# BIOSYNTHESIS OF PROSTACYCLIN IN THE RAT CEREBRAL MICROVASCULATURE

UWE GUSTAV GOEHLERT, B.Sc.

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry
McGill University
Montreal

# PROSTACYCLIN IN RAT CEREBRAL MICROVESSELS

M.Sc. Biochemistry

Biosynthesis of Prostacyclin in the Rat Cerebral Microvasculature

## ABSTRACT

Microvessels, predominantly capillaries, were isolated from rat cerebrum by a modification of published procedures. The morphology and purity of the preparations were monitored by light- and electron-microscopy and by enrichment in alkaline phosphatase and γ-glutamyl-transpeptidase. A reversed-phase high pressure liquid chromatographic method was used in the analysis of prostaglandins after extraction from aqueous incubation solutions. The endogenous biosynthethic capacity of the isolated cerebral capillary fractions for prostacyclin measured as its chemically stable breakdown product, 6-keto-prostaglandin $F_{1\alpha}$, was 11 ng/mg protein/10 min. Choroid Plexus and intact surface vessels synthesized 6-keto-prostaglandin $F_{1\alpha}$ at 37 and 35 ng/mg protein/10 min respectively. The prostacyclin synthesizing enzymes of the cerebral capillaries also converted the exogenously added prostaglandin endo-peroxides to 6-keto-prostaglandin $F_{1\alpha}$. Comparison of the synthesis of prostaglandins 6-keto-$F_{1\alpha}$, $E_2$ and $F_{2\alpha}$ showed that 6-keto-prostaglandin $F_{1\alpha}$ was the major prostaglandin formed in the microvessels, in the larger surface vessels and in the choroid plexus. Prostaglandin $D_2$ was not detected. Endothelial prostacyclin synthesis by the cerebral vasculature is similar to that in other blood vessels and cultured human endothelial cells. Prostacyclin in the cerebral microvasculature, including the choroid plexus, may play an important physiological role particularly in relation to cerebrovascular antithrombogenesis and regulation of cerebral perfusion. The availability of metabolically active microvessels opens up new vistas in the study of the cerebral microcirculation.

## RESUME

Les microvaisseaux principalement les capillaires, du cerveau du rat, étaient isolés par modifications des procédures publiées. La morphologie et pureté des ces préparations étaient vérifiées par microscopie optique et électronique, de même que par l'enrichissement en l'alcaline phosphatase, et le transpeptidase γ-glutamique. Une méthode chromatographique de liquide à haute pression est utilisée pour analyser des prostaglandins aprés l'extraction d'une solution aqueuse. La capacité biosynthétique endogène de ces capillaires isolés pour la prostacycline, mesurée comme le produit stable 6-keto-prostaglandin $F_{1\alpha}$ , est 11 ng/mg protéin/10 min. Le plexus choroidique et les vaisseaux superficiels produisent 6-keto-prostaglandine $F_{1\alpha}$ à 37 et 35 ng/mg protéine/10 min respectivement. Le système enzymique des capillaires cérébraux peut également transformer les endoperoxides de prostaglandine a 6-keto-prostaglandine $F_{1\alpha}$ . Comparaison entre la synthése des prostaglandines 6-keto-$F_{1\alpha}$ , $E_2$ et $F_{2\alpha}$ a démontré que 6-keto-prostaglandine $F_{1\alpha}$ étaient la majeure prostaglandine produite par les microvaisseaux, les vaisseaux superficiels et le plexus choroidique. Le taux synthétique de prostacycline par les parties vasculaires, du cerveau, est similaire a celle des autres vaisseaux de sang et les cellules endothéliales humaines en culture. Prostacycline dans la microvasculature du cerveau pourrait avoir une importante fonction physiologique plus particuliérement en relation à la prevention de thromboses cérébrales et la regulation de la microcirculation dans le cerveau.

## ACKNOWLEDGEMENTS

The research described in this thesis was carried out in the Donner Laboratory of Experimental Neurochemistry, at the Montreal Neurological Hospital and Institute of McGill University.

I wish to express my sincere gratitude and appreciation to my mentor, Dr. L.S. Wolfe, for his continuous enthusiasm, inspiration and stouthearted patience.

I would also like to thank Dr. N.M.K. Ng Ying Kin for his expert technical advice, innumerable informative discussions and help in the HPLC analysis. The excellent technical help of Mrs. K. Rostworowski, in the GC-MS analysis, is gratefully acknowledged.

I am thankful to Dr. F. Coceani and Dr. E. Bishai, of the Hospital for Sick Children, Toronto, for the aid in the synthesis of the prostaglandin endoperoxides, Dr. I. Hüttner of the Department of Pathology, McGill University, for the Electron Microscopic work and Dr. O. Mamer, of Bio-Mass Spectrometry Unit, McGill, for the quantitative mass spectrometry. Mr. C.P. Hodge of the Neurophotography Department, M.N.I., prepared the photographs of all the figures, and I am indebted to Ms. E. Simon for her patience in typing this manuscript.

Prostaglandin standards were generously supplied by Dr. J. Pike, of the Upjohn Company, Kalamazoo, Michigan. I would like to thank the Donner Laboratory and its administrators for financial support throughout the duration of this work.

Most of all, I would like to dedicate this work to my loving parents who created the foundation for me to build upon.

# TABLE OF CONTENTS

## List of Tables



## List of Illustrations

# Chapter I

# INTRODUCTION

## A. Historical Perspective of Eicosanoids.

### 1. Classical Prostaglandins

Coping with the biomedical literature on prostaglandins and allied compounds has become an enormous and almost impossible task. In recent years, prostaglandin research has been one of the fastest growing fields at the center of molecular biology and clinical medicine. This explosion of research is caused not so much by the greater productivity of modern investigators as compared to their predecessors but by the greater number of people fascinated by the potential of these compounds in normal and aberrant physiology and pharmacology. The harbinger of this remarkable scientific development was the discovery, in 1930, by American gynecologists Raphael Kurzrok and Charles C. Lieb that strips of uterine muscle tissue contracted when exposed to seminal fluid (1). This phenomenon had also been observed in uterine tissue during early attempts at artificialinsemination. Unfortunately, due to the lack at that time of adequate pharmacological tools, the significance of that initial observation was not explored and the findings had little impact and were filed away. A few years later a Swedish researcher Ulf S. Von Euler and an English scientist Maurice W. Goldblatt independently observed that human semen and extracts of sheep vesicular glands contained factors that contracted intestinal and uterine smooth muscle and lowered arterial blood pressure in laboratory animals (2,3).

Von Euler (4) had been studying hormones and biogenic amines in seminal fluid at the time of his discovery and was able to show that these potent biological activities were attributable to acidic lipids which he thought derived from the prostate gland. He therefore proposed the name "prostaglandins" for these substances (4). In retrospect one can accumulate many examples of similar biological activities with various names which are now known to be due to mixtures of prostaglandins or prostaglandin-like constituents. Amongst these are Darmstoff, intestinal stimulant acid lipid, irin, menstrual stimulant, vasodepressor lipid, medullin, slow-reacting substance C, and unsaturated hydroxy fatty acid fractions from brain (5).

Little further progress was made in this field during the next two decades due to lack of anaytical methodologies and it was not until Von Euler approached Sune Bergstrom of the Karolinska Institute that work was revived to elucidate the chemical nature of prostaglandins. In the late 50's and early 60's hundreds of kilograms of vesicular glands from young Icelandic sheep were collected to give a moderate quantity of material for purification and structural analysis.

When finally isolation of crystalline material was possible it became apparent that prostaglandin was actually a family of compounds with unique but closely related structures.

The first prostaglandins to be described were prostaglandin $E_1$ ($PGE_1$) and prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) (6). The letters E and F in

PGE and PGF refer to an earlier finding that the E and F compounds could be partially separated by extraction with Ether from phosphate (Fosfat; Swedish) buffer. PGE compounds are extracted preferentially into the less polar ether phase and PGF compounds remain in the more polar phosphate buffer. Soon afterwards a group of six "primary" prostaglandins were characterized, by Bergström and coworkers (5) and a picture appeared of the general chemical structure of the prostaglandins. All six prostaglandins had a twenty (eicosa -) carbon skeleton containing a cyclopentane ring involving carbons numbers 8 to 12. This ring has substituents of either hydroxy-or keto-oxygen functions at positions 9 and 11 depending on whether the prostaglandin was of the E or the F type. The basic structure also included a C-15 hydroxyl group and a carboxylic acid function at position one. Three series of prostaglandins, designated by subscripts 1,2,3 were recognized. The numbers indicated the degree of unsaturation of the hydroxy-octyl and carboxy-hexyl side chains.

In all natural PG's the carbon-carbon double bond at the 13 position is always trans whereas in the prostaglandin 2 and 3 series the additional double bonds at the 5 and 17 position are in the cis configuration. A second subscript of $\alpha$ or $\beta$ in the designation of the F prostaglandins refers to the stereochemistry of the side chains with respect to the C-9 hydroxyl group of the cyclopentane ring.

Almost immediately after the structures of the three series of prostaglandins were announced their kinship with certain essential fatty acids was appreciated.

$PGE_1$ and $PGF_{1\alpha}$ are formed by oxidative bioconversion of 8,11,14 eicosatrienoic acid (homo-γ-linolenic acid), $PGE_2$ and

$F_{2\alpha}$ from 5,8,11,14 eicosatetraenoic acid (arachidonic acid) and $PGE_3$ and $PGF_{3\alpha}$ from 5,8,11,14,17 eicosapentaenoic acid. In 1964, the relationship was proven, independently, by Bergström et al (7) in Sweden and Van Dorp et al (8) in Holland who achieved the biosynthesis of $PGE_2$ from arachidonic acid using microsomal samples from sheep seminal vesicles. Transformations of arachidonic acid into the various eicosanoids is shown in Figure 1.

During the mid 1960's, due largely to the efforts of chemists under the direction of Elias J. Corey at Harvard and those working independently at Upjohn under J.E. Pike, the total chemical synthesis of all the known prostaglandins was accomplished (9). The availability of adequate amounts of pure synthetic prostaglandins and the discovery in 1971 by John Vane that aspirin and related drugs prevented the biosynthesis of prostaglandins (10) gave a strong impetus to research and launched an intense interest in these compounds regarding their roles in fever, pain and inflammation.

2. Endoperoxides

The formation of an unstable cyclic endoperoxide intermediate during the oxygenation of arachidonic acid was postulated by Samuelsson in 1965 (11). Employing $O_2^{16}$, $O_2^{18}$ and mass spectrometry he found that both oxygen atoms present on the cyclopentane ring of prostaglandins were derived from the same molecule of oxygen. It was not until almost ten years afterwards, in 1973, that Hamberg and Samuelsson (12) and Nugteren and Hazelhof (13), in a series of elegant experiments, were able to identify and isolate two labile intermediates in the conversion of arachidonic acid to prostaglandins. The two compounds

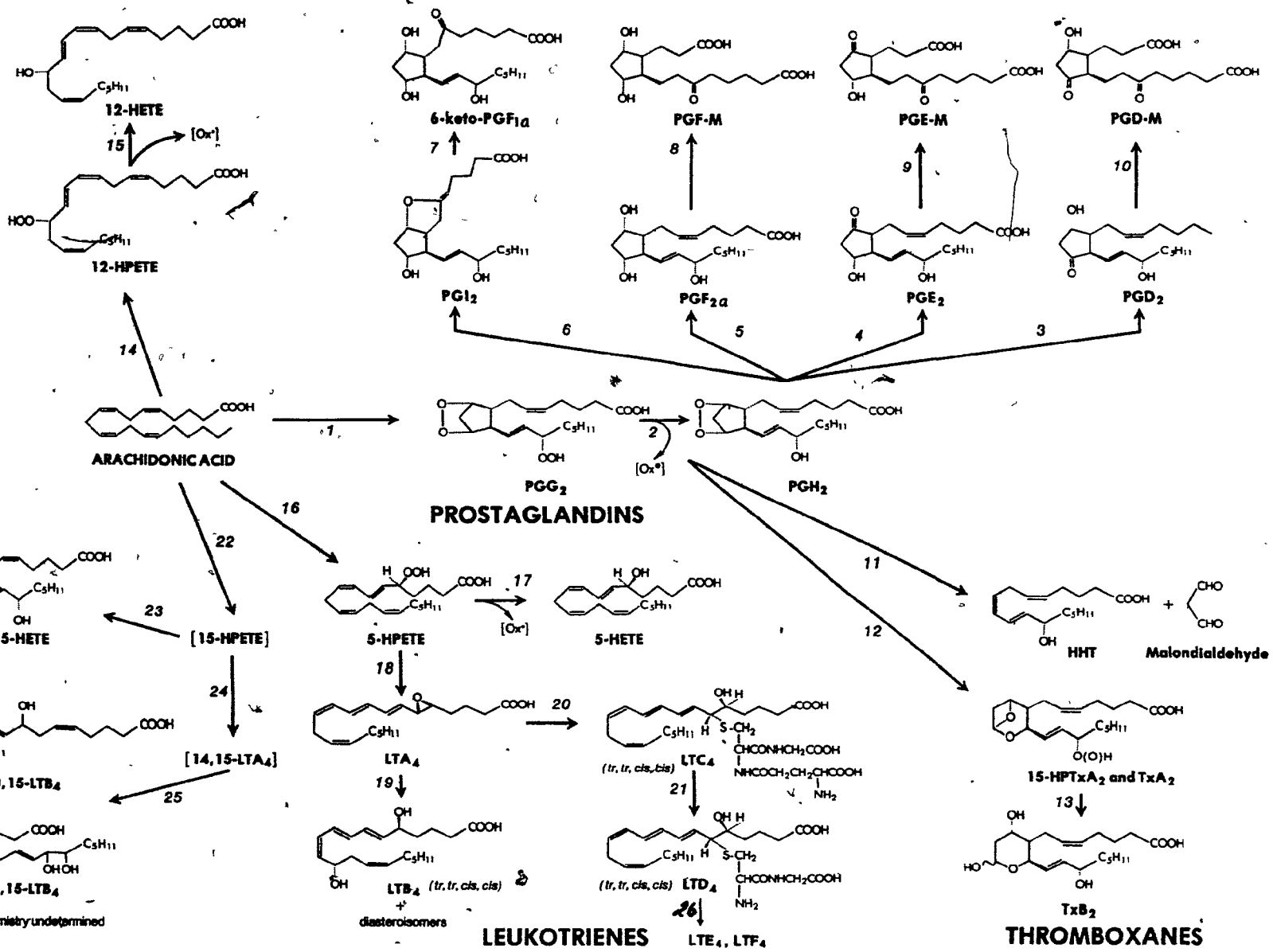
Figure 1. Scheme of Transformations of Arachidonic Acid.

1. Fatty acid cyclo-oxygenase, (Prostaglandin-endoperoxide Synthetase)
2. Prostaglandin Hydroperoxidase (Peroxidase)
3. Endoperoxide D-isomerase
4. Endoperoxide E-isomerase
5. Endoperoxide $F_{2\alpha}$-reductase
6. Prostacyclin Synthetase
7. Nonenzymatic Hydrolysis

8-10. Prostaglandin 15-hydroxydehydrogenase, Prostaglandin 13,14 reductase followed by $\beta$ and $\omega$ oxidations

11. Nonenzymatic Endoperoxide Cleavage
12. Thromboxane Synthetase
13. Nonenzymatic Hydrolysis
14. 12-Lipoxygenase
15. Hydroperoxidase (Peroxidase)
16. 5-Lipoxygenase
17. Hydroperoxidase
18. Leukotriene A Synthetase
19. Enzymatic Hydrolysis
20. Glutathione S-Transferase
21. γ-Glutamyltranspeptidase
22. 15-Lipoxygenase
23. Hydroperoxidase
24. 14,15-Leukotriene $A_4$ Synthetase
25. Enzymatic Hydrolysis
26. Aminopeptidase, γ-glutamyl transpeptidase

# TRANSFORMATIONS OF ARACHIDONIC ACID



were otherwise identical differing only in the substituent on carbon 15. The first derivative named prostaglandin $G_2$ carried a hydroperoxy group at C-15 while in the subsequent compound, prostaglandin $H_2$, this substituent was reduced to a hydroxyl group. With the demonstration of the existence of these labile intermediates a mechanism of prostaglandin biosynthesis was visualized. The initial step consisted of a lipoxygenase-like reaction in which a specific hydrogen at C-13 was removed, the $\Delta^{11}$ double bond was simultaneously isomerized into the $\Delta^{12}$ position, and molecular oxygen (as the diradical) was inserted at C-11. Formation of a cyclopentane ring was a result of a second oxygen attacking at C-15, with shift of the $\Delta^{12}$ double bond, and formation of a new bond between C-8 and C-12 (12). These reactions yielded $PGG_2$ which was then reduced to $PGH_2$. The chemical half-life of the prostaglandin endoperoxides in aqueous media was about 5 min (14) after which the cyclic peroxide had opened to yield prostaglandins E, D and F. In addition two compounds, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde, were identified as by products from fragmentation of the endoperoxides. Three enzymic activities were detected in various rat tissues which converted $PGH_2$ into $PGE_2$, $PGD_2$ and $PGF_{2\alpha}$ (13).

Prostaglandin endoperoxide - $PGE_2$ isomerase was present in the particulate fraction of sheep vesicular gland. Prostaglandin endoperoxide-$PGD_2$ isomerase (detect in some rat tissues) was present in the supernatant after centrifugation at 100,000Xg and thus was considered a soluble protein distinct from the $PGE_2$ forming isomerase.

Prostaglandin $F_{2\alpha}$ was enzymatically formed by a prostaglandin endoperoxide - $PGF_{2\alpha}$ reductase.

3. Thromboxanes

During the 1960's Vane and his colleagues adapted the blood-bathed organ technique (15) to the detection of prostaglandins in biological fluids, a contribution of prime importance for it allowed the continuous monitoring of prostaglandins in blood as well as their quantification. In 1969 Piper and Vane (16) detected the release of an additional substance during anaphylaxis using sensitized guinea-pig lung. The substance was a potent contractor of isolated rabbit aorta and was thus called "Rabbit Aorta Contracting Substance or RCS". RCS was a labile substance with a half-life of 1-2 min in aqueous buffer at room temperature whose release was stimulated by arachidonic acid (17) and inhibited by aspirin-like drugs (16). Because of these properties it was suggested that RCS was an unstable intermediate in the biosynthesis of prostaglandins. Platelet aggregation in vitro was also accompanied by the generation of an RCS (18). The half-lives of RCS (less than 2 min) and prostaglandin endoperoxides (about 5 min) were distinctly different. Furthermore, the relative potencies of endoperoxide versus RCS on platelet aggregating activity and aorta contracting activity were also dissimilar (14). These observations directed Samuelsson and co-workers to search for other substances with RCS activity in platelets. In 1975, Hamberg, Svensson and Samuelsson (19) described the conversion of the endoperoxides into a new group of biologically active compounds. Due to their powerful thrombogenic nature, their tissue specificity (i.e. thrombocytes) and their non-prostanoid

chemical structure, these compounds were called "thromboxanes". The first thromboxane, thromboxane $A_2$ ($TXA_2$) was a highly unstable intermediate witha half-life of 30 sec at 37°C which strongly contracted aortic strips and induced platelet aggregation. The lability was due to the bicyclic oxane-oxetane ring structure. The stable nonenzymatic hydrolysis product was called thromboxane $B_2$ ($TXB_2$) or PHD (19). Before long it became apparent that RCS was a mixture of mainly $TXA_2$ along with some endoperoxide (20). The highest conversion of endoperoxides to $TXA_2$ is in platelets and lung. A specific thromboxane $A_2$ forming enzyme, independent of the cyclo-oxygenase activity, has been described and partially characterized in human and horse platelet microsomes (21). The formation of $TXB_1$, from homo-γ-linolenic acid, has been demonstrated by Falardeau et al (22). Conversion of $PGH_3$ into $TXA_3$ has recently also been reported (23).

