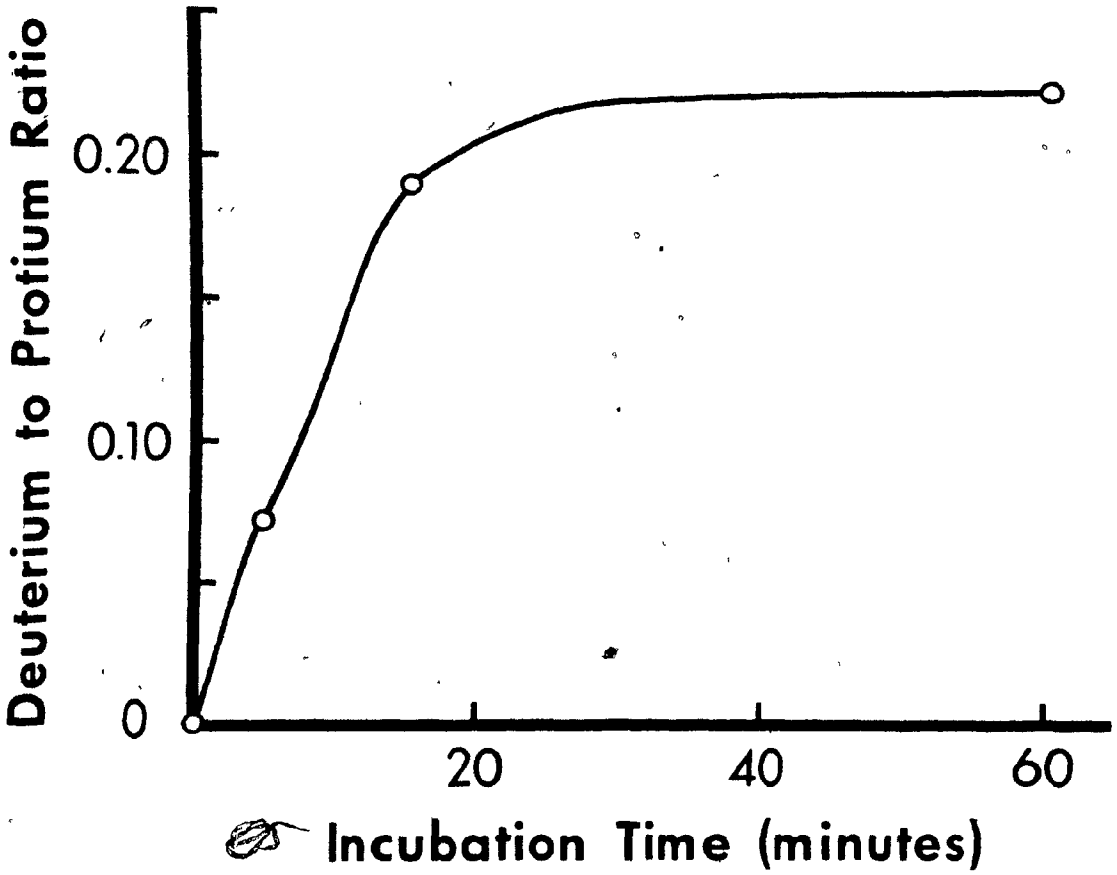
We may divide the D/H in precursor versus time curve into 3 segments bound by the experimental points. The segments may be described approximately by linear or logarythmic functions:

for the first two segments; $D/H = m_i t$ (1)

for the third segment; $D/H = \lambda \ln t$ (2)

Where D/H is the value of the ratio in the precursor at some

Figure 1: The curve relates the deuterium to protium ratio in the free arachidonic acid fraction of washed rat cerebral cortex slices with incubation times. The slices (0.5 mm thick) were incubated up to 60 minutes in the presence of 25μg of [$^{2}H_{8}$]-arachidonic acid per 100mg tissue fresh weight. After incubation, the slices were washed by re-incubation for 5 minutes in buffer containing bovine serum albumin (molar ratio 5 : 1 to total free fatty acid of slice). The values at 0 and 5 minutes are the average of 2 determinations and at 15 and 60 minutes the average of 3 determinations.


Deuterium to Protium Ratio
0.20
0.10
0
20
40
60
Incubation Time (minutes)

point within a segment. The area under the curve could then be given by:

$$A = [\sum_{i=1}^{i=2} 1/2\ (D/H_{t_i} + D/H_{t_{i-1}})\ (t_i - t_{i-1})] + [\lambda(\ln t - 1)t]_{t_2}^{t_3} \qquad (3)$$

Where the last term in (3) is the finite integral of $\lambda \ln t$. D/H_{ti} is the value of the ratio in the precursor at the upper boundary of the i^{th} time segment; the value at the lower boundary is denoted by D/H_{ti-1}. The value of λ in equation (2) was calculated as follows for the third segment:

$$\lambda = 1/2\ [(D/H_{t_{i-1}}/\ln t_{i-1}) + (D/H_{t_i}/\ln t_i)] = 0.06 \qquad (4)$$

The value of the deuterium to protium ratio obtained from equation (2) with $\lambda = 0.06$ was 10% smaller at the lower boundary and 10% larger at the upper boundary when compared to the experimental values. Thus, the area under the third segment of the curve would be close to that under the curve defined by equation (2) with $\lambda = 0.06$.