4. Prostacyclin

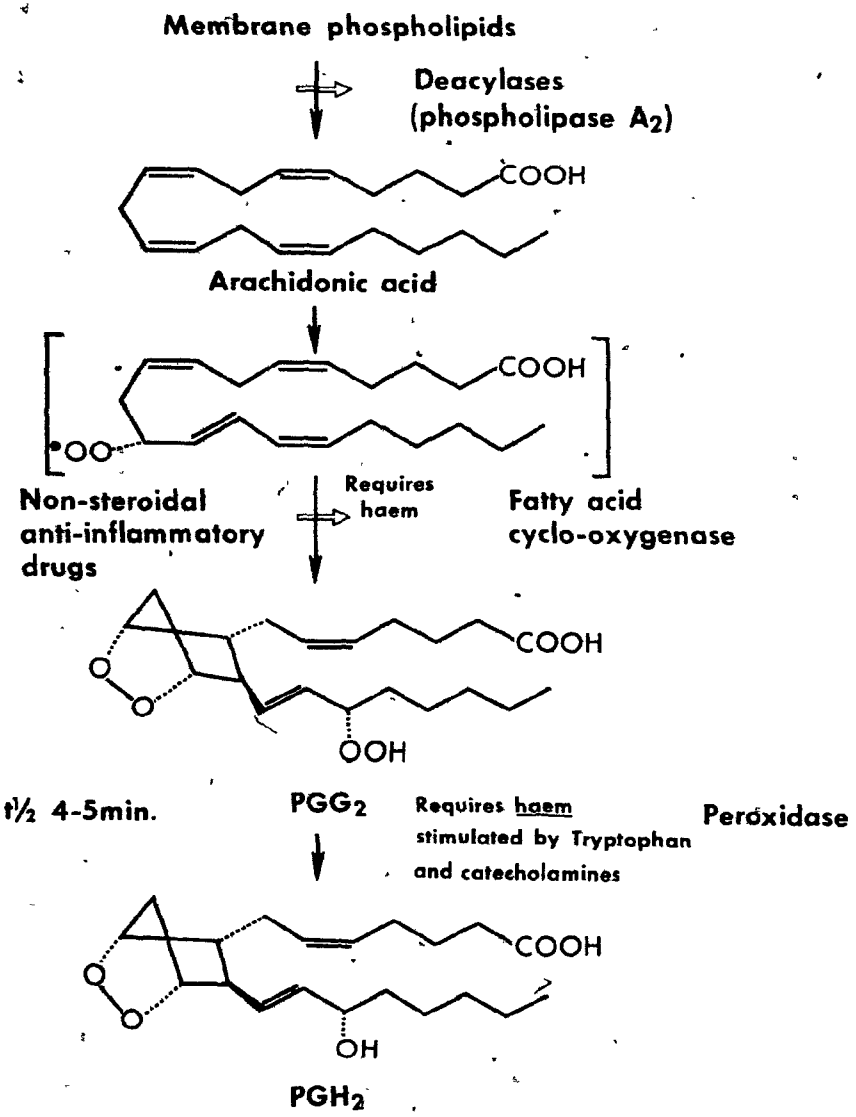
Theevents leading up to the final chemical and biological description of yet another unstable eicosanoid compound began as early as 1970 when Pace-Asciak and Wolfe (24) proposed a novel pathway of arachidonic acid metabolism occurring in rat stomach. They termed this pathway the 6(9)-oxy cyclasepathway. Six years later, while in search for thromboxane synthetase activity in various rabbit tissues, Moncada et al (25) isolated an enzyme from the microsomal fraction of blood vessels that transformed prostaglandin endoperoxides into a labile substance which was a very potent inhibitor of platelet aggregation and relaxed isolated blood vessels. They called this substance prostaglandin X and

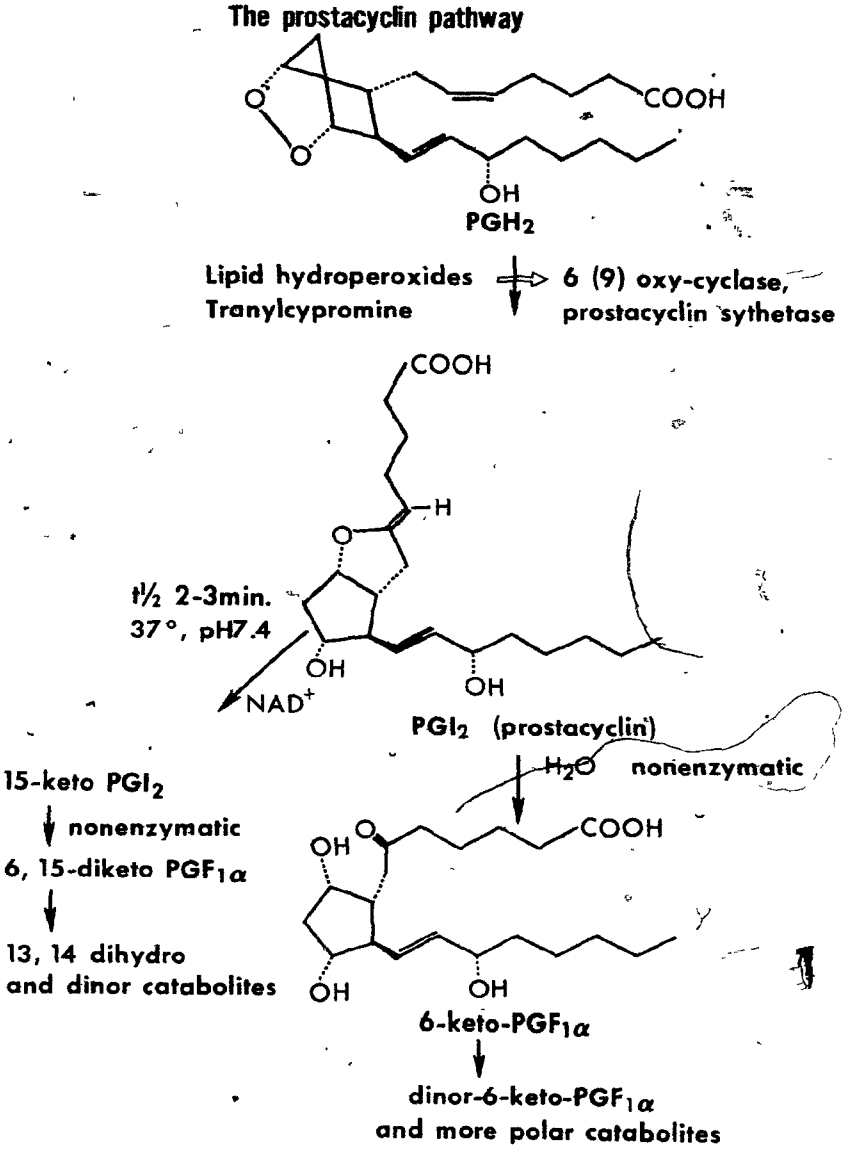
showed that the formation of this unstable compound was the predominant route of arachidonic acid and endoperoxide metabolism in blood vessel walls (26). The only other tissue tested in which PGX was the main product from prostaglandin endoperoxides was the fundic part of the rat stomach (27). This finding suggested that PGX might also be an intermediate in the 6(9) oxy pathway. Subsequent chemical synthesis of PGX by Johnson et al (28) showed the structure of PGX to be equivalent to 6(9) oxy-11,15-dihydroxy prosta-5,13-dienoic acid or 6(9) oxy- $\Delta^5$-$PGF_{1\alpha}$, one of the less abundant isomers isolated in 1971. This biologically active product was then dubbed prostacyclin and abbreviated $PGI_2$ (28). Prostacyclin is a labile bicyclic ether with a half-life of 2-3 min in aqueous solution at 37°C. Earlier in 1976, before the chemical synthesis of prostacyclin was accomplished, Pace-Asciak reported the isolation of another product of the 6(9) oxy pathway (29). This compound was identified by mass spectrometry as 6-keto-$PGF_{1\alpha}$ (30). The relationship of 6-keto-$PGF_{1\alpha}$ to 6(9) oxy- $\Delta^5$-$PGF_{1\alpha}$ (Prostacyclin) was appreciated when it was shown that 6-keto-$PGF_{1\alpha}$ could also exist in a cyclic lactol form (31). The 6-keto-$PGF_{1\alpha}$ was the stable hydration product of prostacyclin which is formed in a sequence of enzymatic reactions starting from arachidonic acid and proceeding via the endoperoxide intermediates (Fig 2).

A corresponding sequence of enzymatic reactions converts eicosapentaenoic acid into $\Delta^{17}$-prostacyclin ($PGI_3$) and $\Delta^{17}$-6-keto-$PGF_{1\alpha}$ (23). The double bond in the 5 position is essential in the synthesis of prostacyclins and 8,11,14 - eicosatrienoic acid (homo-$\gamma$-linolenate) cannot be converted to a prostacyclin (32).

Figure 2. The Prostacyclin Pathway, Consisting of Prostaglandin Endoperoxide Synthetase Complex (Left Plate) and Prostacyclin Synthetase (Right Plate).

## Biosynthesis of Prostaglandin Endoperoxides



Rapid progress in analytical technology and biological assay methods, applied to prostaglandin research, have led to the discovery, chemical characterization, and quantitation of numerous biologically active eicosanoids. The availability of such methods has enabled scientists to discern the metabolic transformations of these substances and unravel their diverse physiological and pharmacological potential.

In the early years of research in this field most methods were based on the potent characteristic activity of a particular prostanoid in some biological system. These were followed by chemical and physical methods such as ultraviolet (UV) spectrometry, gas-liquid chromatography with flame ionization detection, and enzymatic methods utilizing specific prostaglandin dehydrogenases.

Although these techniques were of value in detection, they were lacking adequate sensitivity and specificity to differentiate and quantitate the increasing number of fatty acid derived products.

Better techniques have recently gained widespread use. Radiochemical methods, gas-liquid chromatography with electron capture detection, gas-liquid chromatography-mass spectrometry (GC-MS), high pressure liquid chromatography (HPLC) with UV or radiometric detection, cascade superfusion bioassay and radioimmunoassay are used routinely (33). Radioimmunoassay is today the most routinely employed assay method in prostaglandin research (34). It is inexpensive and quick but relatively less specific and accurate when compared to GC-MS. Therefore, it is essential to validate where possible a radioimmunoassay for a given prostaglandin by quantitative GC-MS (35).

GC-MS is entirely a chemical and thus very specific type of method. Quantitative mass spectrometry with deuterium-labelled compounds and multiple ion detection is presently the most accurate and selective method for prostaglandin analysis (36). It is based on the addition to the sample of a known amount of deuterated analogue which acts as a carrier during the chromatographic purification steps and as an internal standard during mass spectrometry. The small quantity of natural, protium form of the compound can be calculated from the measured proportion between selected ions of the protium and the deuterium forms of the purified compound. The presence of characteristic fragmentation (Table 1) and prominent peaks in the mass spectrum of the 6-keto-$PGF_{1\alpha}$ derivative (Fig 3) makes possible an optimal utilization of the multiple ion detection system (37).

The combination of this quantitative method (GC-MS) with powerful purification technology such as HPLC (38) and octadecylsilyl silica (39) chromatography will yield more reliable data and avoid the shortcomings of the other methods.

5. Lipoxygenase Products and Leukotrienes.

In 1974, at the same time when Hamberg and Samuelsson (40) discovered that platelets produced thromboxane, they also described another novel product derived from arachidonic acid.

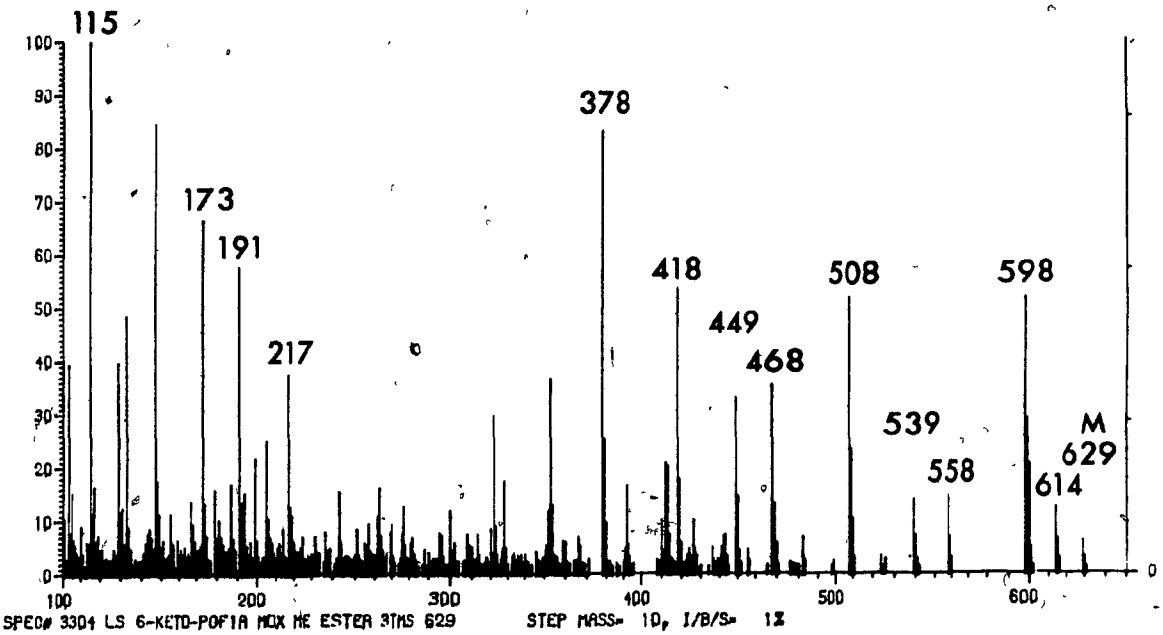
They showed that the chemical structure was 12 (S)-hydroxy-5,8,14-cis, 10-trans-eicosatetraenoic acid (12-HETE) a degradation product of 12(S)-hydroperoxy-5,8,14-cis, 10-trans-eicosatetraenoic acid (12-HPETE) which they thought was formed from arachidonic acid by a lipoxygenase enzyme that was not inhibited by nonsteroidal

TABLE 1.

Prominent Mass Spectral Fragments of 6-keto-Prostaglandin $F_{1\alpha}$

| Fragment Ion | m/e Value |
|---|---|
| Molecular Ion | 629 |
| $M^+ - CH_3$ | 614 |
| $N^+ - OCH_3$ | 598 |
| $M^+ - C_5H_{11}$ | 558 |
| $M^+ - HOTMSi$ | 539 |
| $M^+ - (HOTMSi + OCH_3)$ | 508 |
| $N^+ - (HOTMSi + C_5H_{11})$ | 468 |
| $M^+ - 2(HOTMSi)$ | 449 |
| $M^+ - (2(HOTMSi) + OCH_3)$ | 418 |
| $M^+ - (C_4H_8 \cdot CO_2CH_3 + C_5H_{11} + OCH_3)$ | 412 |
| $M^+ - (2(HOTMsi) + C_5H_{11})$ | 378 Base Peak |
| $M^+ - (HOTMSi + CH_2 \cdot C(=N \cdot OCH_3) + (CH_2)_4 CO_2CH_3)$ | 353 |
| $M^+ - (C_4H_8 \cdot CO_2 - CH_3 + TMSiO^+ = CHOTMSi)$ | 321 |
| $M^+ - TMSiO^+ - C=CH \cdot CH_2 - TMSiO^+$ | 217 |
| $M^+ - TMSiO^+ = CHOTMSi$ | 191 |
| $M^+ - TMSiO^+ = CH \cdot C_5H_{11}$ | 173 |
| $M^+ - C_4H_8 \cdot CO_2CH_3$ | 115 |

Figure 3. Mass spectrum of 6-keto-$PGF_{1\alpha}$ as the methyl ester-methoxime trimethylsilyl ether derivative. The mass spectrometer was operated in the full scan mode to obtain the complete mass spectrum of the compound from 100 m/e to 650 m/e. The spectrum represents 2-3 $\mu$g of material and conditions for GC-MS are described in the text.


115
173
191
217
378
418
449
468
508
539
558
598
614
M
629
100
90
80
70
60
50
40
30
20
10
0
100
200
300
400
500
600
SPEC# 3304 LS 6-KETO-PGF1A MOX ME ESTER 3TMS 629
STEP MASS= 10, I/B/S= 1%

antiinflammatory agents. The platelet arachidonate lipoxygenase was isolated a year later by Nugteren (41). When it was discovered that 12-HETE exhibited chemotactic activity on neutrophils a search for lipoxygenase products of arachidonic acid and other fatty acids in blood cellular components was undertaken. A series of mono-hydroxy-eicosanoic acids were found to be major metabolites of arachidonic acid in the various cell types. Polymorphonuclear leukocytes(PMNL's) produced mainly 5-L-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 8-L-hydroxy-9,11,14-eicosatrienoic acid from arachidonic acid and homo-γ-linolenic acid respectively (42). Rat mast cells convert arachidonate mainly to 11-HETE and 12-HETE while rabbit alveolar macrophages form primarily 5-HETE and 11-HETE (43). The production of 5-,12-,15-,8- and 9- mono-HETE's from arachidonic acid by human neutrophils has also been reported (43,44). More recently novel dihydroxy eicosatetraenoic acids have been described. The first one identified was 5-(S), 12-(R)-dihydroxy-6,14-cis, 8,10-trans-eicosatetraenoic acid (5,12-DHETE) and was found upon incubation of arachidonic acid with rabbit peritoneal PMNL's (45). In the past year isomeric products derived from a 15- instead of a 5- lipoxygenase pathway have been discovered (46). These are 8,15-dihydroxy-5,9, 11,13-eicosatetraenoic acid (8,15-DHETE) and 14,15-dihydroxy-5,8, 10,12-eicosatetraenoic acid (14,15-DHETE). Many other arachidonic acid derived compounds have been described in the prostaglandin literature including trihydroxyeicosatetraenoic (THETE's) and iodo-hydroxy fatty acids (47,48).

The recognition of so many arachidonic acid derivatives brought about a problem of nomenclature. The term "eicosanoids" was coined

by Corey in 1979 to designate the vast number of compounds derived from unsaturated fatty acids containing twenty carbons (49). The increasing number of lipoxygenase products, excluding the mono-hydroxy fatty acids, from various sources have been named "leuko-trienes" (LT's) since they were originally identified in white blood cells and contained a conjugated triene system (50). Hence 5,12,-DHETE, 8,15-DHETE and 14,5-DHETE are called 5,12-$LTB_4$, 8,15-$LTB_4$ and 14,15-$LTB_4$ respectively. The subscript "4" indicates, as in the case of prostaglandin nomenclature, the degree of unsaturation of the compound. The capital alphabetical assignment (A-F) refers to the position the particular leukotriene occupies in the sequential metabolism of arachidonic acid through the lipoxygenase-leukotriene pathway (Fig 1). Leukotriene A ($LTA_4$) was identified as the unstable intermediary 5,6-oxido-7,9-trans; 11,14-cis-eicosatetraenoic acid in the conversion of 5-HPETE to $LTB_4$ (51).

One of the most exciting and rewarding moments in this field came when it was recognized that LTC and LTD had similar chemical and pharmacological characteristics when compared to a substance known as slow-reacting substance of anaphylaxis (SRS-A). This active principle was initially described by Feldberg and Kellaway (52) in 1938, when they detected the release of a material, different from histamine, in a guinea-pig perfused lung preparation. The substance was released by injection of cobra venom into the lung and caused a contraction of guinea-pig jejunum that was slow in onset and of long duration. The agent responsible was therefore referred to as "slow-reacting substance" or "SRS". Two years later the release of a similar SRS

was demonstrated during anaphylactic shock in guinea-pig perfused lungs by Kellaway and Trethewie (43). Brocklehurst in the 1950's while studying the material released during anaphylaxis introduced the term "slow reacting substance of anaphylaxis" or "SRS-A" (54). SRS-A was characterized by strong contraction of various gastro-intestinal smooth muscle preparations and smooth muscle tissue from guinea-pig trachea and human bronchus. The biological role of SRS-A and similar SRS's was studied in detail particularly by Austen, Brocklehurst, Chakravarty, Orange and Uvnas during the 1960's (55,56). It became apparent that SRS-A was immunologically released by lung and other tissue during antigen challenge and that it may be an important etiological agent in anaphylactic bronchospasm of human asthma (57). SRS-A was thought to be a pathophysiological product in clinical asthma and therefore appeared to be an ideal target for antagonistic drug treatment. Although a specific antagonist (FPL 55712) was developed (58) and allowed further assignment of biological activity to SRS-A, the attempts to determine its chemical structure were unsuccessful. The main obstacles in those early days hampering structural elucidation of SRS-A were the minute amounts of material available and the lack of recognizable chemical or physical chemical markers in the purification procedures.

Brocklehurst (55) had characterized SRS-A as a polar lipid. The presence of a sulfur atom (59) and the inactivation of SRS-A by arylsulfatase (60) was demonstrated in 1974. A milestone in the progress towards the ultimate structural elucidation was the

involvement of arachidonic acid in SRS-A biosynthesis. In 1973 Joyce Walker found that aspirin-like nonsteroidal anti-inflammatory drugs enhanced the production of SRS-A (61). She believed that arachidonic acid wasthe precursor of SRS-A, a proposition which was substantiated in 1977 by incorporation of radiolabelled arachidonate into SRS-A (62). The most dramatic point came when Morris et al (63) while purifying SRS-A by HPLC demonstrated that purified SRS-A possessed a characteristic UV spectrum typical of a conjugated triene. One year later when Borgeat and Samuelsson showed production of 5,12-DHETE ($LTB_4$) in rabbit PMNL's it was realized that 5,12-DHETE and SRS-A had similar UV spectra. A partial structure of SRS-A as a sulfur containing lipoxygenase product of arachidonic acid was proposed. A finding which supported this idea was that SRS-A was inactivated by soybean lipoxygenase (64). It was known that many thiols and particularly cysteine stimulated the formation of SRS-A (65). This observation suggested that SRS-A could be a sulfur containing compound like cysteine covalently linked with a fatty acid derivative. The incorporation of $S^{35}$ cysteine into SRS-A by mouse mastocytoma cells and the demonstration that the compound was a 5 HETE derivative carrying a substituent at C-6 via a thio ether link was reported by Murphy et al (66). The substituent at C-6 was later identified as glutathione (glutamyl-cysteinyl-glycine). The structure of mouse mastocytoma SRS was confirmed by chemical synthesis to be 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans,11,14-cis-eicosatetraenoic acid and is now designated leukotriene $C_4$ (LTC4) (67). More recently Morris et al (68) have described a

5-hydroxy-6-S-cysteinylglycinyl-7,9,11,14-eicosatetraenoic acid ($LTD_4$) as the SRS produced by rat basophil leukemia cells. The same investigators showed conversion of $LTC_4$ to $LTD_4$ by the action of γ-glutamyl transpeptidase and suggested that $LTA_4$ and $LTC_4$ were intermediates in the formation of $LTD_4$. Another addition to this family of leukotrienes has been $LTE_4$ which is the corresponding cysteinyl derivative produced by the action of a specific aminopeptidase on $LTD_4$ (69). Immunologically released SRS's including SRS-A are thus a mixture of $LTC_4$, $LTD_4$ and $LTE_4$ with $LTD_4$ having the most potent biological activity (70). The γ-glutamyl transpeptidase reaction is reversible and a γ-glutamyl residue can be added to $LTE_4$ to yield 5-hydroxy-6-S-γ-glutamyl-cysteine-eicosatetraenoic acid or γ-glutamyl-$E_4$ ($LTF_4$) (71).

The number of lipoxygenase products and leukotrienes is increasing exponentially. Leukotrienes derived from all cis 5,8,11-eicosatrienoic acid ($LT_3$'s), arachidonic acid (LT4's) and eicosapentaenoic acid ($LT_5$'s) have been identified (49). Undoubtedly new and related pathways of transformation will be discovered in the very near future.

## B. Metabolic Pathways of Eicosanoids.

### 1. General Occurence of Eicosanoids

Prostaglandins are described, with few exceptions, as being "ubiquitous among mammalian tissues" (5). Seminal plasma, from a variety of mammals including sheep, monkey and man, contains the highest concentration and number of primary prostaglandins. Prostaglandins can also be extracted from tissues of lower vertebrates (chicken, duck, frog) and some invertebrates (mollusc, arthropods, gorgonian coral) (72). Prostacyclin ($PGI_2$), thromboxane ($TXA_2$),

leukotrienes, and a number of lipoxygenase products are generated selectively by animal tissues (73-75).

The types and relative quantities of eicosanoids simultaneously produced may be cell-, tissue-, organ-and species-specific (5,72-75). For example thromboxane $A_2$ is the major product of arachidonic acid metabolism in platelets (19), while the major product in vascular tissue is prostacyclin (25). Leukotrienes and lipoxygenase derivatives are the predominant substances produced from arachidonic acid in whole lung, leukocytes and mast cells (75), and probably have a wider distribution in light of the many different sources of SRS's.

Similarily, the highly selective variations in cell and tissue responses to different eicosanoids may reflect the relative abundance of different specific receptors and the availability of appropriate autacoids in that tissue. At least five types of prostanoid receptors have been described (76). These receptors are characterized by their high affinity for specific prostanoids (ie. PGD,E,F, I) or thromboxane A (77).

Prostanoids are not accumulated or stored intracellularly (78) but are synthesized de novo in response to diverse stimuli such as hormones, neurotransmitters, peptides, trauma, ischemia and inflammation (79). Leukotriene production and release is brought about by immune and allergic reactions (75). Following stimulation, increased levels of eicosanoids can be detected in plasma, urine and other biological fluids (80).

Prostaglandins are derived from twenty carbon polyunsaturated fatty acids. Arachidonic acid (20:4) is the most abundant of these

acids and is the precursor of the predominant (dienoic) type of prostaglandins (5). Recent interest in the (trienoic) type of prostaglandins has been aroused by the finding that the substrate for prostaglandin biosynthesis in Greenland Eskimos was predominantly eicosapentaenoic acid (20:5) and that these people had ischemic heart disease only rarely (32).

In either case the precursor fatty acids are usually found in no more than trace amounts as free acids and the bulk of the material is esterified with other acids to cellular lipids (81). Arachidonic acid is most concentrated in phospholipids but may also be present as a triglyceride or cholesterol ester. Release of precursor fatty acid, upon appropriate stimulation, is carried out by one or several of a group of membrane bound enzymes generally referred to as acylhydrolases. Phospholipases, particularly pospholipase $A_2$, have been considered to play important roles in rate limiting the biosynthesis of prostaglandins because they regulate substrate availability (79). Two enzymes have been implicated in the release of arachidonic acid from the phosphatidylinositol of platelet membranes (82). The first activity is a phosphatidylinositol-specific phospholipase C which results in the arachidonic acid containing diglyceride. This substrate is now acted upon by a diglyceride lipase to yield free arachidonic acid and the corresponding monoglyceride. Mechanisms involving diglyceride lipase activity may be important in brain where the enzyme is particularly abundant (83). In the ovary, arachidonic acid can be derived from the large amounts of cholesterol arachidonate by the action of a cholesterol esterase after stimulation by

luteinizing Hormone (84). Hirata and Axelrod (85) have described a more complicated mechanism of arachidonic acid release from membrane phospholipids referred to as the "transmethylation pathway".

The liberation of arachidonic acid by phospholipase $A_2$ can be stimulated by various hormones and is $Ca^{++}$ dependent (86). The anti-inflammatory corticosteroids produce their effects by reducing the amount of arachidonic acid available for prostaglandin biosynthesis (87). It has been shown by Flower and Blackwell (88) that the mechanism responsible for this action consists of an induced biosynthesis of a phospholipase $A_2$ inhibitor.

Once released the substrate fatty acid, arachidonate, is rapidly metabolized to oxygenated unstable and then stable products by two distinct enzymatic pathways, the cyclo-oxygenase and the lipoxygenase pathways.

2. Cyclo-oxygenase Pathways.

The cyclo-oxygenase pathway is a series of sequential enzymatic transformations of arachidonic acid resulting in three classes of compounds; the primary prostaglandins ($PGE_2$, D2 and $F2\alpha$), thromboxane ($TXA_2$) and prostacyclin (Fig. 1).

This fatty acid cyclo-oxygenase (EC1.14.99.1) is also known as prostaglandin endoperoxide synthetase since its immediate reaction products are the unstable endoperoxides (14). Cyclo-oxygenase activity is found in the microsomal fraction of nearly all mammalian cells. The enzymes which convert these PG-endoperoxides into the

various primary prostaglandins, prostacyclin and thromboxane have individual names and are, in general, tissue specific. Thus, the distribution and quantity of individual products, in various cells and tissues, reflects the distribution of the different enzymes metabolizing the endoperoxides and is not due to the presence of different cyclo-oxygenases. One exception has been recently noted by Lysz and Needleman (90) who provided evidence that $PGE_2$ and $PGF_{2\alpha}$ biosynthesis in rabbit brain is accomplished via two distinct cyclo-oxygenases. They arrived at this conclusion after observing an abrupt halt in $PGE_2$ production while $PGF_{2\alpha}$ biosynthesis continued.

Cyclo-oxygenase activity has been purified to homogeneity from bovine (91) and sheep (82) vesicular glands. The catalytic activity of prostaglandin endoperoxide synthetase has been resolved into two reactions (Fig. 2). The first reaction is a lipoxygenase-like, bis-dioxygenation of the unsaturated fatty acid resulting in the formation of $PGG_2$. The second is an inherent peroxidase type of activity which converts the 15-hydroperoxy group of $PGG_2$ to a 15-hydroxy group in $PGH_2$ (89). Heme either as hematin or hemoprotein is required as cofactor for both the cyclo-oxygenase and hydroperoxidase reaction (93). Tryptophan or other aromatic compounds stimulate the conversion of $PGG_2$ to $PGH_2$. The peroxidase activity is believed to cause an irreversible inactivation of the cyclo-oxygenase through the generation of oxygen radicals during the reduction reaction (93). The more detailed biochemical and biophysical characteristics of this enzyme are reviewed by Samuelsson et al (89).

Reversible and irreversible inhibitors of cyclo-oxygenase have been discovered and evaluated (89, 90). Analogs of the natural fatty acid precursors are competitive inhibitors of the cyclo-oxygenase (94). Among them is the acetylenic analog of arachidonic acid, 5,8,11,14-eicosatetraynoic acid (93).

Many nonsteroidal anti-inflammatory agents (eg. aspirin, indomethacin etc.) inhibit the cyclo-oxygenase reaction (95). Aspirin (acetylsalicylic acid) irreversibly inactivates the cyclo-oxygenase enzyme by acetylation of its active site (96). Recovery from irreversible inactivation of cyclo-oxygenase requires biosynthesis of new enzyme. Nucleated cells such as endothelial cells, when compared to platelets, are thus more able to recover from the inhibitory effectsof aspirin (97). The prostaglandins are formed from $PGH_2$ by specific PG-endoperoxide isomerase and reductases (93). The conglomerated groups of enzymes producing primary prostaglandins are generically termed "prostaglandin synthetases".

Prostaglandin $D_2$ is formed from $PGH_2$, in the presence of glutathione, by a soluble $PGH_2$-11-keto-isomerase (Fig. 1, (3)). A microsomal $PGH_2$-9-keto-isomerase, also requiring glutathione, forms $PGE_2$. (Fig. 1, (4)), while a $PGH_2$ reductase yields $PGF_{2\alpha}$ (Fig. 1, (5)). $PGF_{2\alpha}$ and $PGE_2$ and their metabolites can be interconverted in many tissues by a Prostaglandin-9-Keto-reductase (98) and a 9-hydroxy-dehydrogenase (100).

Immediately after the prostaglandins have exerted their biological effects they are rapidly catabolized in tissues (primarily lung, liver and kidneys) to more polar, inactive metabolites which are then excreted (93,99).

Four main degradative biotransformations are well recognized: 1. $NAD^+$ or $NADP^+$ dependent oxidation of the 15-Hydroxy-group to the 15-keto-group catalyzed by 15-hydroxyprostaglandin dehydrogenases. 2. Reduction of the 13,14-trans-double bond to give the dihydro keto derivative. 3. Chain shortening, to form dinor or tetranor metabolites, is accomplished by $\beta$-oxidation, and 4. Omega ($\omega$) oxidation results in dicarboxylic acid derivatives of prostaglandins.

The initial degradative step has been most extensively studied and involves cytosolic 15-hydroxyprostaglandin-dehydrogenase (99). The enzyme is most concentrated in the lung, spleen and renal cortex and is very low in mammalian brain (79). Nonsteroidal anti-inflammatory drugs, loop diuretics and probenecid inhibit 15-hydroxy PG dehydrogenase (99).

The first two reactions in the degradation of prostaglandins occur very rapidly and produce 13,14-dihydro-15-keto-PG derivatives, major metabolites in the peripheral circulation, which then undergo $\beta$ and/or $\omega$ oxidation by hepatic microsomal enzymes to yield shorter mono- and dicarboxylic acids, the predominant metabolites observed in urine. Variable permutations of the four major transformations in different species and individuals results in a large number of urinary metabolites. In man for instance the major products from PGF's is 5$\alpha$, 7$\alpha$-dihydroxy-11-keto tetranor-prostane-1,16-dioic acid (Fig. 1, (8)), and from PGE's is 7$\alpha$-hydroxy-5,11-diketo-tetranor prostane-1,16-dioic acid (Fig. 1, (9)).

Sex differences, in amounts of prostaglandins excreted, also exist with the males excreting more per 24 hr. period than females (79).

Thromboxane synthetase has been demonstrated in many tissues (74,89) and has been purified from human (101) and bovine (102) platelet microsomes.

It has been resolved into two components, the prostaglandin endoperoxide synthetase and an enzyme producing $TXA_2$ from $PGH_2$. TX $B_2$ was originally discovered as a product of arachidonic acid metabolism in human platelets. This biologically inactive substance is the spontaneous hydrolytic reaction product of the bioactive $TXA_2$ (Fig. 1, (12,13)). Thromboxane synthetase also produces HHT through a dismutase type of reaction (93). Selective inhibitors of thromboxane synthetase have been actively sought due to thromboxane $A_2$'s platelet aggregatory and vasoconstrictor activity. To date imidazole, dipyridamole, azo- and epoxymethano-analogs of $PGH_2$ as well as a few other compounds have been identified as selective thromboxane synthetase inhibitors.

Prostacyclin synthetase is distributed throughout various tissues (89,103) and is particularly enriched in vascular structures such as heart, aorta, ductus arteriosus, and other adult and fetal arteries (104). It is also abundant in lung and rat stomach. As with the other enzymes involved in arachidonic acid metabolism there is species-, sex-and age-related variation in prostacyclin synthetase activity (105). Biosynthetic activity decreases with age (106) and is stimulated by estradiol (107) in the rat. Three possible mechanisms for the biosynthesis of $PGI_2$ from prostaglandin endoperoxides have been investigated (108,109). Peroxidase-dependent, oxygen radical mediated, inactivation of prostacyclin synthetase but not thromboxane synthetase has been reported (110). Prostacyclin is rapidly inactivated by

hydrolytic conversion to 6-keto-$PGF_{1\alpha}$ or other metabolic routes. Catabolism of $PGI_2$ is species-specific and also depends on which vascular bed is studied (105). In Rhesus Monkey prostacyclin is metabolized mainly to 6, 15-diketo-$PGF_{1\alpha}$ (111). Rabbit produces primarily 7,9-dihydroxy-4,13-diketo-dinor-prostanoic acid (112) while cat produces mainly 6,15,diketo-13,14-dihydro-$PGF_{1\alpha}$ (113). The major urinary metabolite in rat and man was dinor-6-keto-$PGF_{1\alpha}$ (114,115) indicating that the main pathways of metabolism of prostacyclin and 6-keto-$PGF_{1\alpha}$ is via $\beta$ and $\omega$- oxidation and not via the 15-hydroxy-prostaglandin dehydrogenase. This is compatible with the observation that prostacyclin is not metabolized to any extent in lung which has one of the highest concentrations of 15-hydroxy PG-dehydrogenase (93). Blood vessel microsomes can convert $PGG_2$ into 6,15-diketo $PGF_{1\alpha}$ (116) and cytoplasmic fractions of mesenteric (117) but not cerebral (117) vasculature can metabolize $PGI_2$ via the 15-hydroxy-PG dehydrogenase. More recently an active hepatic metabolite of prostacyclin indentified as 6-keto-$PGE_1$ has been reported (119). This could be generated via the 9-hydroxy prostaglandin dehydrogenase pathway (100). No such activity has been found in brain or its vasculature.

Prostacyclin synthetase can be selectively inhibited by lipid peroxides such as 15-hydroperoxy arachidonic acid and by the monoamine oxidase inhibitor tranylcypromine (120,121). In relation to prostacyclin's clinical pharmacology (122) there is presently an intense interest in developing more specific and powerful prostacyclin analogs, antagonists and synthetase inhibitors.

3. Lipoxygenase Pathways.

Nonenzymatic lipid peroxidation is the oxidative deterioration of polyunsaturated lipids. Lipid peroxidation in vivo is of basic importance in aging, atherosclerosis, some forms of hepatic drug injury and in oxygen toxicity (123). The occurence of lipoxygenases in plants and the mechanism of lipid peroxidation has long been known. Eicosanoic fatty acids are converted to their corresponding 15-hydroperoxy- acids by Soybean lipoxygenases (124).

Mammalian lipoxygenases that catalyse the oxidation of polyunsaturated fatty acids such as arachidonate have been detected only relatively recently (40,41). Lipoxygenase activity, in contrast to cyclo-oxygenase, has been so far only detected in some tissues. Platelet lipoxygenase, isolated from the soluble fraction of platelet homogenates, produces the 12-hydroperoxy- and 12-hydroxy-isomer of eicosatetraenoic acid. The platelet lipoxygenase can be inhibited by acetone phenylhydrazone and 5,8,11-eicosatriynoic acid (125). Neutrophil lipoxygenases can produce many other (eg. 5-,8-9,11-,15-) hydroperoxy- and hydroxy-isomers of eicosanoic acids (43).

In each case the "Hydroperoxide Synthetase" consists of a position specific lipoxygenase and a peroxidase (hydroperoxidase) analogous to the prostaglandin endoperoxide synthetase (126). Of these pathways the 5-,12-, and 15-lipoxygenase pathways are the most common (Fig. 1, (16,14,22)). Since both lipoxygenase and cyclo-oxygenase pathways utilize the same precursor, selective inhibition of either one of the pathways will result in greater availability of substrate for the uninhibited other route. Some hydroperoxy fatty acids produced by

neutrophils (eg 5-,15-HPETE) can form oxido-triene intermediates called leukotriene A's(LTA's) which are subsequently metabolised to sulfido-peptidyl eicosatetraenoic acids or enzymatically hydrolysed to dihydroxy compounds (Fig. 1, (18,19,20,24,24)). The fatty acid structural requirements for leukotriene synthesis is a $\Delta^{5,8,11}$ unsaturation in a polyenoic fatty acid (127). Information on the enzymology of leukotriene pathways is sparse but it is thought that the pathway is a combination of lipoxygenase metabolism of arachidonate together with the glutathione detoxification pathway (128).

Leukotriene $A_4$ is converted by glutathione-S-transferase to LTC4 (Fig. 1, (20)). The glutamyl-residue is then removed by the action of γ-glutamyl-transpeptidase (EC 2.3.2:2.), yielding the cysteinyl-glycine eicosanoid derivative ($LTD_4$). This enzyme is very highly localized in the CNS to cerebral microvessels (129,130) and is believed to function as a mediator in amino acid translocation across cellular membranes (131). Choroid plexus also contains large amounts of this enzymatic activity but the levels in CSF are extremely low (132).

Leukocytes exhibit extremely high levels of this enzyme compared to the other formed elements in blood such as erythrocytes and platelets (133). The biophysical parameters for γ-glutamyl transpeptidase isolated and purified from beef, hog and human kidney have been published (134). Lipoxygenase or γ-glutamyl transpeptidase inhibitors block the biosynthesis of leukotrienes C and D (135). $LTD_4$ is enzymatically converted to $LTE_4$ by the action of an aminopeptidase (69). This enzyme activity has not been well studied and is probably a nonspecific hydrolytic removal of the glycine residue from the cysteinyl-glycine

dipeptide. $LTF_4$ is formed by a reversal of the γ-glutamyl transpeptidase reaction in which the leukotriene now becomes the acceptor of the γ-glutamyl residue from glutathione to yield the C-6-γ-glutamyl cysteine derivative of $LTE_4$ (71).

Further transformations involving oxidative or hydrolytic degradation of leukotrienes will most likely be discovered when the enzymology of this pathway is better understood.

C. Prostaglandins and Thromboxanes in Brain.

1. Cerebral Parenchyma

Prostaglandins and thromboxanes in brain have been the subject of several reviews over the past few years (49,79,136-140).

Samuelsson (141) in 1964 demonstrated the occurence of $PGF_{2\alpha}$ in ox brain. Following this initial finding, Prostaglandins $E_2$, $F_{2\alpha}$, $D_2$, 6-keto-$F_{1\alpha}$ and thromboxane $B_2$ were identified in brain tissue of different species (142-146). $PGF_{2\alpha}$ had been considered the major prostaglandin in brain for many years but it is now apparent that $PGD_2$ is the most prominent PG in some species (147), i.e. rat and mouse. The major prostaglandin in rabbit and guinea pig is $PGF_{2\alpha}$ while in cat $PGE_2$ is formed in large amounts (146). Prostaglandin 6-keto-$PGF_{1\alpha}$ could be identified in brain homogenates of mouse, rat and rabbit but was a very minor component when compared with the other PG's (145). Thromboxane $B_2$ was formed in excess, of the prostaglandins $F_{2\alpha}$ and $E_2$, only in guinea pig cerebral cortex slices and homogenates (144).

Prostaglandins are widely distributed throughout brain and differences inthe pattern of formation by various brain regions have

been observed (148). Total amounts of PG's varied in the different regions studied with the limbic system and cerebral cortex having quantitatively the greatest while the cerebellum and medulla had the least (146). Of twenty-four regions studied in rat brain the highest concentration of PGE and PGF, based on protein content, was the median eminence (148).

Grey matter appears to have the highest concentration of PG's (142) which suggests their localization to neurons. Subcellular fractionation of rat cerebral cortex homogenate shows the concentration of prostaglandins in cholinergic and noncholinergic nerve endings (149). Only brain microsomal fractions exhibited significant ability to synthesize prostaglandins. These two observations led the authors to conclude that the nerve endings were a storage site for PG's whereas the microsomes were the site of synthesis, observations which have yet to be confirmed.

Prostaglandins can be measured from intact cerebral cortex slices, brain homogenates, and superfusates of cerebral cortex, cerebellum and spinal cord preparations (136). The levels can be augmented by increased neuronal activity and electrical stimulation of various brain structures, application of neurotransmitters, including 5-Hydroxytryptamine, Dopamine and Noradrenaline, and exposure to analeptic drugs (79). Barbiturates and chlorpromazine diminish the amount of prostaglandins released (136). Free arachidonic acid supplied from exogenous sources to brain tissue in vitro does not significantly stimulate prostaglandin or thromboxane synthesis (150). The origins of the arachidonate used to synthesize PG's are membrane phospholipids especially phosphatidylinositol and phosphatidylcholine (151) and as in all other tissues, prostaglandins

are not stored but synthesized de novo upon exposure to an appropriate stimulus.

The catabolism of prostaglandins, including prostacyclin and 6-keto-$PGF_{1\alpha}$, by brain tissue is negligible (152,153). Brain levels of 15-hydroxy-PG dehydrogenase, the limiting enzyme in the catabolic sequence, are exceedingly low or not detectable in rat brain homogenates and blood vessels (145). Very low levels of $\Delta^{13}$-PG-reductase activity have been detected in monkey and swine, but not rat brain (154,155). A 9-keto-reductase activity has been reported in monkey, pigeon and rat brain (156) but may be the resultant action of non-specific reductases uncovered when high concentrations of PGE are used (157). Prostaglandins produced by brain parenchyma are mostly released into the extracellular and cerebrospinal fluids to be removed into the circulation and metabolized by extraneuronal tissues (158,159).

The possible physiological functions of prostaglandins in the brain have been recently discussed (140). They may play important roles in temperature regulation, body water balance, regulation of food intake, cerebrovascular homeostasis and maintenance of cerebral perfusion (49, 79,136). One of the most important physiological properties of prostaglandins is their ability to modulate cyclic nucleotide levels and affect the mobilization of calcium in many cell types (79,136). Influx of calcium into nerve terminals and receptor-adenylate cyclase interaction is an absolute requirement in synaptic transmission. Prostaglandins of the E series inhibit the effector responses elicited by sympathetic nerve stimulation (160). Hedqvist proposed a negative feedback-control theory in which endogenous $PGE_2$ formed by the postsynaptic effector membrane

inhibits norepinephrine release from presynaptic sympathetic nerve terminals (136,160). Thus PGE's may play an important role in regulation of autonomic neurotransmission. Prostaglandins have not been implicated in cholinergic transmission either at sympathetic ganglia or neuromuscular junctions (79).

Prostaglandins of the E and D types increase intracellular cyclic adenosine monophosphate (cAMP) in many tissues including platelets (161) and neural tissue (162). Although $PGI_2$ is the most potent stimulator of adenylate cyclase in platelets (163) it has no effect on adenylate cyclase in neurons (79).

$PGF_{2\alpha}$ increases cellular cyclic guanosine monophosphate (cGMP) and may facilitate excitatory cholinergic and serotoninergic pathways in brain (136). In most cellular systems cAMP and cGMP have antagonistic inverse effects on intracellular events. Since the two major PG's in brain, $PGD_2$ and $PGF_{2\alpha}$, stimulate adenylate and guanylate cyclase respectively, a "dualistic" control theory known as the "Yin-Yang" Hypothesis has been formulated (89,136).

Prostaglandins have many other effects on CNS function such as modulation of cardioregulatory and respiratory reflexes, spinal cord reflexes and behavioral responses (136,164). These effects are very variable and complex, sometimes even contradictory, and many more experimental data must be accumulated in order to clarify which prostaglandins have a real physiological significance in CNS function.

2. Cerebral Blood Vessels

Soon after prostaglandin synthesis was demonstrated by peripheral vascular tissue (165), Pickard and collaborators (166) reported the

first indications that cerebral arteries may also synthesize these compounds. They observed the release of a substance from bovine cerebral arteries which had actions similar to prostaglandins in a rat stomach bioassay. Although not chemically characterized, the release of this substance was abolished by indomethacin treatment, and it was thus suggested that the material was most likely a prostaglandin (166). Since then biosynthesis of prostaglandins by cerebral blood vessels has been unequivocally demonstrated in a number of different species, including rat (145), pig (167), ox (168,169) and man (170). The principal prostaglandin formed in the cerebral blood vessels of all these species was $PGI_2$. In bovine cerebral arteries (163) $PGE_2$ was the second most prominent prostaglandin formed followed by $PGF_{2\alpha}$. These investigators also reported minor synthesis of $PGD_2$ and $TXB_2$ which probably derived from residual intraluminal platelets. In rat (145) and human (170) cerebral arteries no $PGD_2$ or $TXB_2$ was detectable and $PGF_{2\alpha}$ was the second major prostaglandin formed after $PGI_2$.

The effects of prostaglandins on cerebrovascular reactivity are very difficult to interpret. Comparison of experimental results must be done with extreme caution since intracranial and extracranial blood vessels may have different responses to the same prostaglandin. Species differences, variations in blood vessel size and experimental methods (eg. *in vivo* or *in vitro* preparations) all contribute to the great variability of effects observed when prostaglandins are applied to cerebral blood vessels (136).

$PGF_{2\alpha}$ causes consistent vasoconstriction in all species studied (171,172,181). The effects of $PGE_1$ and $PGE_2$ are variable and depend on the cranial blood vessels studied and which species is employed. In dog epicerebral arteries of less than 700 microns $PGE_1$ causes powerful contraction (172,173). $PGE_2$ causes dilation of cat cerebral arterioles (174) but induces spasm of the basilar artery in the dog (175). $PGD_2$ and $PGG_2$ also produce dilatation in cat (174). $PGI_2$ is the most potent vasodilator of cerebral blood vessels in all species examined (140,174,177,178,179).

The prostacyclin synthetase inhibitor, 15-hydroperoxy arachidonic acid, causes vasoconstriction of dog basilar artery (49,180). The most potent cerebral vasoconstrictor is $TXA_2$ which caused contraction of bovine middle cerebral artery to 150% of the reference response (176). The synthesis and vasoactivity of prostaglandins and prostacyclin in cerebral arteries has implicated them in normal and pathologic responses of the cerebral circulation (49,79,136,140).

Prostaglandins, especially $PGF_{2\alpha}$, and $TXA_2$ are thought to be involved in the complex pathophysiology of cerebral ischemia, vasospasm and stroke. $PGE_2$ has also been implicated in the pathogenesis of migraine headaches (182,183). Prostacyclin formed in cerebral blood vessels may play an important role in cerebral blood flow regulation (184). Regulation of the cerebral circulation is a complex interaction of neurogenic, metabolic and chemical factors (185-189), and it is likely that prostacyclin interacts with one or more of these in the normal control of cerebral blood flow. For example, it has been demonstrated that a prostaglandin, most probably prostacyclin, is

required for hypercapnia to produce full cerebral vasodilation (190), and that indomethacin reduces cerebral blood flow in the normoxic but not the hypoxic state (191).