If the total free arachidonic acid fraction in the slice is the sole precursor pool of $PGF_{2\alpha}$ synthesis, the expected deuterium to protium ratio in the latter product after 60 minutes of incubation would be given by:

$$A/t = 1/60(1/2[0.07x5] + 1/2[0.18+0.07]10 + [0.06(\ln t - 1)t]_{15}^{60}) = 0.17$$

However, the D/H of the $PGF_{2\alpha}$ produced by the slice was 0.24 after 60 minutes of incubation in the presence of $[^2H_8]$-arachidonic acid. The discrepancy between the observed value and the expected value suggests that the total tissue-bound unesterified arachidonic acid may not be the only

precursor pool used for $PGF_{2\alpha}$ synthesis by rat cerebral cortex slices. On the other hand, the difference may result from the enrichment in deuterium of the products over the precursor (see appendix C).

APPENDIX C

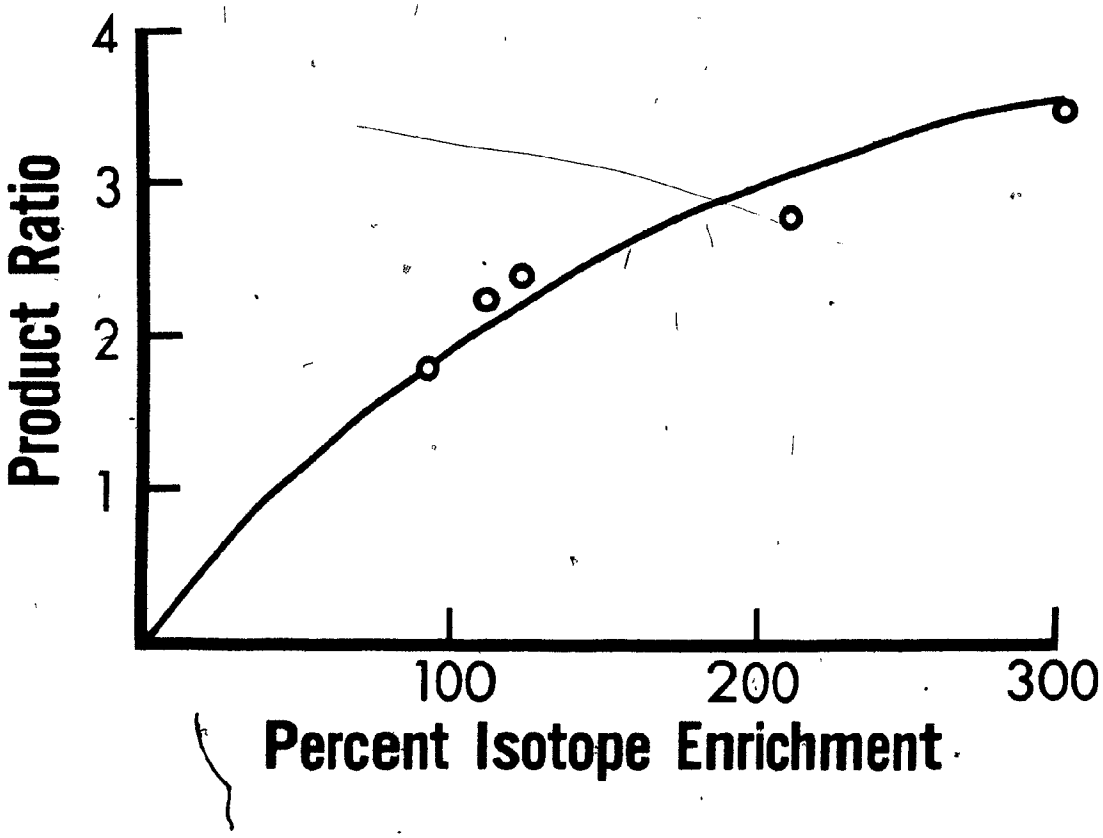
Estimation of the Enrichment in Deuterium of $PGF_{2}\alpha$ Relative to its Precursor in Cerebral Cortex Tissue *In Vitro*

The ratio of PG endoperoxide products which require carbon to hydrogen bond breaking to those that do not require it for their synthesis determined the degree of enrichment in deuterium of $PGF_{2}\alpha$ relative to a precursor mixture of arachidonic acid and octadeutero-arachidonic acid (115). In brain, the major PG endoperoxide products requiring carbon to hydrogen bond breaking for their synthesis are PGE_{2} and PGD_{2} (47,239). PGI_{2} production appears to be low in this tissue (28,38,41). The major products not requiring carbon to hydrogen bond breaking are $PGF_{2}\alpha$ and TxA_{2} (47,238,239); hydroxyheptadecatrienoic acid is not detected in brain (238).