Cerebral ischemia following head trauma may also involve arachidonic acid metabolites, since direct application of $PGG_2$ and hydroperoxy arachidonate cause vascular damage through the generation of oxygen radicals during their metabolism (180,192). Vascular intimal damage by products of the cyclo-oxygenase or lipoxygenase pathways ultimately leads to reduced prostacyclin production which predisposes the cerebral vasculature to ischemia, vasospasm and intravascular thrombosis.

3. Cerebro-Spinal Fluid

The choroid plexus is a prominent structure in the ventricles of the developing brain (193). It is a highly convoluted richly vascularized tissue in which fine capillaries are encased in connective tissue elements. These microvessels are surrounded by a monocellular layer of specialized epithelial cells derived from the ependyma of the ventricles. The choroid plexus forms about 65% of the total CSF and is responsible for the clearance of solutes and weak organic acids (eg. prostaglandins) from the brain CSF back into cerebral blood across the "Blood-CSF Barrier" (159). The physiology and mechanism of CSF secretion have been excellently reviewed in Davson's classic monograph (194) and more recently by Wright (195).

In the early 1960's Ramwell (196) analysed CSF for new pharmacologically active substances. He detected a substance, which

was not a polypeptide or biogenic amine, that contracted rat uterus smooth muscle and suggested that it was a prostaglandin. The occurence of prostaglandins $F_{2\alpha}$, $E_2$, $D_2$, 6-keto $F_{1\alpha}$ and thromboxane $B_2$ in human CSF has been reported (197-201). Prostaglandin 6-keto $F_{1\alpha}$ was the major prostaglandin followed by $PGF_{2\alpha}$, and $PGE_2$ was detected in lower amounts while $PGD_2$ was below the limit of detection (200).

Prostaglandin levels in CSF increased considerably as a result of neurological disease. $PGF_{2\alpha}$ concentrations in CSF of patients suffering from epileptic seizures, meningitis or cerebrovascular trauma are markedly elevated (197,199). $PGE_2$ in CSF is higher than normal in febrile conditions brought about by a variety of pyrogens (136,202). $PGE_2$ may also be involved in the pathogenesis or symptomatology of schizophrenia (201) but this association may be onlycursory.

The changes in CSF 6-keto-$PGF_{1\alpha}$ levels of patients with neurovascular diseases have not been extensively studied. The only two clinical conditions in which 6-keto $PGF_{1\alpha}$ in CSF is above normal control values are intracranial hypertention and acquired hydrocephalus (200). This interesting finding is consistent with the fact that prostacyclin, a potent hypotensive agent, is produced in greater quantities by spontaneously hypertensive rats (206,207). Furthermore, this increased capacity of prostacyclin synthesis by stroke-prone and stroke-resistant spontaneously hypertensive rats is drastically reduced following the development of stroke (208). The increased production of CSF by choroid plexus, observed in

hydrocephalus, may somehow also be responsible for its increased biosynthesis of prostacyclin.

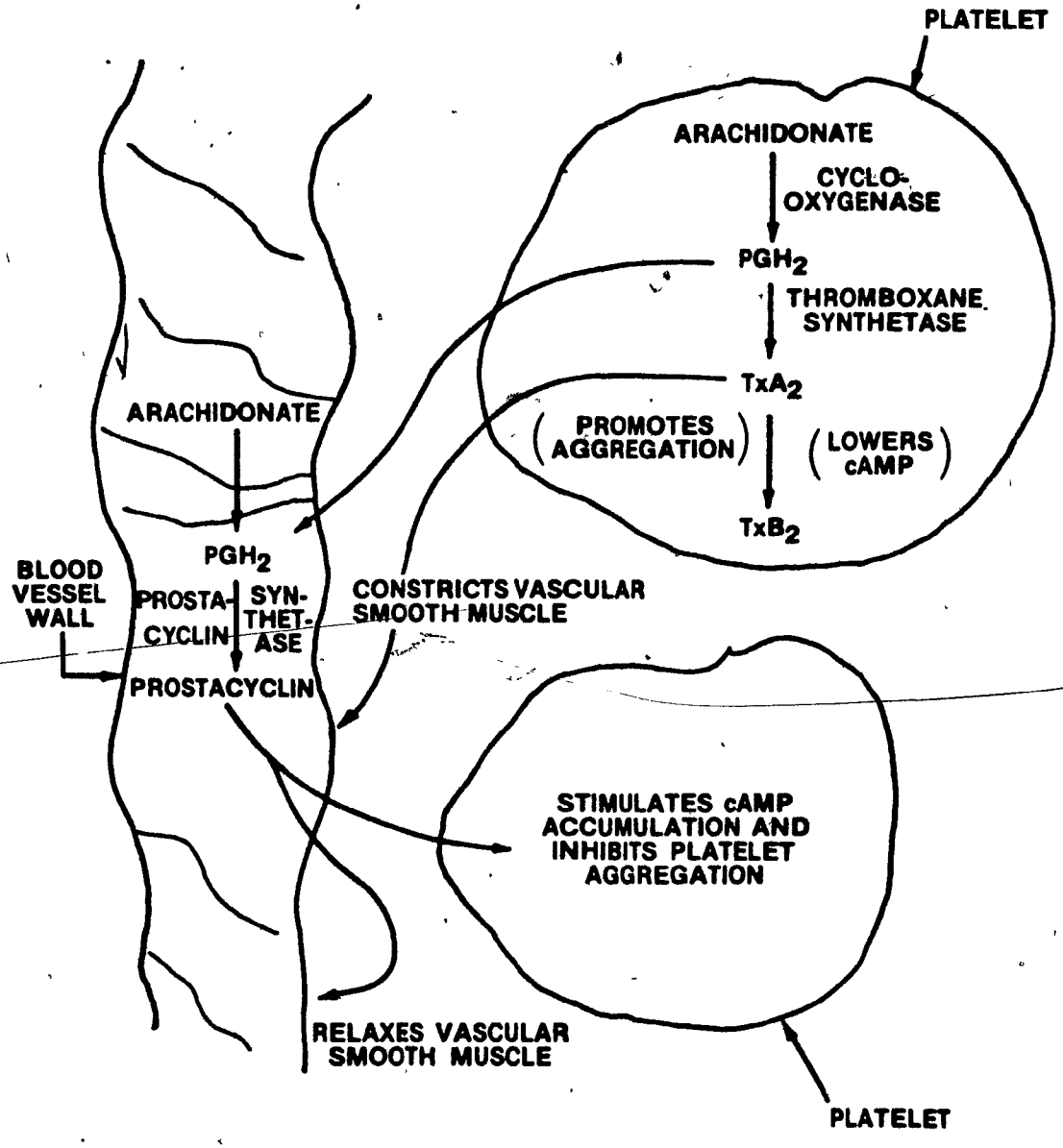
Prostaglandin $F_{2\alpha}$ levels measured in patients during the course of their illness have been shown to return to basal levels with the resolution of the neurological pathology (204).

D. Prostacyclin and Vascular Endothelium.

1. Prostacyclin, Physiology and Pharmacology

The physiology and pharmacology of prostacyclin in the various organ systems (cardiovascular, respiratory, renal, gastrointestinal and endocrine-reproductive) have been selectively and adequately reviewed in the recent past (80,105,122,209,210). The most important physiological role of prostacyclin, however, is in the hematologic system. Prostacyclin and thromboxane $A_2$ are thought to play primary roles in modulation of platelet function (211,212). Interactions between platelets and blood vessel walls in conditions of hemostasis and thrombosis are mainly the result of the antagonistic properties of prostacyclin derived from blood vessel endothelium and $TXA_2$ released from aggregating platelets (213). Moncada and Vane (214) proposed that prostacyclin synthesized by the intimal endothelium was the physiological mechanism that protects the vessel wall from deposition of platelet aggregates which were the result of increasing platelet $TXA_2$ levels. They envisaged a reciprocal regulation (Fig. 4) of platelet aggregability and vascular tone by the balance between prostacyclin and thromboxane $A_2$. Under normal healthy conditions platelets in the blood stream coming into contact with the vessel wall would be prevented from adhering to the luminal surface and

Figure 4. Reciprocal Regulation of Platelet Aggregation and Vascular Tone by Prostacyclin and Thromboxane $A_2$. Note Prostacyclin Synthetase can use both endothelial and platelet derived endoperoxide. (Taken from Ref.214)


PLATELET
ARACHIDONATE
CYCLO-OXYGENASE
$PGH_2$
THROMBOXANE SYNTHETASE
$TxA_2$
PROMOTES AGGREGATION
LOWERS cAMP
$TxB_2$
ARACHIDONATE
$PGH_2$
BLOOD VESSEL WALL
PROSTA-CYCLIN
SYN-THET-ASE
PROSTACYCLIN
CONSTRICTS VASCULAR SMOOTH MUSCLE
STIMULATES cAMP ACCUMULATION AND INHIBITS PLATELET AGGREGATION
RELAXES VASCULAR SMOOTH MUSCLE
PLATELET

aggregating with each other by the cantinuous release of prostacyclin from the endothelium. In the event of intravascular damage (eg. atherosclerosis, vascular deformations and trauma, aneurysms etc.) there would be endothelial destruction with concomitant reduced prostacyclin synthesis leading to the predisposition of thrombus formation. Depending on the extent of the vascular lesion there may only be platelet adhesion without thrombus formation since prostacyclin prevents aggregation at a much lower concentration than is required to prevent adhesion (213). This property is beneficial because the adhering platelets, in close proximity to the endothelial surface, may donate their endoperoxide to enhance vessel prostacyclin synthesis thereby preventing further platelet aggregation and thrombus formation.

If the lesion is overwhelmingly large the subendothelial collagen causes the formation of a large thrombus which may embolize producing vascular occlusion. Prostacyclin can disperse platelet aggregates once they are formed and the high production of prostacyclin in lung vasculature and lack of its metabolism may be a defensive mechanism in the prevention of pulmonary thromboembolic events (213, 214).

The vasodilator property of prostacyclin is responsible for its hypotensive actions in vivo (105,122), and the increased synthesis in hypertensive states may be an adaptive measure in response to these clinical states (200,207).

The therapeutic implications of stable, orally active, prostacyclin analogs and potent selective thromboxane inhibitors in

vascular homeostasis have been the subject of intense research over the past few years and clinical trials of prostacyclin used as an antithrombotic agent during extracorporeal circulation have proved very promising (122).

2. Cultured Endothelium

The morphology, embryology and metabolism of vascular endothelium in culture and in vivo have been explored during the past 25 years by many investigators (215). Much has been learned about the fine structure of the microvasculature and the function of endothelial cells under normal physiological and pathological conditions. The study of prostaglandin metabolism by cultured endothelium is relatively new and was initiated by Gimbrone in 1975 (216). He demonstrated the release of immunoreactive material resembling PGE from human umbilical vein endothelial cells which was inhibited by indomethacin and stimulated by angiotensin II.

The first demonstration that endothelial cells produced a substance which inhibits platelet aggregation was made two years earlier by Saba et al (223). These investigators noted that after incubation of endothelial cells with platelets, subsequent platelet aggregation induced by several stimuli (eg. ADP, epinephrine, collagen) was inhibited. It was not until recently that the importance of this finding was appreciated. Prostacyclin is the major prostaglandin produced by cultured endothelium derived from many species including pig (218), ox (217) and man (219-222). Prostacyclin is the major product from arachidonic acid and PG endoperoxide with the latter precursor being converted more efficiently (219). Several

investigators have studied the influences of chemical agents on the synthesis and release of $PGI_2$ in cell cultures. Stimulation of prostacyclin production was observed after incubation of endothelial cells with the calcium ionophore A23187 (5 $\mu$M), but not by preincubation with ADP (5 $\mu$M), epinephrine (0.9 $\mu$M), bradykinin (100 ng/ml) and angiotensin II (10 ng/ml) (218,220). Thrombin and trypsin also stimulate prostacyclin production in some incubation systems. The cyclo-oxygenase inhibitors aspirin and indomethacin inhibit prostacyclin synthesis by cultured endothelial cells (218,219,221). Specific inhibitors of prostacyclin synthetase eg. tranylcypromine and 15HPAA completely abolished endothelial prostacyclin production (218,217). The regulation of prostacyclin production in endothelial cells is still unclear but with more specific inhibitors and increasing experimental data using more biological test substances (dibutyryl cAMP, adenosine etc.) it will eventually be possible to understand and manipulate prostacyclin production in endothelial cells.

3. Isolated Endothelium (Microvessels)

Advances and improvements in biochemical fractionation techniques have made it possible to isolate pure metabolically active capillary fragments from the cerebral cortex of a number of different species (227,229-236). Eisenberg and Suddith (225) have edited a monograph exclusively dedicated to studies involving isolated cerebral microvessels. Many enzymes have been assayed in isolated microvessels, including those involved in glycolysis, glycogen metabolism, pentose phosphate pathway, amino acid and

biogenic amine metabolism (228,236,247,249). Carrier mediated glucose transport (243), amino acid transport (237), sodium and potassium transport (244,245) and uptake of adenosine (241) and $S^{35}$ cystine (242) by isolated brain capillaries has been documented. The enzymes metabolizing histamine (228) and acetylcholine (225) have also been studied in microvessel preparations. Renin-like activity (246) and the properties of Angiotensin-Converting Enzyme (240) were studied in rabbit isolated microvessels. The phospholipid composition from rat (234), bovine and human (229) isolated microvessels have been determined. Diet has been shown to alter the fatty acyl composition of phopholipids in isolated rat cerebral capillaries (234,226), with the greatest change occurring in the plasmalogen ethanolamine fraction.

The study of prostaglandin metabolism by these microvessel preparations is novel with the first reports appearing in 1980. To date prostaglandin biosynthesis has been studied only in rat (251,254) cat (250) and bovine (252,253) isolated microvessels. Microvessels produce $PGE_2$, $F_{2\alpha}$ and 6 keto $F_{1\alpha}$ but not thromboxane $A_2$. Prostacyclin is apparently the major prostaglandin produced in all three species but conflicting results have been reported by Gerritsen et al (251,252) in both rat and ox microvessels. In one report using rat microvessels (251) this group failed to detect significant amounts of $PGI_2$ formation when the microvessels were incubated with $PGH_2$ and showed instead that $PGD_2$ was the major prostaglandin formed. This observation is in contradiction with other reports in which no $PGD_2$ was detectable after incubation of cerebral microvessels with and without arachidonic acid (249, 250,253,254). In the study by Gerritsen et al (252) using bovine

isolated cerebral microvessels it was concluded that $PGE_2$ was the major metabolite of PG endoperoxide and that prostacyclin formation, measured as 6-keto $PGF_{1\alpha}$, only became significant at higher microvessel protein concentrations. Maurer et al (253), however, showed conclusively that prostacyclin was the major prostaglandin produced by isolated bovine microvessels. Further studies on arachidonic acid metabolism by isolated microvessels from many species will hopefully clarify these discrepancies and provide information on the metabolic regulation of cyclo-oxygenase and lipoxygenase pathways in health and disease.

E. Aims and Scope of Present Work

As a result of the impact on cardiovascular research and the central role of prostacyclin in preventing thrombogensis and regulating vascular tone it has become of importance to determine if cerebral blood vessels, both large and small, as well as structures producing cerebrospinal fluid can synthesize this compound.

Although much is known about metabolism and effects of prostacyclin, as well as other prostanoids and thromboxanes in the peripheral circulation (165) their formation and effects in the cerebral microcirculation have not been extensively studied (250-254 ).

With recently developed methods for the isolation of metabolically competent cerebral microvessels, predominantly capillaries, small precapillary arterioles, and postcapillary venules, it is now feasible to study previously well known phenomena such as blood brain barrier function and factors affecting cerebral blood flow regulation in vitro.

In this work the endogenous synthesis of prostacyclin, and prostaglandins $E_2$ and $F_{2\alpha}$, by cerebral microvessels and choroid plexus isolated from rat brain was investigated. The ability to utilize exogenously added prostaglandin endoperoxides as substrate for the biogenesis of prostacyclin was also studied in these preparations.

Data from experiments performed using surface cerebral blood vessels and those obtained from studies of prostacyclin synthesis

in a peripheral large artery and intimal endothelial cells in culture were collated. Considering all of the known important functions of the cerebral microvasculature and choroid plexus, and possibly those not yet appreciated, the definitive demonstration that these structures can synthesize prostacyclin, and that its synthesis can be altered by vasoactive hormones and drugs, would strongly suggest that this biogenic lipid may have a prominent function in blood brain barrier mechanisms and in cerebrovascular homeostasis, particularly antithrombogenesis and blood flow regulation.

# Chapter II

## MATERIALS AND METHODS

### A. Isolation of Microvessels, Choroid Plexus and Surface Vessels.

Rat brain cerebral microvessels were prepared essentially by the method of Goldstein et al (232) with a number of modifications. Typically, six or eight male Wistar rats weighing 200-300 gms (Canadian Breeding Laboratories, Québec) were decapitated and the brains rinsed with ice-cold oxygenated 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (Hepes) buffer (15 mM, pH 7.4) containing NaCl (147 mM), KCl (4.0mM), $CaCl_2$ (3.0 mM), $MgCl_2$ (1.2 mM), L-glucose (5.5 mM) and 1% (W/v) Cohn fraction V bovine serum albumin. All isolation procedures following decapitation were performed in the cold (0-4°C). Cerebral hemispheres were separated from cerebellum and brain stem and the choroid plexus and surface pial vessels were isolated under a Zeiss dissecting microscope and kept on ice until ready for use. The cleaned hemispheres were finely chopped on a glass plate with a razor blade and then homogenized (3 brains/20 ml buffer) in a Potter-Elvejhem homogenizer with a motor driven teflon pestle for ten strokes at 300 rpm. The homogenate was then passed through a series of nitex nylon screens (Kressilk, Thompson Ltd., Montreal, Quebec) supported in microsyringe filter holders (32 mm diameter, Millipore Corp., Mass.) starting with 2x670 µm, 670/335 µm, and finally 335/118 µm size mesh. The collected filtrate was centrifuged at 900 g for 10 min in a Sorvall RC-3 refrigerated centrifuge using a SS 34 rotor, the supernatant discarded and the upper loose portion of the pellet was removed and retained, while the lower compacted mass was rehomogenized (6 strokes, 150 rpm) and again

centrifuged as above. This final pellet was resuspended with the first loose upper layer and centrifuged once more at 1500 g for 10 min. The supernatant was discarded and the pellet dispersed in 10 ml of the above buffer of identical composition except that now it was 25% (w/v) Cohn fraction V bovine serum albumin (pH 7.5). The resulting suspension was centrifuged at 3000 g for 15 min following which a floating cap of myelin and neuronal elements appeared. The cap and infranatant were collected, redispersed using a vortex mixer and centrifuged again at 3000 g for 15 min. The small red pellets from both centrifugations containing microvessels and blood components were carefully cleaned with wet cotton tipped swabs and resuspended in a small volume of buffer without Cohn Fraction V bovine serum albumin. The material was applied to a pre-cooled glass bead column (Glasperlen, 0.25-0.30 mm diameter, Braun Melsurgen AG, West Germany), occupying a volume of 1.0 to 1.5 ml in a 5 ml Plastipak syringe. The glass bead column with adhering microvessels was washed with 50 ml of freshly oxygenated buffer without Cohn fraction V to remove excess bovine serum albumin (Cohn V), erythrocytes, neuronal and glial contaminants. The glass beads were then extruded with the same buffer into a small beaker and the microvessels, predominantly capillaries, were recovered by agitation of the beads, the beads allowed to settle and the supernatant centrifuged at 3000 g for 10 min to give a final microvessel preparation.

B. Preparation for Light Microscopy.

Dried smears of microvessels or pieces of choroid plexus were fixed in 10% neutral formalin and stained for 5 min with Harris' hematoxylin. The tissue was washed by quick immersion three times

in distilled water, once in tap water, once in (0.3%) ammonia water and again once in distilled water. The tissue was then stained for 3 min with eosin solution* and dehydrated by submersion in 95% ethanol/water (2 times) followed by 100% ethanol (2 times). The tissues were cleared with xylene and mounted with Permount. Photomicroscopy was performed using a Zeiss Standard RA photomicroscope with an automatic 35 mm camera attachment.

C. Preparation for Electron Microscopy.

Microvessels freshly isolated were immersed in a fixative containing 2% freshly prepared paraformaldehyde and 2.5% purified glutaraldehyde in 0.1 N sodium cacodylate buffer (pH 7.4) with sucrose (final osmolality 900 mOSmoles) overnight at 4°C. The tissue was washed in 0.1 N sodium cacodylate buffer (pH 7.4) containing 11.25% sucrose (final osmolality 380 mOSmoles); this was followed by fixation for 90 min at 4°C with 1% $OSO_4$ in Palade buffer (pH 7.4) containing 4.9% sucrose (final osmolality ~430 mOSmoles).

The microvessels were then treated for two hours at 4°C with 2% uranyl acetate in sodium hydrogen maleate-NaOH buffer, (0.05 M, pH 6.0), followed by a short washing in maleate buffer (pH 5.2); the tissue was then dehydrated in graded ethanols and embedded in Epon 812. Silver to gray sections were cut with a diamond knife on an LKB III microtome and examined following lead citrate staining with a Philips EM-300 microscope.

D. Enzyme Assays.

Alkaline phosphatase (EC 3.1.3.1) activity in microvessels and initial cerebral cortex homogenates was determined by adding 1 to 4 mg

*Eosin solution; 50 ml of 2% aqueous eosin Y plus 30 ml potassium dichromate (0.5 g) is added to 10 ml saturated picric acid and 10 ml absolute ethanol.

protein to a reaction mixture of final volume 1 ml containing p-nitrophenyl-phosphate (7 mM), $MgCl_2$ (0.5 mM), and glycine buffer (50 mM, pH 10.5) (255). After incubation for 30 min at 37°C, the reaction was terminated by the additionof 10 ml 0.02 N NaOH and mixing. The absorbance of the mixture was read at 410 nm using a Zeiss PMQ II spectrophotometer. Absorbance remaining after addition of 0.1 ml concentrated HCl to each sample was subtracted from the first reading and the activities calculated from a calibration curve using p-nitrophenol. γ-Glutamyl transpeptidase (EC 2.3.2.2) was assayed in a similar manner using L-γ-glutamyl-p-nitroanilide as the substrate (256). The reaction mixture contained 0.1 to 4 mg protein, γ-glutamyl-p-nitroanilide (4.6 mM), glycyl-glycine (0.1 M) and tris-HCl buffer (0.1 M, pH 9.0) in a final volume of 0.55 ml. After incubation for 20 min at 37°C the reaction was stopped by additionof 2 ml acetic acid (1.7 N) and the liberated p-nitro-aniline was diazotized with 1 ml of aqueous sodium nitrite solution (0.1%). Exactly three minutes following diazotization, 1 ml of ammonium sulfamate solution (1% w/v) was added, followed 3 min later by 1 ml of N-(1-Naphthyl)-ethylenediamine (3 mM). The overall reaction sequence of this assay is based on the initial transfer of the glutamyl residue from L-glutamyl-p-nitroanilide to glyclyglycine catalysed by γ-glutamyl transpeptidase:

1. L-Glutamyl-p-nitroanilide + Glycylglycine $\xrightarrow{GGTP}$ p-Nitroaniline + Glutamyl-gly-glycine
2. p-Nitroaniline + $NaNO_2$ $\xrightarrow{Acid}$ Diazo Compound
3. Diazo Compound + N-(1-Naphthyl)-ethylenediamine → Pink Azo-Dye.

The absorbance of the final mixture was read at 540 nm. Enzyme activities were calculated from a calibration curve in which the chromophore was prepared from standard known amount of p-nitroaniline.

Protein was determined by the method of Lowry et al (257) using whole bovine serum albumin as standard.

E. Preparation of Prostaglandin Endoperoxides

Prostaglandin endoperoxides ($PGG_2$ and $PGH_2$) were biosynthesized and isolated essentially according to the established protocol of Hamberg et al. ( 12) and Nugteren and Hazelhof ( 13) with modifications as previously described (258).

Frozen ram seminal vesicles (20 g) were partially thawed, denuded of fat and connective tissue, then diced and homogenized (1/5, w/v) in ice-cold potassium phosphate buffer ( 0.1M, pH 7.4) using a Brinkman Polytron with a PT 10 ST generator at a pulse frequency of 7300 cps for 30 sec. The homogenate was centrifuged at 8000xg for 15 min (Beckman J-21, 0-4°C) to remove nuclei, mitochondria and cell debris, following which the resultant supernatant was decanted and centrifuged at 100,000xg for 60 min to obtain the microsomal enriched pellet. This microsomal fraction was then used as the source of the prostaglandin endoperoxide synthetase.

The microsomal pellet was resuspended by homogenization in 20 ml of potassium phosphate buffer containing 1 mM p-chloromercuribenzoate (Sigma Chemical Co.). The suspension was equilibrated at 37°C for two minutes prior to its addition to warm (5,6,8,9,11,12,14,15-$H^3$) arachidonic acid 150 µCi (Amersham,

Oakville, Ont.) diluted with 500 μg of unlabelled material (Nucheck Inc., Elysian, MN), dissolved in 50 μl of ethanol (final Sp. Activity 91.5 μCi/μmole).

Following 90 seconds of incubation (37°C, 100% $O_2$), the aqueous medium was rapidly extracted with ice-cold diethyl ether (50 ml) plus 1.0 M citrate (2 ml). The ether extract was then backwashed and neutralized with ice-cold water (5 ml) and the organic phase separated from the aeqeous phase by centrifugation and then dried over anhydrous magnesium sulfate.

The ethereal extract after evaporation of the solvent under vacuum (-20°C) was dissolved in a small amount of cold (-20°C) petroleum ether-diethyl ether (8:2 v/v) for silicic acid column chromatography.

Silicic acid (2 g, Bio-Sil HA-minus 325 mesh, Bio-Rad Laboratories, Mississauga, Ont.) was introduced as a slurry in petroleum ether into a glass column (15 cm, 1.4 cm. O.D.) to a height of 10 cm. The column was washed with 50 ml of petroleum ether at room temperature and subsequently placed in the cold room (0-4°C) overnight. The sample was fractionated at -20°C on this column with petroleum ether:diethyl ether mixtures (8:2, 6:4, 4:6 by vol) and methanol in that order. The products in the initial ethereal extract from the incubation and the purity of the fractions from silicic acid chromatography were assessed by thin layer chromatography (Brinkmann, precoated 5 cm x 20 cm silica gel G plates) performed at -20°C for 90 min using dry diethyl ether as developing solvent. Prostaglandin standards were visualized

with 10% phosphomolybdate in ethanol and radioactivity monitored using a Packard Model 7220/21 Radiochromatogram Scanner.

The petroleum ether-diethyl ether eluates 6:4 and 4:6 which contained respectively $PGG_2$ and $PGH_2$ were evaporated to dryness and the residues then dissolved in dry diethyl ether (5 ml) for storage. At -80°C the endoperoxides proved to be stable for several months. The identity of the endoperoxides was confirmed by their chemical conversion to $PGF_{2\alpha}$ after mild reduction with stannous chloride in ethanol (12.5 mg/5 ml).

F. Incubation Conditions

1. Incubation using endogenous precursors.

Freshly isolated cerebral microvessels (3-6 mg protein) or surface vessel segments (35-45 mg wet weight) or isolated choroid plexes (12-15 mg wet weight) were incubated in 1 to 2 ml Krebs-Henseleit bicarbonate buffer pH 7.4 at 37°C for 10 min in an atmosphere of 95% $O_2$/5% $CO_2$.

2. Incubations using exogenous precursors.

Initial studies were performed using (1-$C^{14}$) arachidonic acid (New England Nuclear, Boston, Mass., 2.6 µg, 0.5 µCi) and the conditions described above. Subsequently, incubations with synthetic tritiated prostaglandin endoperoxides were carried out. Prostaglandin endoperoxides (3-5 ug, 1 µCi) were incubated with fresh tissues either for 10 min in Krebs-Henseleit buffer or aerobically for 90 sec in $KH_2PO_4$/NaOH buffer (0.1 M, pH 7.4). Following incubations for 90 sec, residual endoperoxide was reduced by addition of stannous chloride in ethanol (12.5 mg/5 ml). The stannous chloride was allowed to react, with vigorous stirring, for two minutes, after

which the mixture was worked up by standard extraction and purification procedures. Non-enzymatic decomposition of endoperoxides was assessed by control incubations performed with boiled tissue (3 min, 100°C) or in buffer alone.

G. Purification and Analysis of Prostaglandins

1. Extraction from incubation mixture.

All incubation mixtures underwent a standard extraction procedure. In quantitative analytical experiments, deuterated prostaglandin internal standards and carrier (1 or 2 ug) were added prior to acidification.

Typically, immediately following the incubation at 37°C for the appropriate times the mixture was cooled in an ice-water bath and acidified to pH 2.5-3 with 1 M HCl. This mixture was then partitioned three times with 5 volumes of diethyl ether, the ether phase backwashed twice with distilled water to neutrality and then evaporated *in vacuo* or under a stream of dry nitrogen. The residue was reconstituted in the solvents required for the subsequent purification step.

2. Thin layer chromatography.

A qualitative assessment of distribution of radioactivity in a sample was routinely carried out by thin layer chromatography using pre-coated silica gel 60/F-254 plates (E. Merck A.G., Darmstadt, Germany) in an ascending solvent system consisting of the organic phase of ethyl acetate:2,2,4-trimethyl pentane:acetic acid: water (55:25:10:50). Development was at ambient temperature for 1 hr, after which the plates were allowed to dry and then developed

again ( 1 hr) in the same system. The plates were sprayed with phosphomolybdate (10%) in ethanol and after heating for 5 min, the prostaglandins appeared as dark blue spots on a greenish-yellow background. Radioactivity was monitored by a Packard Model 7220/21 Radiochromatogram Scanner.

3. Octadecylsilyl Silica ($C_{18}$ SepPak) Chromatography

Chromatography of arachidonic acid and its metabolites using octadecylsilyl (ODS) silica ($C_{18}$ SepPak, Waters Associates, Milford, MA) was investigated initially with radioactive standards and a number of closely related solvent mixtures - ethanol:diethyl ether, methanol:diethyl ether, and ethylacetate:diethyl ether. Mixtures with decreasing polarity ratios (1:1, 1:2, 1:4, 1:8) and diethyl ether alone were tested.

All SepPak cartridges were pretreated and activated by passing 5 ml ethanol through them followed by 10 ml of distilled water. The samples to be chromatographed were dissolved in 20% ethanol-water (pH 3) and applied to the cartridge through a 20 ml glass syringe. The cartridge was washed with neutral aqueous ethanol (10 ml) and then benzene (5 ml).

Prostaglandins were subsequently eluted with 10 ml of diethyl ether, the most suitable of the tested solvents, and subjected to further purification or analysis.

4. Silicic acid column chromatography

Group separation of prostaglandins for quantitative analysis was accomplished by silicic acid column chromatography. Silicic acid (Bio Sil HA, minus 325 mesh, Bio-Rad Laboratories) was activated

for 1 hr at 110°C. After cooling in a desiccator, a slurry was made of silicic acid in diethyl ether and poured into a 2 ml pipet having a glass wool plug. Silicic acid was allowed to sediment to a height of 6 mm in the glass pipet. The column was then washed with chloroform until all the silicic acid became transparent. Sample residues from incubate extractions or ODS chromatography were applied to the silicic acid column in chloroform.

The column was cleansed with 5 ml of chloroform followed by 2% methanol in chloroform. Prostaglandins $E_2$, $D_2$, and 6-keto-$F_{1\alpha}$ were eluted with 5% methanol in chloroform and $PGF_{2\alpha}$ with 10% methanol in chloroform.

5. High pressure liquid chromatography.

High pressure liquid chromatography (HPLC) analysis was performed using a Waters Associates Model M-6000A solvent delivery system equipped with a Model U6K manual injector and Model 450 Ultraviolet absorbance detector with variable wavelength capacity. The separations were performed isocratically on a Waters Associates Fatty Acid Analysis column (30 cm x 4 mm i.d.) at room temperature. Modifications of the solvent system described by Russel et al. (38) were employed as the mobile phase with flow rates of 1 or 2 ml per minute. Tetrahydrofuran (HPLC grade), acetonitrile (Pesticide grade) and acetic acid (Reagent grade) were purchased from Fisher Scientific Co., Montreal, Que. Distilled, deionized water was thoroughly degassed prior to mixing with other solvents and the final solvent system was briefly evacuated.

The compositions of the solvent mixtures (by volume) were as follows:

| | Tetrahydrofuran | Acetonitrile | Water | Acetic acid |
|---|---|---|---|---|
| System A | 45 | 58 | 157 | 0.25 |
| System B | 45 | 62 | 153 | 0.25 |

Prostaglandins standards (1 µg of each) were detected at 210 nm and radioactivity from samples was monitored in the column eluate by counting 0.1 ml aliquots in 10 ml liquid scintillation fluid (Scintiverse, Fisher Scientific Co., Montreal) with an Intertechnique Liquid Scintillation Spectrometer. In some cases HPLC elution profiles were plotted directly by a microprocessor unit of a Beckman LS 8100 Programmable Liquid Scintillation Spectrometer.

6. Gas-liquid chromatography and mass spectrometry

a) General derivatization procedures

Methoximation - Samples containing prostaglandins with carbonyl functions (i.e. $E_2$, $D_2$, $B_2$ or 6-keto-$F_{1\alpha}$) were methoximated in silanized glass vials (Reacti-Vial, 0.3 ml, Pierce Chemical Company, Rockford, Ill.) by addition of 30 µl of 2% methoxylamine-HCL in pyridine (Pierce Chemical Co.) to the dry residue. The vessel was capped with a screw top containing a teflon septum and kept at 80°C for 1 hr. At the completion of the reaction the sample was dried under a stream of nitrogen and methylated.

Methoximation is performed first because of the fact that 6-keto-$PGF_{1\alpha}$ can coexist in a lactol form. The hemi-ketal function can be immobilized by methylation thus rendering the ketal inaccessible to subsequent reaction with methoxylamine.

Methylation - Carboxyl functions of prostaglandins and arachidonic acid were converted to the methyl ester derivative in Reacti-vials by dissolving the dried sample in 10 µl of methanol followed by 90 µl of freshly prepared ethereal diazomethane.

Diazomethane was generated from N-methyl-N'-nitro-N-nitroso guanidine (Diazald, Aldrich Chemical Co., Milwaukee, Wisc.) in 18 N KOH/2-(2 ethyethoxy)-ethanol solution and trapped in diethyl ether. The sample in the closed Reacti-Vial was held at room temperature in the dark for 20 min, after which the evolved gas was released and the ether removed by a stream of dry nitrogen.

Trimethylsilylation - Immediately prior to gas liquid chromatography (GLC) with flame ionization detection (FID) or mass spectrometry (MS), all samples containing hydroxylated compounds (except $PGE_2$ in GLC-MS analysis) were trimethylsilylated with 10 µl of N,O-Bis(trimethylsilyl)-acetamide (BSA, Pierce Chemical Co.) and 10 ul of Tri-Sil Z (Pierce Chemical Co.) heated for 5 min at 60°-70°C. For GLC-MS analysis, the O-methyloxime methyl ester derivative of $PGE_2$ was first converted to its $B_2$ analogue by treatment with 10 ul of piperidine at 70°C for 5 min and then silylated with 10 ul trimethylsilylimidazole (TSIM, Pierce Chemical Co.) for 5 min at 60°-70°C. Trimethylsilylation was always performed immediately preceding injection into the gas chromatogram.

b. Quantitative analysis

Gas chromatographic mass fragmentographic analysis was carried out on a LKB 9000 instrument using the multiple ion detector unit

to monitor alternatively the intensities of the protium and tetra-deuterated prostaglandins in the same injection sample.

The gas chromatograph was equipped with a 6 foot glass column packed with 1% OV-101 on chromasorb WHP and operated at an oven temperature of 220°C. Ion source temperature was 290°C with electron source energy of 70 eV and trap current of 60 μA. Under these conditions the 6-keto-$PGF_{1\alpha}$ derivative had a retention time between 4-5 min.

The spectral fragments focused for multiple ion detection depend on absence of contaminants in that mass range and their relative intensities in a full mass spectrum (Table 1 and Fig. 3).

Both protium and corresponding deuteroprostaglandin spectra are very similar except that ions containing the deuterated side chain have m/e values 4 units higher.

The ions monitored were m/e 598 and 602 for the methyl (me)-ester-methoxime (MOX)-trimethylsily (TMS) ether derivative of 6-keto-$PGF_{1\alpha}$ ; m/e 321 and 325 for $PGE_2$ as the Me ester-TMS derivative of $PGB_2$; and m/e 423 and 427 for the Me ester-TMS derivative of $PGF_{2\alpha}$ . Standard curves were constructed from known mixtures of reference ($^2H$) and ($^1H$) prostaglandins and the minute amount of protium in the deuterated standards was subtracted from sample measurements.

# Chapter III

# RESULTS

## A. Morphological Evaluation of Isolated Microvessels

Microvessel preparations routinely evaluated by light microscopy revealed predominantly capillaries and some small fragments of arterioles and venules (Figure 5 A,B). Typically, the microvessels appeared as fine strands or tubules composed of oval elongated nuclei regularly spaced along a branching network with discernible but sparse and ill defined cytoplasm. At higher maghification occasional intraluminal erythrocytes were seen along the course of the vessels. The purity of the preparations was estimated to be greater than 90% as visualized in randomly chosen fields and absence of gross contamination by neuronal and neuroglial elements. Indeed electron microscopy (Fig 6A-C) confirmed the absence of gross contamination by glia, nerve cells, synaptosomes and myelin. A red blood cell was sometimes seen in the lumen (Fig 6C). Bits of residual shards of astrocytic vascular foot processes were found adhering to the basement membrane. The microvessels consisted of endothelial cells forming a partially collapsed lumen surrounded by a continuous basement membrane which often included a pericyte (Rouget cell)(Fig 6A). The endothelial cells and constituent organelles, primarily mitochondria were mildly swollen but the plasma membranes were continuous and the intracellular junctions, zona occludens were well preserved (Fig 6 B,C). Interestingly incubation of a microvessel preparation in the presence of EDTA, a calcium chelator, disrupted the junctional complex.

Figure 5. Photomicrographs of fixed wet mount preparations of isolated microvessels stained with Hematoxylin/Eosin illustrating the capillary network and its uniform tubular configuration. The regular array of focal dark nodules localize the endothelial and pericyte nuclei (N). Occasional erythrocytes (E) are also visible.

(A) Original x 200, (B) Original x 600, (C) Original x 1250.

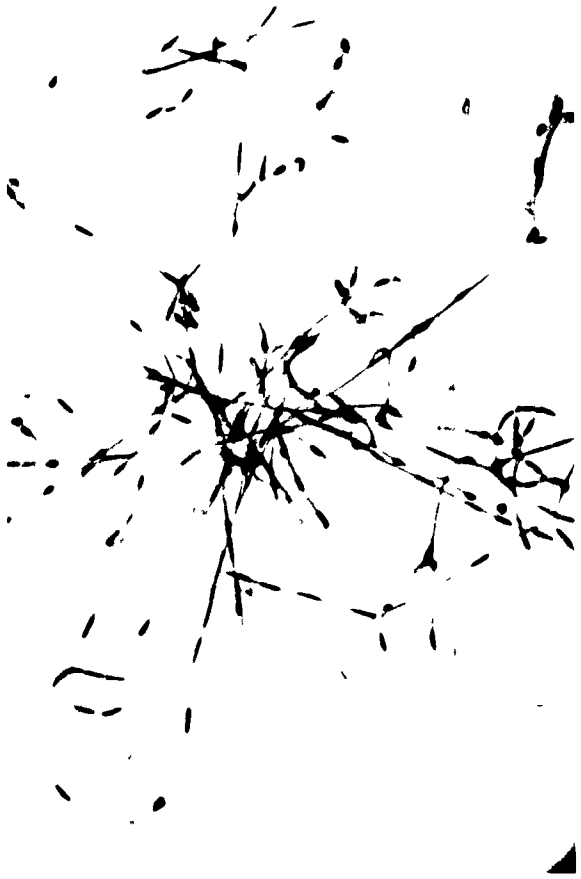
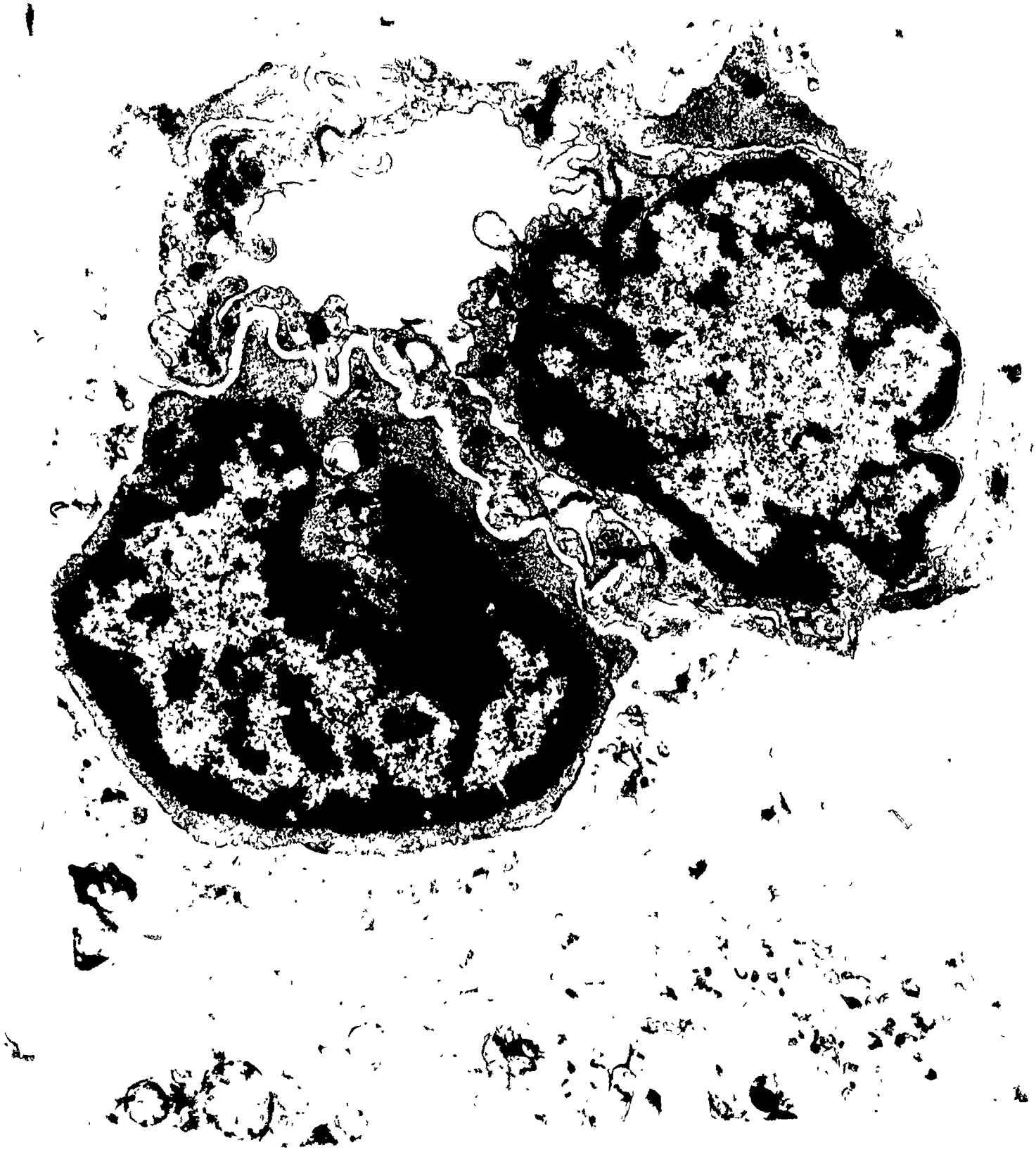