A curve relating the product ratio to the percent enrichment of $PGF_{2}\alpha$ in deuterium (Figure 1) was made using the data of Woldawer and Samuelsson (115). These authors worked with sheep seminal vesicle microsomes and the product ratio which they used was PGE_{2} + PGD_{2} to $PGF_{2}\alpha$ + HHT. Since the above tissue was incubated in the presence of glutathione, PGI_{2} would not be formed in significant amounts (378). The production of TxA_{2} by this tissue could not be demonstrated (379).

The PG endoperoxide product ratio for rat brain homogenates was calculated from the data of Abdel-Halim *et al* (239). The homogenates had been kept at room temperature in phosphate buffer for 18-45 minutes. The PGE_{2} + PGD_{2} to $PGF_{2}\alpha$ + TxB_{2} ratio was 3.07 in this case. The curve of Figure 1 indicates that this ratio would correspond to more than a 200%

Figure 1: The curve relates the product ratio PGE_2 + PGD_2 to $PGF_{2\alpha}$ + HHT and the percent of deuterium isotope enrichment in the $PGF_{2\alpha}$ produced by sheep seminal vesicles incubated in the presence of a mixture of arachidonic acid and octadeuteroarachidonic acid. The curve was derived from the data of Woldawer and Samuelsson (115).


Product Ratio
4
3
2
1
100
200
300
Percent Isotope Enrichment

deuterium enrichment in the $PGF_{2\alpha}$ relative to its precursor pool of arachidonic acid.

In our experiments, the deuterium to protium ratio in the $PGF_{2\alpha}$ produced by homogenates of rat cerebral cortex slices incubated for 60 minutes in Ringer-Glucose-Bicarbonate buffer was equal to the ratio found in the total free arachidonic acid fraction. Since a large isotope enrichment of $PGF_{2\alpha}$ is likely to have occurred as seen above, the precursor pool used for $PGF_{2\alpha}$ synthesis may not correspond to the total arachidonic acid fraction. However, different incubation conditions will result in different ratios of PG endoperoxide products. Thus the magnitude of the isotope enrichment of $PGF_{2\alpha}$ must be determined for each situation.

CLAIMS TO ORIGINAL RESEARCH

1- Pre-labelling of rat brain lipids *in vivo* by intracerebroventricular injections of $[^3H_8]$-arachidonic acid has been used to identify the origin of the arachidonic acid released post-mortem in this tissue.

2- A mixture of phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine has been implicated as the major source of the arachidonic acid released post-mortem in rat forebrain. Phosphatidic acid and phosphatidylserine have been excluded as significant components. Triglycerides and diglycerides have also been excluded as a source of the released arachidonic acid. Phosphatidylinositol was singled out as the major contributor to the free arachidonic acid pool bound to the microsomal fraction prepared from rat forebrain.

3- Comparison of the deuterium to protium ratios in the prostaglandin $F_{2\alpha}$ to those in the arachidonic acid of various lipid fraction from rat cerebral cortex slices incubated in the presence of $[^2H_8]$-arachidonic acid has been used to study the compartmentation of the arachidonic acid available to the prostaglandin synthetase of this tissue.

4- A standard line has been made for known amounts of $[^2H_8]$-prostaglandin $F_{2\alpha}$ versus the amounts measured by gas chromatography-mass spectrometry using $[^2H_4]$-prostaglandin $F_{2\alpha}$ as internal standard. This line was used to quantitate the $[^2H_8]$-$PGF_{2\alpha}$ produced by rat cerebral cortex slices and homogenates from $[^2H_8]$-arachidonic acid.

5- It was shown that exogenous arachidonic acid penetrates poorly into rat cerebral cortex slices. However, the use of exogenous arachidonic acid by the damaged surfaces of cortex slices to produce prostaglandin $F_{2\alpha}$ as well as the stimulation of the prostaglandin synthetase at that location by norepinephrine has been demonstrated.

6- A probable isotope enrichment effect suggests that endogenous arachidonic acid is preferentially used for prostaglandin $F_{2\alpha}$ synthesis in the homogenate of rat cerebral cortex although the deuterium to protium ratio of the total free arachidonic acid was similar to that of the prostaglandin $F_{2\alpha}$ in this preparation.

7- No complex lipid was found to act as an intermediate in the delivery of exogenous arachidonic acid to the prostaglandin synthetase of rat cerebral cortex in vitro.

8- The level of the major free fatty acids in rat cerebral cortex slices has been measured at various times post-mortem and arachidonic acid was found to be released initially at the fastest rate.

9- The selective release of arachidonic acid in rat forebrain during carbachol induced convulsions has been demonstrated. A correlation has been established between the release of arachidonic acid and the synthesis of prostaglandin $F_{2\alpha}$ but not thromboxane B_2 by rat forebrain during drug induced convulsions. Under these conditions, the production and disappearance of the prostaglandin endoperoxide were very rapid.

10- It was found that phenytoin decreased the post-mortem levels of free arachidonic acid and stearic acid in rat forebrain when injected intraperitoneally *in vivo*.

11- The choroid plexus was established as a major site of incorporation of arachidonic acid into rat brain triglycerides following intraventricular injection of the fatty acid. The choroid plexus triglycerides had a fatty acid composition distinct from that of forebrain and cerebral cortex.

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