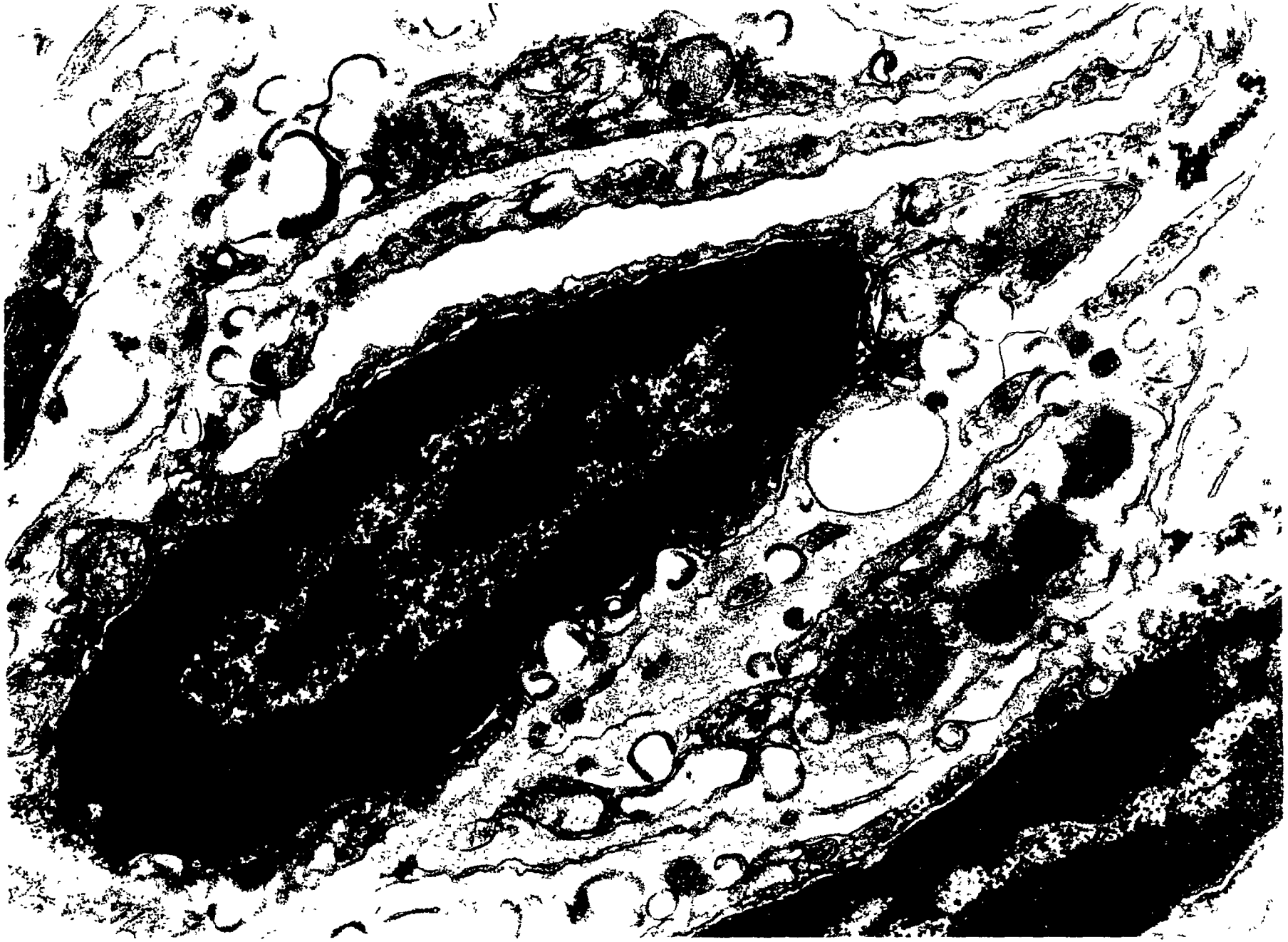
Fig 5A

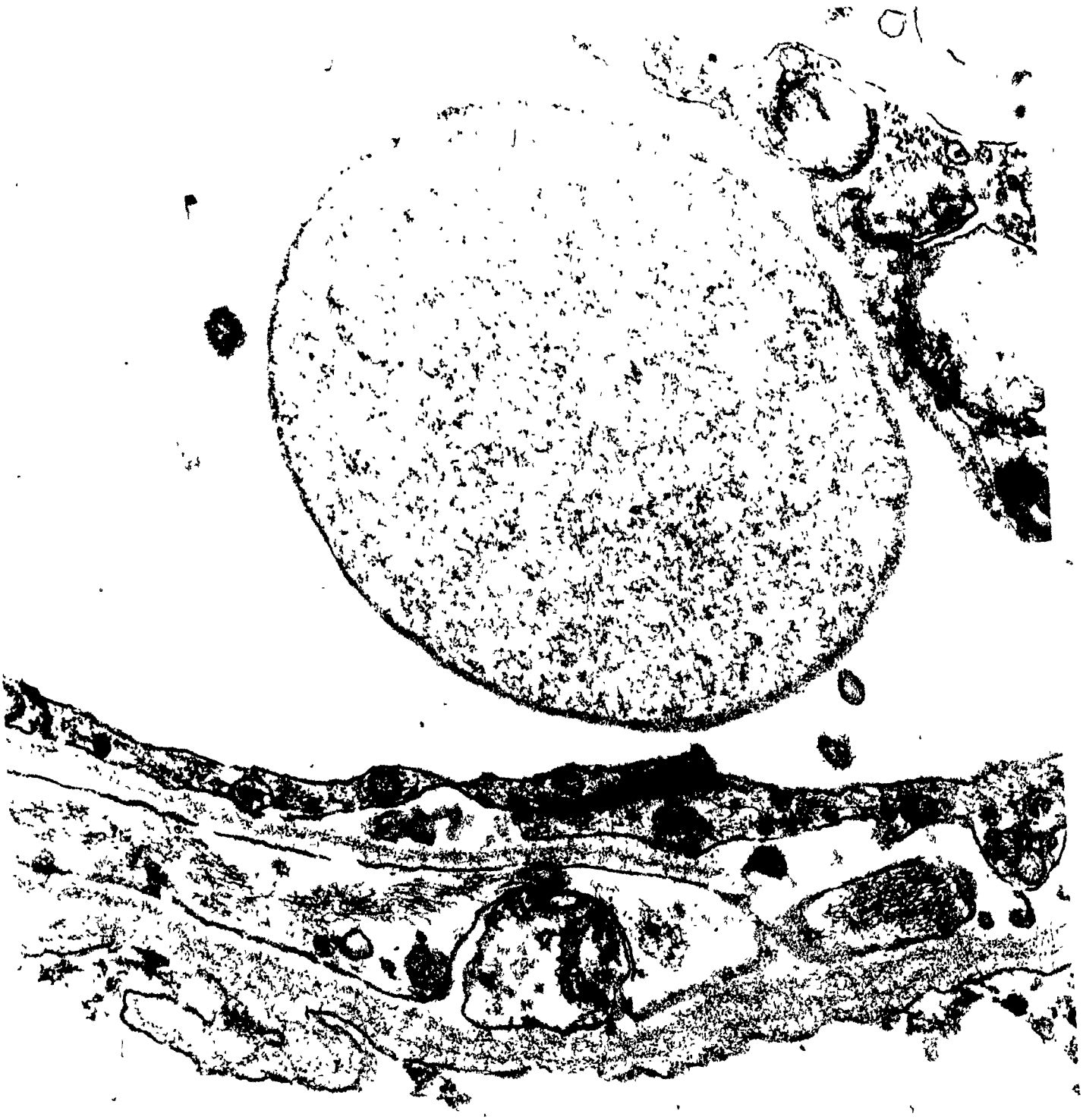
Fig. 5B


Fig. 5C

Figure 6. High power transmission electron micrographs of capillary preparations in cross section. (A) Capillary with the lining endothelial cell, investing basement membrane and a pericyte. Astrocytic foot process debris is evident in the bottom of the picture. (B) Capillary endothelial cell nucleus with the lumen collapsed showing the continuous plasmalemma and preserved intercellular junctional complexes. (Original x 57,500) (C) Higher magnification of a typical tight junction of isolated microvessels.







B. Enzyme Enrichments

The activities of enzymes considered as specific markers for microvessels were routinely measured to follow the purification efficacy, and compared with their activities in crude cerebral hemisphere homogenates (Table 2).

Alkaline Phosphatase showed an enrichment of 14 fold and γ-glutamyl transpeptidase yielded a 34 fold increase over crude cerebral homogenate. The most spectacular enrichment was seen with prostacyclin synthetase which demonstrated a 193 fold enrichment in the microvessels versus the crude homogenate. Enzymic activities in choroid plexus were hundreds of times higher than in the homogenate, and it was also prostaglandin synthetase which had the higher relative enrichment of the three enzyme activities determined.

C. Characterization of Prostaglandin Endoperoxides

The distribution of radioactivity following TLC of the product isolated from the incubation of tritiated arachidonic acid with ram seminal vesical microsomes showed that conversion to $PGG_2$ and $PGH_2$ was 35% and 33% respectively (Fig 7A). After silicic acid chromatography, the radiochemical purity of PGG2 was calculated to be 83% and that of $PGH_2$ was 92% with cross contamination being the major interference (Fig 7B and C). Further evidence for the identification of the endoperoxides was the conversion of the radiolabelled reaction products to $PGF_{2\alpha}$ after mild $SnCl_2$ reduction and characteristic retention times in three standard solvent systems. Final yield of $PGG_2$ was 149 μg (91.5 μCi/μmole) and of $PGH_2$ was 104 μg (91.5 μCi/μmole). The endoperoxides were found to be stable for longer than three months

Table 2. Activities and enrichment of enzymes in isolated rat microvessels

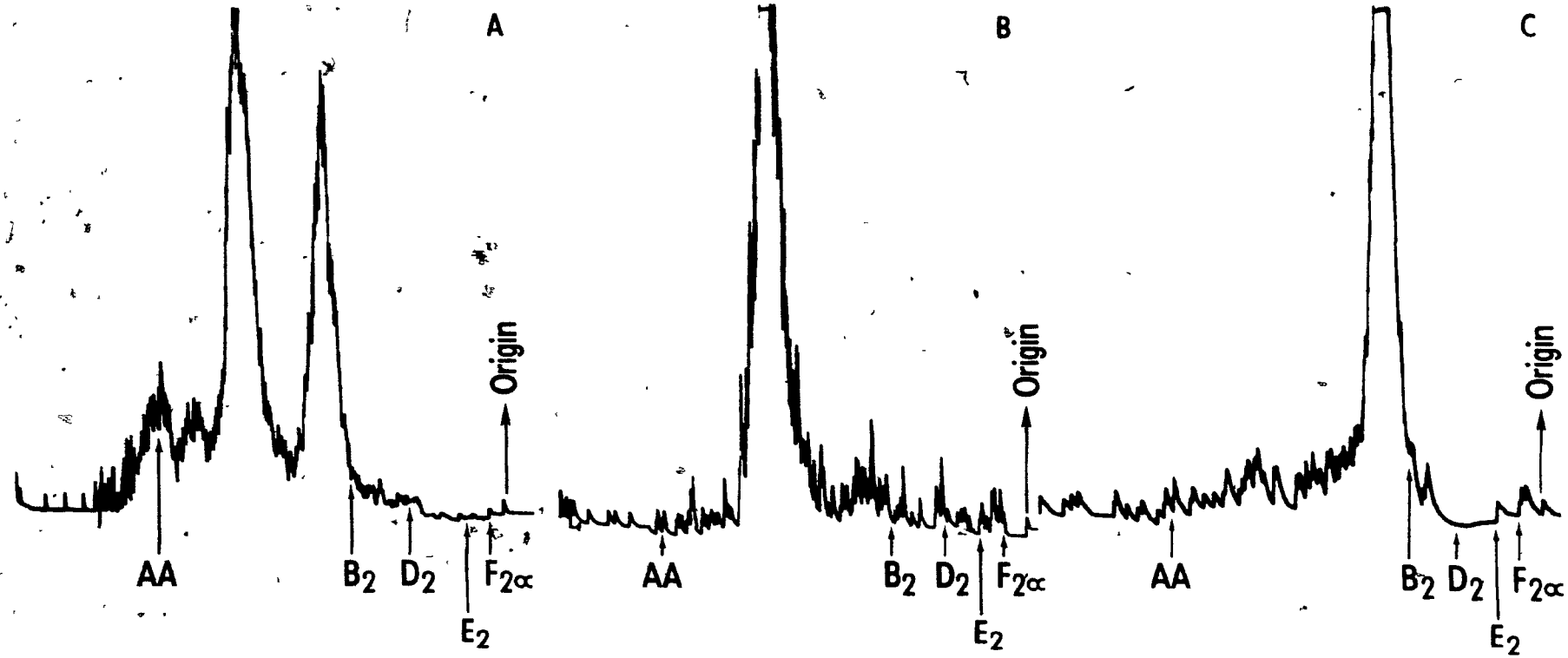
| Enzyme | Activities | | Enrichment factor |
|---|---|---|---|
| | Cortex | Microvessels | |
| Alkaline phosphatase[a] (4) | 0.55 ± 0.25 | 7.6±2.0 | 14 |
| γ-Glutamyl-transpeptidase[a] (6) | 1.0 ± 0.5 | 35.2±18.5 | 34 |
| Prostacyclin synthetase[b] (5) | 0.057±.023 | 11.0± 0.4 | 193 |

Enzymes assayed as described in Methods. Values are mean ± S.D. with number of determinations in brackets.

[a] Aliquots of 80-100 µg of capillary protein incubated with either p-nitrophenyl phosphate (30 min) for alkaline phosphatase or L-γ-glutamyl-p-nitroanilide (20 min) for γ-glutamyl-transpeptidase; µmole substate released/mg protein/min.

[b] Derived from GC-mass fragmentographic data using 2 to 5 mg capillary protein; ng of 6-keto-$PGF_{1\alpha}$ produced/mg protein/10 min.

Figure 7. Radiochromatogram tracings of (A) ether extract of ram seminal vesicle microsomes after incubation with tritiated arachidonic acid for 90 seconds, showing conversion to $PGG_2$ and $PGH_2$; (B) $PGG_2$ and (C) $PGH_2$ after purification by silicic acid column chromatography. Development of thin layer plates was performed in diethyl ether at -20°C for 90 min. Relative mobilities of prostaglandin standards $F_{2\alpha}$, $E_2$, $D_2$, $B_2$ and arachidonic acid (AA) are shown.


A
B
C
Origin
Origin
Origin
AA
B2
D2
E2
F2α
AA
B2
D2
E2
F2α
AA
B2
D2
E2
F2α

when stored at -80°C. Incubation solutions were prepared by dilution with buffer just prior to individual experiments.

D. Prostaglandins Generated from Endogenous Sources.

Prostanoid production, when compared in various specific vascular components of the rat, unquestionably shows that prostacyclin is the major endogenous metabolite of arachidonate in all instances and that in the microvessels the synthetic capacity is about 1/3 that of the larger vessels (Table 3). Aortic rings, brain basal arteries and choroid plexus had equal synthetic rates and $PGF_{2\alpha}$ was the next most abundant prostaglandin made in these larger vessels. In microvessels however the trend was reversed and $PGE_2$ was synthesized at about twice the rate of $PGF_{2\alpha}$. Another interesting finding was that the ratios of $E_2$/6-keto-$F_{1\alpha}$ and $F_{2\alpha}$/6-keto-$F_{1\alpha}$, i.e. the relative amounts of the other prostaglandins to prostacyclin, was the highest in the microvessels. There was also an indication that in general microvessels in themselves have a greater capacity to synthesize $PGE_2$ and $PGF_{2\alpha}$ when compared to aortic rings and choroid plexus. $PGD_2$ the major prostaglandin formed in whole rat brain homogenates and also used as a test of purity for microvessel preparations was not detected in our capillaries in agreement with other (249).

E. Prostaglandins Generated from Exogenous Sources

Prostacyclin measured as 6-keto-$PGF_{1\alpha}$ was formed enzymatically from added labelled $PGH_2$ by both choroid plexus and microvessels (Figs. 8 and 9). Control incubations of $PGH_2$ in buffer alone

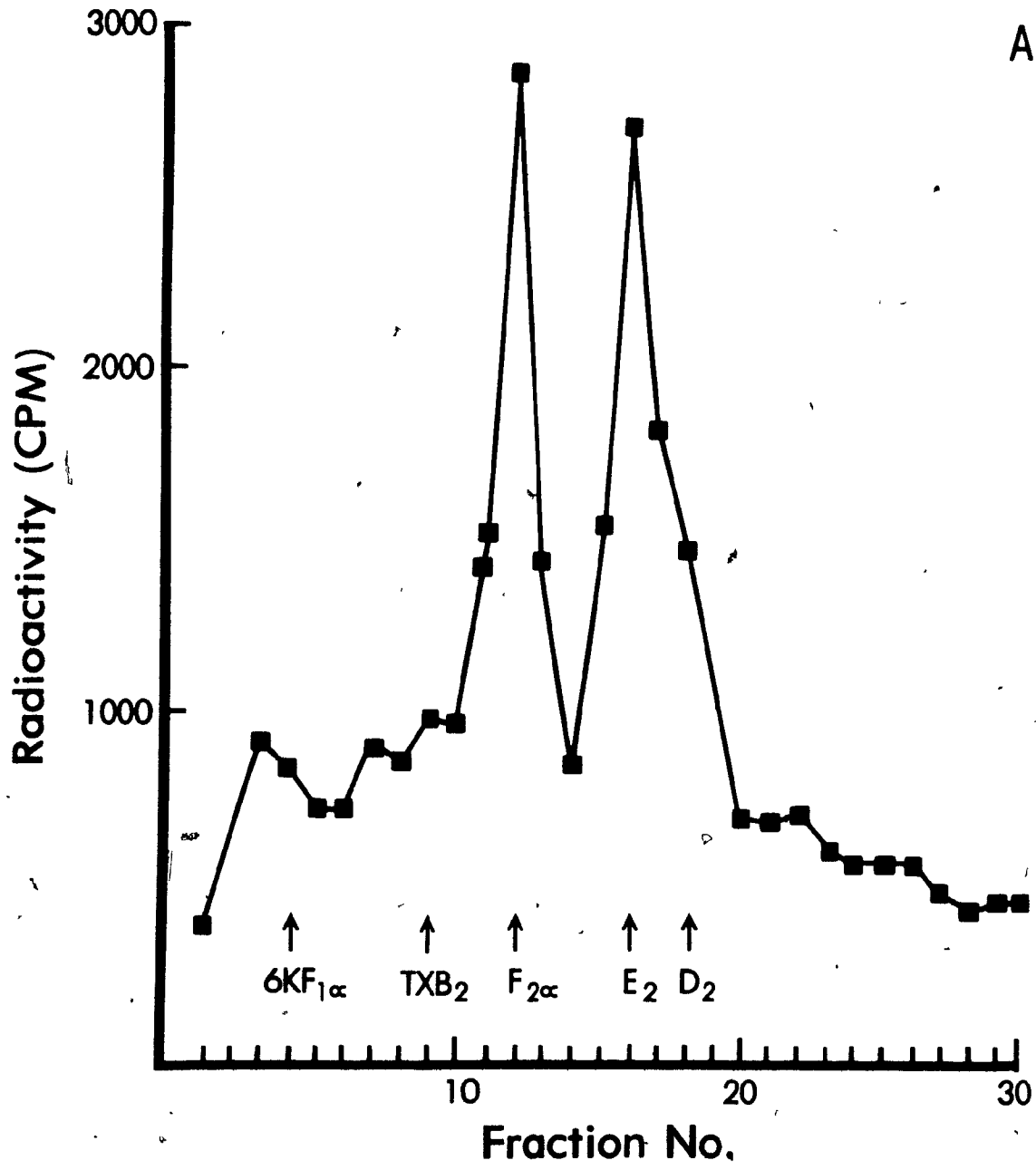
Table 3. Formation of Prostaglandins by rat vascular tissue

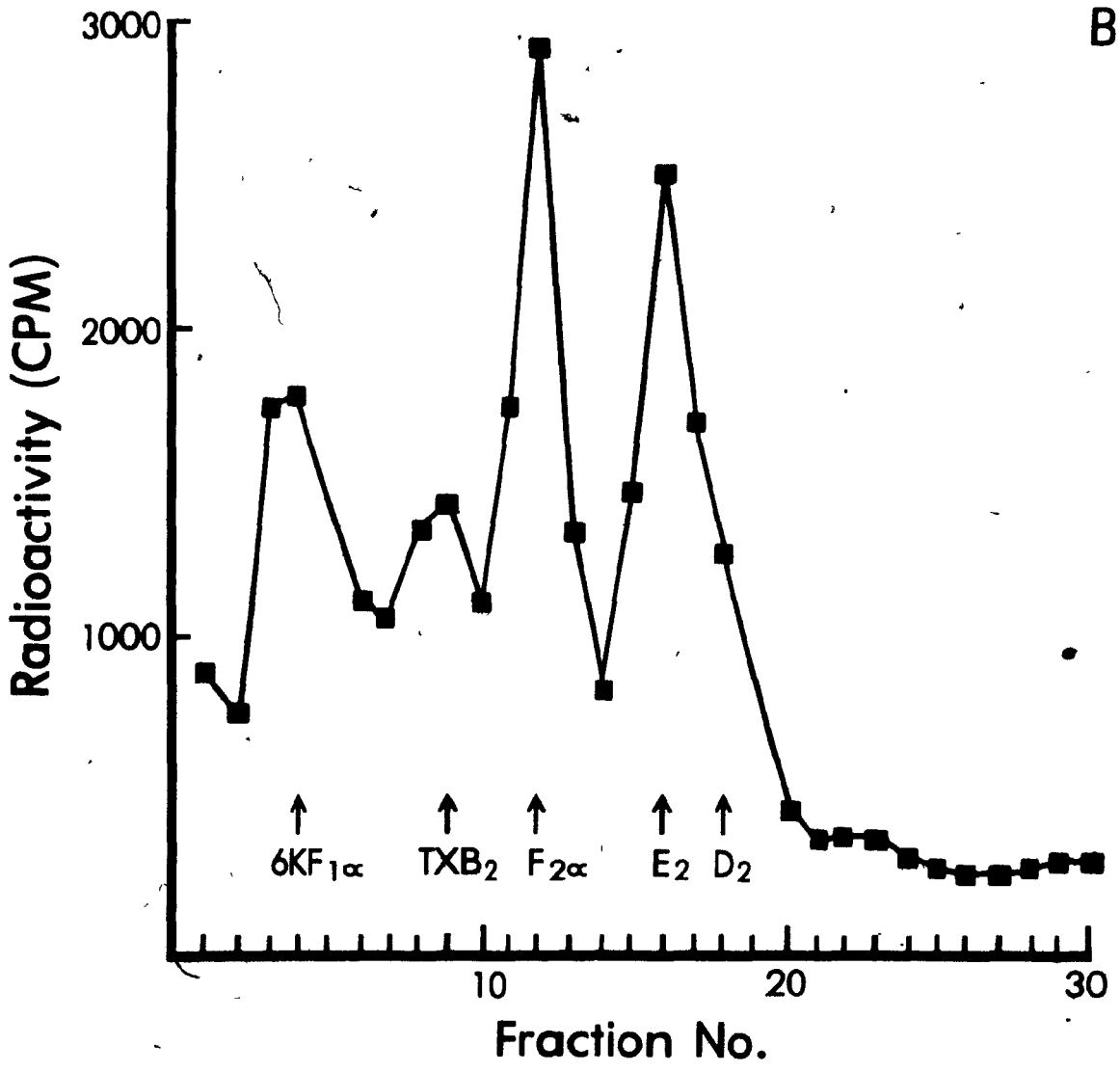
| Vascular Tissue | Prostaglandins (ng/mg protein) | | |
|---|---|---|---|
| | 6-keto-$PGF_{1\alpha}$ | $E_2$ | $F_{2\alpha}$ |
| Aortic rings | 36.4 ± 5.2 (12) | 0.93 ± 0.05 (3) | 1.1 ± 0.3 (3) |
| Basilar artery and Circle of Willis | 35.2 ± 5.4 (4) | - | 4.2 (1) |
| Isolated cerebral microvessels | 11.0 ± 0.4 (5) | 3.24 ± 1.39 (3) | 1.30 ± 2.26 (3) |
| Chorold plexus | 36.8 ± 3.1 (4) | 1.2 ± 0.1 (3) | 2.6 ± 0.2 (3) |

Values are means ± S.D. Number of experiments in brackets.

Quantitation by GC-mass fragmentography as described in Methods.

Figure 8. Metabolism of $PGH_2$ in isolated capillaries and choroid plexus. Incubations for 10 min at 37°C in Krebs-Henseleit bicarbonate buffer pH 7.4, (A) control, no tissue added; (B) choroid plexus, 12-15 mg wet weight; (C) cerebral capillary preparation, 4-5 mg protein. HPLC conditions: solvent THF: $CH_3CN$: HOAc (45:58:157:0.25 by vol.), flow rate 2 ml/min. *Radioactivity monitored in column effluent.


A
3000
2000
1000
Radioactivity (CPM)
6KF1α
TXB2
F2α
E2
D2
10
20
30
Fraction No.


B
3000
2000
1000
Radioactivity (CPM)
6KF1α
TXB2
F2α
E2
D2
10
20
30
Fraction No.

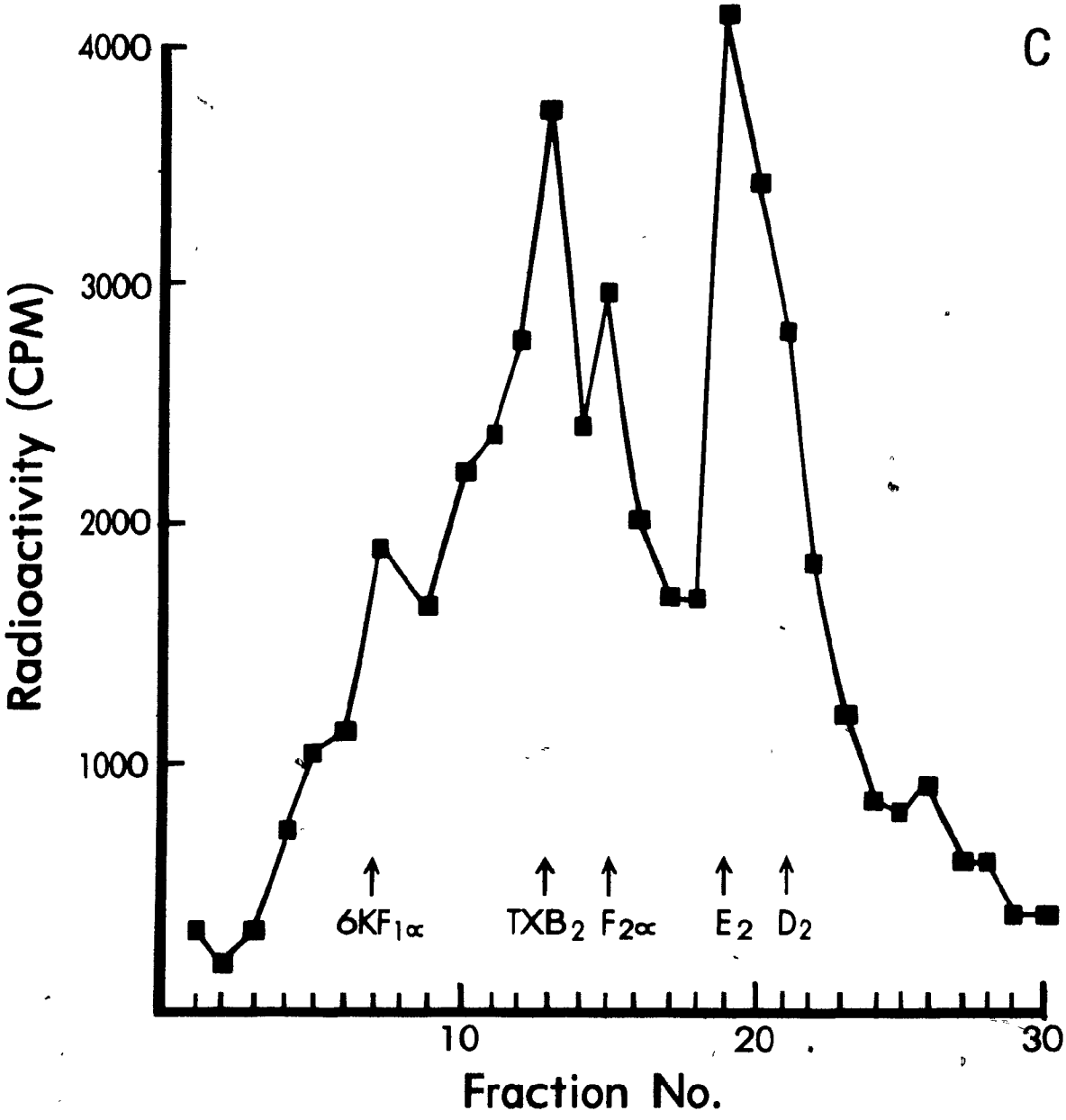
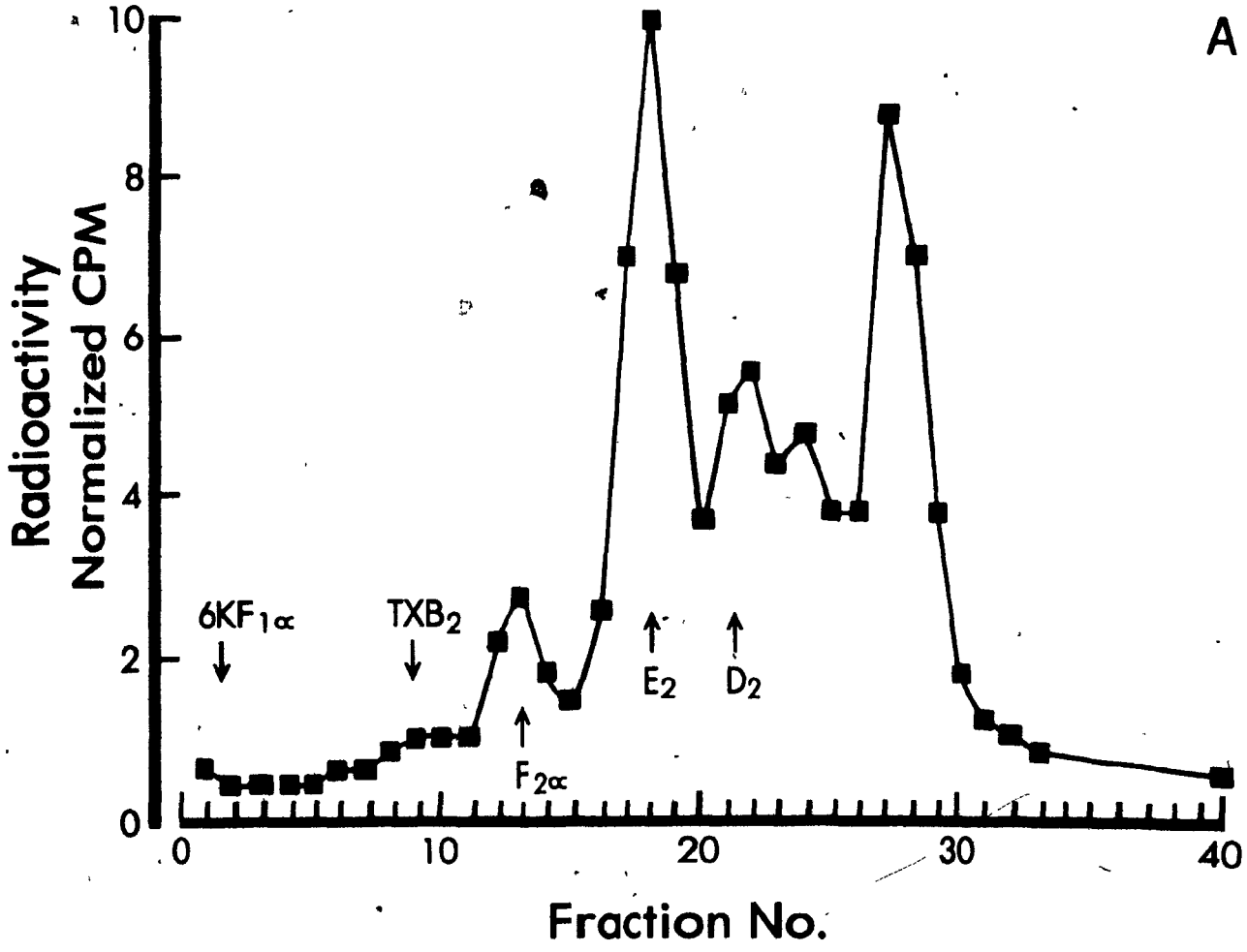
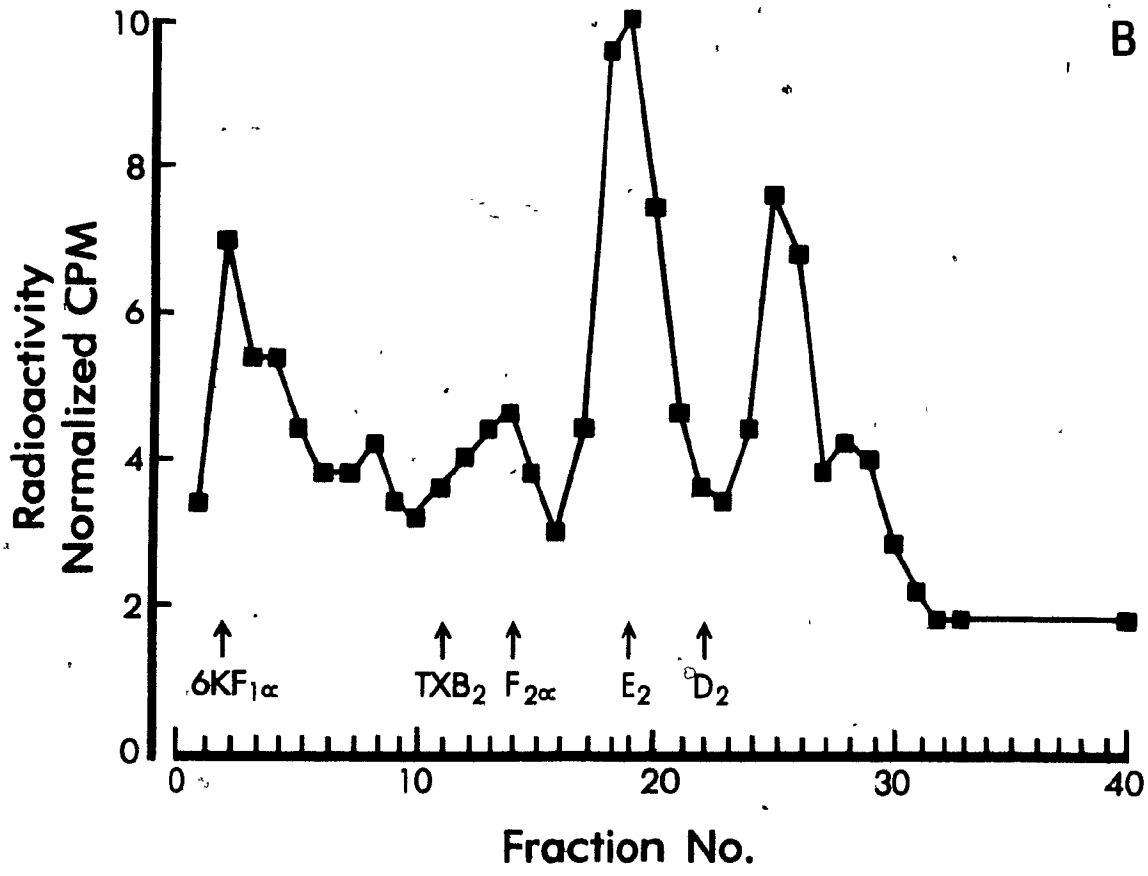

C
4000
3000
2000
1000
Radioactivity (CPM)
6KF1α
TXB2
F2α
E2
D2
10
20
30
Fraction No.

Figure 9. Radio-HPLC profiles of reaction products following 90 sec incubation of $PGH_2$ with (A) boiled capillary tissue and (B) 2-3 mg of fresh tissue. Excess endoperoxide was removed as $PGF_{2\alpha}$ by reduction and silicic acid chromatography. HPLC conditions: solvent THF:$CH_3CN$:$H_2O$:HOAc (45:62:153:0.25 by vol); flow rate 1 ml/min. Radioactivity monitored in column effluent.


A
Radioactivity
Normalized CPM
10
8
6
4
2
0
6KF1α
TXB2
F2α
E2
D2
0
10
20
30
40
Fraction No.


B
Radioactivity
Normalized CPM
10
8
6
4
2
0
6KF1α
TXB2
F2α
E2
D2
0
10
20
30
40
Fraction No.

(Fig 8A) or with boiled tissue (Fig 9A) showed nonenzymic hydrolysis to $PGF_{2\alpha}$, $PGE_2$ and $PGD_2$. Radio-HPLC analysis of choroid plexus and isolated capillaries showed that after a 10 min incubation (Fig 8B and C) $PGH_2$ had decomposed mainly to $PGE_2$ and $PGF_{2\alpha}$ with a concomitant noticeable production of 6-keto-$PGF_{1\alpha}$ (Fig 8B).

Taking into account the nonenzymic production of prostaglandins in choroid plexus, it is clear that 6-keto-$PGF_{1\alpha}$ was the major prostaglandin formed enzymatically from exogenously supplied endoperoxide.

In microvessels, 6-keto-$PGF_{1\alpha}$ was also the major enzymatic prostaglandin (Fig 9B). There was also a significant amount of thromboxane $B_2$ present in microvessels incubated for 10 min with labelled $PGH_2$ (Fig 8C). A possible explanation may involve residually platelets in the lumen or those adhering to the cut ends of the microvessels.

To reduce the contribution of nonenzymic prostaglandins, incubation of $PGH_2$ and isolated capillaries was shortened to 90 seconds and the excess endoperoxide reduced and removed as $PGF_{2\alpha}$. Under these circumstances capillaries showed large production of 6-keto-$PGF_{1\alpha}$ with no significant production of thromboxane $B_2$ (Fig 9B). Under identical conditions, rat cerebral surface vessels synthesized mainly 6-keto-$PGF_{1\alpha}$ and some thromboxane $B_2$. Studies with the hydroperoxy endoperoxide $PGG_2$ were much more variable in the nonenzymic breakdown patterns.

Qualitative results by Radio-HPLC were confirmed by quantitative analysis using GC-mass spectrometry. The results summarized in Table 4 indicate that in the presence of microvessels or choroid

Table 4. Metabolism of prostaglandin endoperoxide $H_2$ in microvessels and choroid plexus to prostaglandins 6-keto-$F_{1\alpha}$, $E_2$ and $F_{2\alpha}$ measured by GC-mass fragmentography

| Tissue | Protein (mg) | +$PGH_2$ (µg) | %Conversion to Prostaglandins | | |
|---|---|---|---|---|---|
| | | | 6-keto-$PGF_{1\alpha}$ | $E_2$ | $F_{2\alpha}$ |
| Microvessels | 2.2 | 5.2 | 2.2 | 52.3 | 7.5 |
| | 0 | 5.2 | 1.5 | 56.5 | 2.1 |
| | 1.7 | 3.5 | 2.5 | 14.8 | 3.6 |
| | 0 | 3.5 | 0.5 | 19.7 | 0.3 |
| Choroid plexus | 1.5 | 5.2 | 8.3 | 25.1 | 13.7 |
| | 0 | 5.2 | 1.5 | 56.5 | 2.1 |
| | 1.2 | 3.5 | 4.3 | 5.6 | 4.3 |
| | 0 | 3.5 | 0.5 | 19.7 | 0.3 |

*Results of four experiments with appropriate controls, are expressed as percentage of the added endoperoxide. Incubation period of 10 minutes.

plexus the conversion to 6-keto-$PGF_{1\alpha}$ from exogenous $PGH_2$ is increased an average of 5 fold. The highest increase was seen in choroid plexus in which 4.3% of added endoperoxide was converted to 6-keto-$PGF_{1\alpha}$ versus 0.5% without tissue, a 9 fold augmentation. Small amounts of 6-keto-$PGF_{1\alpha}$ was detected in incubations without tissue but the major nonenzymic product in both experimental and control situations was $PGE_2$. Because of the variability between experiments in the breakdown pattern of endoperoxides to prostaglandins and other hydroxy fatty acids and recovery of the three major prostaglandins, it is not possible to decide with these data alone whether the presence of enzyme causes shunting of the endoperoxide from the major nonenzymatic pathway or whether enzymatic conversion is superimposed on the nonenzymic one.

# Chapter IV

# DISCUSSION

## A. Prostacyclin as the Major Prostanoid in the Rat Cerebral Microvasculature

Since the first report of prostaglandin synthesis by blood vessels (165), later highlighted by the discovery of the predominantly vascular prostaglandin ( 25), prostacyclin, an enormous amount of experimental data on vascular production and actions of these compounds has been accumulated. While most of these studies have dealt with peripheral blood vessels (259) few and only recent studies have focused on production and effects of arachidonic acid metabolities in the larger vessels of thecerebrovasculature (174,175 ). Knowledge of arachidonic acid derived substances and their activities in the cerebral microvasculature is practically nonexistent with few literature reports to date ( 250-254).

It has been shown that both brain parenchyma (157 ) and brain blood vessels (170) from a number of species synthesize a variety of prostaglandins, including prostacyclin and thromboxanes. The capacity to synthesize these eicosanoids and their relative preponderance is a function of the tissue type and regional differences of the brain (146). In neuronal elements of some species the major prostaglandin produced is PGD2 (147) which is a powerful stimulator of adenylate cyclase and is also produced by neuroblastoma cells, hence it has been proposed to function as a neuromodulator (260).

Prostacyclin is the principal prostaglandin produced by blood vessels ( 25). It is the most potent platelet antiaggregatory substance known (259) and is a powerful vasodilator (261 ). This has

led to the postulation of an important role for this substance in vascular homeostasis (214). The production of $PGI_2$ by cerebral arteries has been demonstrated in a number of species (146) including man (170). Skidgel and Printz 1978 (262) as well as other (171) have suggested greater occurrence of prostacyclin in arteries and arterioles than in veins and venules, which suggests that prostacyclin may have its effects at the microcirculatory level. In support of this hypothesis it has been found that inhibition of $PGI_2$ formation in the microcirculation enhances platelet aggregation (171).

The suspicion that in the vasculature $PGI_2$ is derived mainly from the endothelium was probably brought about by the high content of this prostanoid in perfusates of isolated organs of high endothelial cell content, ie. lung, heart and kidney (80). Continuous release of $PGI_2$ by the lung has even been demonstrated (263), leading some to hypothesize that prostacyclin may function as a circulating hormone (264). Definitive evidence for the origins of endogenous prostacyclin in blood vessels was reported by Moncada et al. (259) who studied prostacyclin formation in different layers of rabbit aorta. These investigators compared $PGI_2$ synthesis by intimal cell suspensions with that of internal elastic lamina, media and adventitia, and noted that the distribution of prostacyclin synthetase was not uniform throughout the vessel wall. They concluded that the intima of rabbit aorta represents approximately 5% by weight of the total arterial tissue, whereas it produces about 40% of the total endogenous $PGI_2$.

Absolute proof that endothelial cells were the source of vascular prostacyclin was the synthesis of prostacyclin from arachidonic acid and prostaglandin endoperoxides by cultured endothelial cells (219) and the minute if any synthesis by cultured aortic media smooth muscle cells and adventitial fibroblasts (218). The earliest report of prostaglandin synthesis by cultured endothelial cells was that of Gimbrone who also showed that $PGE_2$ production can be stimulated by angiotensin II (216). Since then production of prostaglandins and particularly $PGI_2$ has been demonstrated in; human umbilical vein, bovine aortic (217) and porcine aortic (218) cultured endothelial cells. Recognition of hormone and neurotransmitter receptors on cultured vascular endothelial cells (221) has led to the further understanding of regulation of prostacyclin synthesis in endothelial cells. Many substances (eg. thrombin, trypsin, $Ca^{++}$ ionophores) stimulate prostacyclin synthesis by cultured endothelial cells (220). Macintyre et al (218) also reported that cell free plasma from human, dog and rat stimulated $PGI_2$ production by cultured porcine cells. Epinephrine, Norepinephrine and ADP, however did not stimulate $PGI_2$ production. The enhanced synthesis of prostacyclin was abolished by mepacrine ($PLA_2$ inhibitor), by the known cyclo-oxygenase inhibitors aspirin and indomethacin (221) as well as the specific prostacyclin synthetase inhibitors tranylcypromine and 15-hydroperoxyeicosatetraenoic acid (217). The inhibitory effect of aspirin on vascular cultured cells was temporary and recovered in 1 to 2 hours, presumably as a result of new cyclo-oxygenase protein synthesis (222). Furthermore, it was shown that

indomethacin pretreated cultured endothelial cells exposed to stimulated platelets could use the exogenous $PGH_2$ to synthesize $PGI_2$ in a phenomenon termed "endoperoxide steal" (219). Prostacyclin synthesis in cultured cells from arachidonic acid was only 1.6% and from $PGH_2$ about 11.9% (221) indicating probably that the amount of $PGH_2$ present is the critical factor. This seems logical if we think of the events of platelet aggregation in which large amounts of $PGH_2$ are produced and the pivotal role it plays in the production of thromboxane $A_2$ and prostacyclin. Although endothelial cells in culture have no doubt increased our knowledge of prostacyclin metabolism they have some serious drawbacks. In particular they may lose their prostacyclin synthetase activity after many passages in culture (218).

With the advent of methods to isolate fresh metabolically active cerebral microvessels (225) we can now study prostaglandin metabolism under the most physiological conditions now attainable, since techniques for the in vivo study of intracerebral microvasculature have not been adequately developed. Brain capillary endothelium is unique in morphology and enzymatic activity (236). The hydrolytic enzymes alkaline phosphatase and γ-glutamyl transpeptidase are present in high concentrations and have been used as markers for isolated capillary preparations (225). The choroid plexus also shows very high enrichment of γ-glutamyl transpeptidase (265).

In vessel fractions from rat brain used in this study the activities of alkaline phosphatase and γ-glutamyl transpeptidase marker enzymes

were 14 and 34 times higher than in total homogenates, indicating that our preparations contained mainly capillaries (Table 2). Goldstein et al (232) found enrichment of alkaline phosphatase and γ-Glutamyl transpeptidase to be 15 and 20 times that of crude homogenates, whereas Brendel et al (231) achieved an 8.2 fold enrichment of γ-glutamyl transpeptidase and only 3.8 fold increase of alkaline phosphatase. Others (233,236 ) have also obtained different enrichment factors but all were consistently lower or equal to those obtained with our microvessels. These variations between the different capillary preparations are probably attributable to primary species differences as well as the size differences in the spectrum of microvessels isolated since γ-glutamyl transpeptidase is concentrated mainly in the capillaries of the brain whereas most of the alkaline phosphatase appears to be localized in the larger microvessels (266). The very high enrichment of γ-glutamyl transpeptidase together with the morphology (Fig. 5) indicates that the isolates were mainly the smaller microvessels almost exclusively capillaries.

1. Comparison with arterial tissue

Prostacyclin was the major prostaglandin produced by rat isolated capillaries, choroid plexus and surface vessels such as basilar arteries and arteries of the circle of Willis (Table 3). It is the predominant prostaglandin generated endogenously and from exogenously added endoperoxide in the rat cerebral microvasculature. It was also the principle prostaglandin in peripheral vascular tissue with a comparable production in aortic rings as reported by others (259 ).

Microvessels produced 11 ng of 6-keto- $PGF_{1\alpha}$ /mg protein/10 min and had a capacity of approximately 31% of whole aortic rings (36 ng/mg protein/10 min.). Data from Moncada et al (259), who showed that the aortic intima was responsible for 40% of the synthetic capacity of the whole aortic tissue on a weight basis (1 ng/mg wet weight/5-30 min), in conjunction with the assumption that 10% of tissue weight is protein, is in good agreement with these result (Table 2).

2. Comparison with cultured endothelium

Comparison of the results of prostacyclin biosynthesis in cultured endothelial cells (219) also shows relatively good agreement with that obtained in rat isolated microvessels. Prostacyclin production, as 6-keto $PGF_{1\alpha}$, from arachidonic acid was about twice that of $PGE_2$ production, the second most abundant prostaglandin generated. $PGF_{2\alpha}$ was quantitatively the lowest of the three and $PGD_2$ was not detected. Incubation of cultured endothelial cells with $PGH_2$ for 5 min, revealed a conversion to prostaglandins very similar to that found in isolated microvessels (Table 5). In cultured endothelium, of the total endoperoxide converted to $PGF_{2\alpha}$, $PGE_2$ and 6-keto-$PGF_{1\alpha}$, 26% was 6-keto $PGF_{1\alpha}$, whereas in isolated microvessels it was 24%.

Investigators using cultured cells also reported the presence of $PGD_2$ and HHT when $PGH_2$ was used. HPLC analysis of $PGH_2$ metabolites in isolated capillaries, choroid plexus, buffer (Fig 8), and boiled capillary tissue (Fig 9) also indicated the presence of $PGD_2$, but as in cultured endothelium this was due to the nonenzymatic

Table 5. Comparison of endoperoxide conversion in cultured endothelium and in isolated microvessels

| | Cultured endothelium[a] | | Isolated endothelium[b] | |
|---|---|---|---|---|
| Prostaglandin | + $PGH_2$ | Control | + $PGH_2$ | Control |
| 6-keto-$PGF_{1\alpha}$ | 26 | 2 | 24 | 2.5 |
| $PGE_2$ | 62 | 94 | 46 | 95 |
| $PGF_{2\alpha}$ | 12 | 4 | 30 | 2.5 |

[a] Calculated from Marcus et al, 1978 (219).

[b] Average result of two incubations for 10 min at 37°C with $PGH_2$. All results expressed as relative percentage of $PGH_2$ converted into 6-keto-$PGF_{1\alpha}$, $PGE_2$ and $PGF_{2\alpha}$.

degradation of $PGH_2$ in the presence of protein ie. albumin (267).

Prostaglandin endoperoxides, as is well known, spontaneously decompose into a number of compounds mainly $PGE_2$, $F_{2\alpha}$, $D_2$, and HHT. These studies in cultured endothelial cells and in rat isolated microvessels also demonstrated a minute (< 1%) spontaneous production of 6-keto-$PGF_{1\alpha}$ (Table 4). The larger less polar product in the HPLC profiles when $PGH_2$ was used is probably HHT (Fig 9).

The results summarized in Table 4 indicate that during incubation of $PGH_2$ with both microvessels and choroid plexus, there is an increase in 6-keto-$PGF_{1\alpha}$, (in general larger than that of $PGF_{2\alpha}$), accompanied by a concomitant decrease of $PGE_2$ when compared to controls. This trend was also reported for cultured endothelium (219). Because of the variability between experiments in the recovery of the three major prostaglandins and the effect of albumin on $PGH_2$ breakdown, it is not possible from this data alone to decide whether the presence of the enzymes causes shunting of the endoperoxide from the major nonenzymatic pathway or whether enzymatic conversion is superimposed on the nonenzymatic one.

3. Comparison with other isolated microvessels

Recently other reports of prostaglandin metabolism by isolated microvessels have appeared (250). Maurer et al (253) using isolated bovine microvessels and radioimmunoassay also detected 6-keto-$PGF_{1\alpha}$ as the major prostaglandin but found higher levels of $PGF_{2\alpha}$ than $PGE_2$. In contrast Gerritsen et al. (252) found $PGE_2$ to be the major product from $PGH_2$ when using bovine microvessels but pointed out that this occurred only when the conditions were: low tissue

protein concentrations (<1 mg/ml) and reduced glutathione was present. With higher protein concentrations 6-keto-$PGF_{1\alpha}$ was the major product.

Isolated cat cerebral microvessels (250) showed a 6 fold enrichment of 6-keto-$PGF_{1\alpha}$ when compared to homogenates. These researchers also stated that it was the major prostaglandin formed followed by $PGF_{2\alpha}$ and $PGE_2$. The only report on prostaglandin metabolism in isolated rat cerebral microvessels proposed that $PGD_2$ is the major prostaglandin produced from $PGH_2$ in the cytosol and that although there is synthesis of 6-keto-$PGF_{1\alpha}$ there is no enrichment of $PGI_2$ synthetase in microvessels when compared to whole cortex homogenate (251). In the same study production of 6-keto-$PGF_{1\alpha}$ from $PGH_2$ was less than $PGE_2$ and $PGF_{2\alpha}$. An explanation for the discrepancies observed in Gerritsen's work both in the studies using bovine and rat tissue, as compared to the other studies (250,253,254) including this one which have shown the lack of $PGD_2$ and have unanimously shown prostacyclin to be the major product, is the amount of microvessel protein present in the incubations.

In this study (see methods) 2-10 mg of capillary protein is used while Maurer et al (253) uses 5-10 mg protein and Birkle et al (250) used microvessels isolated from one whole cat brain.

These amounts are in sharp contrast with those used by Gerritsen in both her studies (5-100 μg). The lack of sufficient prostacyclin synthase protein in the incubation mixture is likely since 1. the enzymic activity and relative enrichment of γ-glutamyl

transpepidase is low, 2. there is no prostacyclin synthase enrichment and 3. in the same report 6-keto-$PGF_{1\alpha}$ formation increased significantly at protein concentrations greater than 50 μg and becomes the major product at 200 μg of protein (251).

In summary, prostacyclin is the major prostaglandin produced by cerebral microvessels obtained from cat, ox and rat.

B. Prostacyclin in Choroid Plexus and CSF.

Unlike most other tissues of the body, mammalian brain and its vasculature have a very negligible capacity to accumulate or catabolize prostaglandins (140). No evidence of metabolic transformation of 6-keto-$PGF_{1\alpha}$ to its 15-keto-analogue, 15-keto-13, 14-dihydro analogue or the 6-keto $PGE_1$ compound was found in either brain homogenates or cerebral blood vessels (including choroid plexus) of the rat (146). Due to the high biosynthetic rates of prostaglandins, especially $D_2$ and $F_{2\alpha}$ in brain parenchyma and prostacyclin in brain blood vessels and the lack of the prime pathways for their inactivation, a dynamic neutralization mechanism operable in vivo has been proposed. The stable prostaglandins and the inactive products from the labile eicosanoids ie. 6-keto-$PGF_{1\alpha}$ and $TXB_2$ are not taken up by neurons or glia (159) but are cleared fromthe extracellular fluid by an active transport system across choroidal (158) and extra-choroidal (159) components of the blood-cerebrospinal fluid and blood-brain-barriers.

These acidic lipids are naturally occurring constituents of normal CSF and appear in perfusates of the cerebral cortex (136) from which they reach the venous circulation of the brain and then systemic organs to be metabolized. Ramwell (196) initially detected prostaglandin-like activity in CSF using bioassay.

Prostaglandins $F_{2\alpha}$, $E_2$, $D_2$, 6-keto-$F_{1\alpha}$ (200) and thromboxane $B_2$ (201) have been measured in human CSF from healthy and ill persons. The most abundant prostaglandin was 6-keto-$PGF_{1\alpha}$ (120-1500 pg/ml) and prostaglandin $D_2$ was below the limit of detection (<50 pg/ml) (200). $PGE_2$ is also normally low or undetectable. Thromboxane $B_2$ is present in a concentration of about 400 pg/ml (201). The level of $PGF_{2\alpha}$ in healthy human subjects is usually less than 100 pg/ml (197). The existence of lipoxygenase products such as hydroxyeicosatetraenoic acids and leukotrienes has not yet been demonstrated in CSF.

The assumption that the origin of 6-keto-$PGF_{1\alpha}$ in CSF was the choroid plexus was based on preliminary observation of 6-keto-$PGF_{1\alpha}$ synthesis by this tissue (170). Quantitative measurements of prostaglandins including prostacyclin synthesis by isolated choroid plexus has not been documented. In this study rat choroid plexus incubated for 10 min synthesized 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and $PGE_2$ in amounts of 36.8 ng, 2.6 ng and 1.2 ng per mg protein, respectively (Table 3).

The occurrence of choroidal $PGF_{2\alpha}$ as the second most abundant prostaglandin and the lower amount of $PGE_2$ as well as the absence of $PGD_2$ is consistent with the reports of the trends these prostaglandins exhibit in CSF. The discrepancies in magnitude of PG's in choroid plexus versus CSF are most probably a result of dilution and it is reasonable therefore to assume that human choroid plexus would be capable of synthesis in the nanogram range. In one early study of $PGF_{2\alpha}$ and $PGE_2$ CSF content, it was reported that on the average men had slightly higher $PGF_{2\alpha}$ levels than women and that there was a

significant correlation between CSF $PGE_2$ and the age of the men and CSF $PGF_{2\alpha}$ and the age of the women (198). The fundamental defect of the conclusions reached by these investigators was that some of the CSF used in that study was from patients afflicted with CNS pathology. In the light of more recent evidence of prostaglandin levels in pathological CSF samples, this previous study should be viewed with skepticism or probably completely discounted.

Cerebrospinal fluid is unique in its diagnostic potential concerning diseases of both nervous and vascular tissues of the CNS because of its easy accessibility and varied content. Most substances produced by the brain eg. biogenic amines and their metabolites can be found in the CSF and mirror to some extent the brain's state of health.

Changes in CSF content of various prostaglandins has been observed in a variety of neurological disorders.

Large increases of $PGF_{2\alpha}$ have been documented in patients with epilepsy, inflammatory conditions such as meningitis and meningoencephalitis, and cerebral trauma from accident and surgery (197). Vascular disorders of the CNS such as cerebral and subarachnoid hemorrhage (204), cerebral infarction and ischemia (199), and hydrocephalus (197) are also associated with variable increases of $PGF_{2\alpha}$ and $PGE_2$.

The implication of prostaglandins of the E series in the genesis of fever is based on a number of related observations. One is the high PGE content of CSF observed after pyrogen or endotoxin induced fever (202). Other supporting facts are the antipyretic activity of powerful prostaglandin synthesis inhibitors such as

aspirin and the pyretic effects of PGE's when administered intraventricularly or iontophoresed into the preoptic-anterior hypothalamus (136). A direct cause and effect relationship between prostaglandins and fever, however, has not been proven. Cranston et al (203) contends that augmented levels of $PGE_2$ in CSF is not essential in the genesis of fever but merely a side effect of the pyretic agent. Further studies of prostaglandin in pathogenesis of fever are clearly necessary.

Another unproven association involves an increase of the PGE concentration in CSF of patients with clinically diagnosed schizophrenia (201). It is of interest in this condition that phenothiazines and several other psychotropic agents inhibit the prostaglandin synthetase system (205). These researches also showed no change in $TXB_2$ levels of schizophrenics when compared to controls and stated that their data are inconclusive and more experimental evidence is required.

Only very recently has human CSF been re-examined for the presence of 6-keto-$PGF_{1\alpha}$. Extremely high levels of this compound have been measured by GC-MS in patients who have intracranial hypertension (200). Prostaglandin 6-keto-$F_{1\alpha}$ status in CSF of patients with other cerebrovascular disorders like SAH resulting from ruptured aneurysms and in patients with cerebral stroke has not been evaluated. Considering the localization of this compound to the vasculature, including the choroid plexus, the levels of this stable end product in CSF would act ideally as a general indicator of the integrity of all the cerebral blood vessels. This would complement

a similar function for $PGF_{2\alpha}$ with respect to the brain parenchyma.

When one interprets and compares quantitative data of prostaglandin levels in CSF it must not be forgotten that; 1. there are species differences in prostaglandin synthesis, 2. the anatomical site where CSF is obtained (eg. ventricular, cisterna magna or lumbar portion of the CNS) influences the concentration measured (202), 3. the different assay methods employed, particulary radioimmunoassay, radioisotope dilution and gas-chromatographic-mass spectroscopy, each have their advantages and disadvantages, and most importantly 4. very stringent and reliable criteria must be employed in selecting and defining patients or experimental animals with these various conditions as well as their representative controls.

In the review of CNS diseases associated with changes of these autacoids in the CSF it is evident that although no specific disease entity can be directly linked with increases or decreases of one or more particular prostanoid it will at least narrow and direct our concentration to a specific component of the CNS.

C. Possible Physiological Roles of Prostacyclin in the Cerebrovascular and Cerebroventricular Systems.

The possible contributions of the classical prostaglandins in the functioning of the nervous system have been investigated for some time (136). The roles of prostacyclin in brain have been confined to the cerebral circulation (140) particularly regulation of vascular hemodynamics and prevention of thrombosis.

Prostacyclin may function as a "protective hormone" in regards to cerebral thrombogenic and thromboembolic insult (214). Similarly,

production of prostacyclin by choroid plexus may serve to inhibit thrombosis in the cerebroventricular system (170). It may also act as an antihypertensive agent in the CNS since its production is increased in humans with intracranial hypertension (140) and is also elevated in spontaneously hypertensive rats (206).

Prostaglandins $F_{2\alpha}$ and $E_2$ constrict cerebral vessels (140). The development of migraine headaches may also involve $PGE_2$ (182). Thromboxane $A_2$ formed by platelets, but not cerebral blood vessels, is the most potent vasoconstrictor influencing the cerebral vasculature (176) and has been regarded as the prime, but not only, candidate in the genesis of cerebral vasospasm subsequent to apoplexy and subarachnoid hemorrhage (49). Stable prostacyclin analogues as well as specific thromboxane inhibitors may be of invaluable therapeutic use in these lethal conditions.

Prostacyclin may have a definite role in cerebral blood flow regulation and prevention of ischemia. Findings which add credence to this hypothesis is that $PGI_2$ infusion increases CBF by 71% (184), and indomethacin reduces cerebral blood flow (166) and the only prostaglandin producing vasodilation in cerebral blood vessels is prostacyclin (140). Pickard and his associates (190) have accumulated evidence <u>in vivo</u> that $PGI_2$ is required for hypercapnia to produce thorough cerebral vasodilation. Prostacyclin synthesis in the microcirculation where the majority of the vascular resistance occurs may thus provide a means of maintaining capillary patency thereby controlling regional cerebral perfusion as well as cerebral blood

pressure. The potency, short biological half-life and the site of synthesis make prostacyclin a firm contender for these physiological functions.

As in all biological systems the ultimate outcome of normal and pathological responses to internal or environmental stimuli is a result of innumerable complex factors. Prostacyclin most probably interacts with and complements other vasoactive agents such as $CO_2$, angiotensins and kinins etc, in fulfillment of its biological effects in vivo. Continuing research with more specific prostacyclin inhibitors and stable prostacyclin mimetics will undoubtedly clarify the position of this potent vascular prostanoid in the cerebral circulation.

D. Future Considerations

With the availability of metabolically active isolated cerebral microvessels and isolated choroid plexus, which both demonstrate an active prostacyclin synthesizing system, it will be possible to study biochemical and physical factors affecting $PGI_2$ synthesis in vitro. The effects of various cardiovascular drugs, nitroglycerine, Verapamil etc., on prostaglandin biosynthesis in microvessels would be particularly interesting. Conversely the effect of prostacyclin or stable analogues on other biochemical parameters in microvessels could be investigated.

The capacity to produce or metabolize lipoxygenase products such as the hydroxy fatty acids and leukotrienes could be studied in these preparations. This seems a reasonable proposition since 12-hydroxyeicosatetraenoic acid has been shown to be present in gerbil brain (49), leukotriene C can effect cerebellar Purkinje neurons (49) and enzymes involved in synthesis (Glutathione-S-

transferase) and degradation (γ-glutamyl-transpeptidase) of leukotrienes are present in different amounts in microvessels. Subcellular fractionation of components of microvessels has already been accomplished and studies using the membrane fractions have revealed differences in the enzymology of the luminal versus the abluminal endothelial membranes (238). With purified membrane fractions receptors for peptide hormones and vasoactive drugs can be examined. Prostacyclin, and possibly leukotriene receptors may be discovered on the endothelial membranes of microvessels. Insulin receptors on microvessel membranes have been demonstrated,(239) and the usefulness of these in vitro systems in understanding the etiology and treatment of microangiopathy accompanying advanced diabetes is ready for assessment. Estrogen receptors are known to exist in endothelial cells (215) and with these microvessels prostacyclin-hormone interactions can be probed. Basement membranes from microvessels have also been analysed (225). The potential of isolated microvessel preparations in the study of the anatomical and biochemical functions of the blood-brain-barrier is unlimited.

## Claims to Original Research

Prostacyclin was shown quantitatively to be the principle prostaglandin produced endogenously and from exogenous PG endoperoxide by rat cerebral microvessels and choroid plexus. It was also discovered that prostaglandin $E_2$ was the second major prostanoid produced in microvessels, a result which is in agreement with data from cultured endothelial cells. Choroid plexus however demonstrated greater amounts of $PGF_{2\alpha}$ which is also consistent with the higher levels of $PGF_{2\alpha}$ versus $PGE_2$ detected in CSF. Prostaglandin $D_2$ was not detected in either tissue.

REFERENCES

1. Kurzrok R. and Lieb C.C., Proc. Soc. Exptl. Biol. Med., 28:268-272, 1930.

2. Goldblatt M.W., J. Soc. Chem. Ind.(London), 52:1056-1057, 1933.

3. Euler, Von U.S., Arch. Exptl. Pathol. Pharmacology, 175:78-84, 1934.

4. Euler, Von U.S., Klin. Wschr., 14:1182-1183, 1935.

5. Bergstrom S., Carlson L.A. and Weeks J.R., Pharmacol. Rev., 20: 1-48, 1968.

6. Bergstrom S., Ryhage R., Samuelsson B., and Sjovall, H., Acta. Chem. Scand., 16:501-502, 1962.

7. Bergstron S. et al., Biochem. Biophys. Acta., 90:207-210, 1964.

8. Van Dorp D.A. et al., Biochem. Biophys. Acta., 90:204-207, 1964.

9. Bindra J.S. and Bindra R., Prostaglandin Synthesis, Academic Press, New York, 1977.

10. Vane J.R., Nature (New Biol.), 231:232-235, 1971.

11. Samuelsson B., J. Am. Chem. Soc., 87:3011-3013, 1965.

12. Hamberg M. and Samuelsson B., Proc. Natl. Acad. Sci. U.S.A., 70:899-903, 1973.

13. Nugteren D.H. and Hazelhof E., Biochem. Biophys. Acta., 326:448-461, 1973.

14. Hamberg M., Svensson J., Wakabayashi T. and Samuelsson B., Proc. Natl. Acad. Sci. U.S.A., 71:345-349, 1974.

15. Ferreira S.H. and Vane J.R., Nature (London), 216:866-873, 1967.

16. Piper P.J. and Vane J.R., Nature (London), 223:29-35, 1969.

17. Vargaftig B.B. and Dao N., Pharmacol., 6:99-101, 1971.

18. Vargaftig B.B. and Zirinis P., Nature (New Biol.), 244:144-116, 1973.

19. Hamberg M., Svensson J. and Samuelsson B., Proc. Nathl. Acad. Sci., U.S.A., 72:2994-2998, 1975.

20. Svensson J., Hamberg M. and Samuelsoon B., Acta. Physiol. Scand., 94:222-226, 1975.

21. Needleman P., Moncada S., Bunting S., Vane J.R., Hamberg M. and Samuelsson B., Nature (New Biol.), 261:558-559, 1976.

22. Falardeau P., Hamberg M. and Samuelsson B., Biochem. Biophys. Acta., 441:193-197, 1976.

23. Needleman P., Raz A., Minkes M.S., Ferrendelli J.A. and Sprecher H., Proc. Natl. Acad. Sci. U.S.A., 76:944-948, 1979.

24. Pace-Asciak R.C. and Wolfe L.S., Biochemistry, 10:3657-3664, 1971.

25. Moncada S., Gryglewski R., Bunting S., and Vane J.R., Nature (London) 263:663-665, 1976.

26. Moncada S., Higgs E.A. and Vane Jr., Lancet, 1:18, 1977.

27. Gryglweski R.J., Bunting S., Moncada S., Flower R.J. and Vane J.R., Prostaglandins, 12:685-714, 1976.

28. Johnson R.A. et al, Prostaglandins, 12:915-928, 1976.

29. Pace-Asciak C.R., Experientia, 32:291-292, 1966.

30. Pace-Asciak C.R., J. Am. Chem. Soc., 98:2348-2349, 1976.

31. Pace-Asciak C.R. and Carrara M.C., Prostaglandins, 16: 397-410, 1978.

32. Dyerberg J. and Bang H.O., Scand. J. Clin. Lab Invest., 40:589-593, 1980.

33. Granstrom E., in Advances in Prostaglandin and Thromboxane Research, Vol. 6, (Samuelsson B., Ramwell P. and Paoletti R., eds.), Raven Press, New York, 1980, pp. 69-76.

34. Granström E., Prostaglandins, 15: 3-17, 1979.

35. Granstrom E. and Kindahl H., in Advances in Prostaglandin and Thromboxane Research. Vol. 5 (Frolich J.C. ed.), Raven Press, New York, 1978, pp. 119-125.

36. Axen U., Green K., Horlin D. and Samuelsson B., Biochem. Biophys. Res. Commun., 45:519-524, 1971.

37. Pace-Asciak C.R., Anal. Biochem., 81:251-255, 1977.

38. Russel F.A. and Deykin D., Prostaglandins, 18:11-18, 1979.

39. Powell W.S., Prostaglandins, 20:947-957, 1980.

40. Hamberg M. and Samuelsson B., Proc. Natl. Acad. Sci, U.S.A., 71: 3400-3404, 1974.

41. Nugteren H., Biochem. Biophys. Acta., 380:299-307 , 1975.

42. Borgeat P., Hamberg M. and Samuelsson B., J. Biol. Chem., 251: 7816-7820, 1976.

43. Goetzl E.J. and Sun F.F., J. Exp. Med., 150:406-411, 1979.

44. Borgeat P. and Samuelsson B., Proc. Natl. Acad. Sci., U.S.A., 76:2148-2152, 1979.

45. Borgeat P. and Samuelsson B., J. Biol. Chem., 254:2643-2646, 1979.

46. Jubiz W., Radmark O., Lindgren J.A., Malmsten C., and Samuelsson B., Biochem. Biophys. Res. Commun., 99:976-986, 1981.

47. Bryant R.W. and Bailey, J.M., Prostaglandins, 17:9-18, 1979.

48. Boeynaems J.M. and Hubbard W.C., J. Biol. Chem., 255:9001-9004, 1980.

49. Wolfe L.S., J. Neurochem., 38:1-14, 1982.

50. Samuelsson B., Borgeat P., Hammarstrom S. and Murphy R.C., Prostaglandins, 17:785-787, 1979.

51. Borgeat P. and Samuelsson B., Proc. Natl. Acad. Sci., U.S.A., 76:3213-3217, 1979.

52. Feldberg W. and Kellaway C.H., J. Physiol., 94:187-226, 1939.

53. Kellaway C.H. and Trethewie E.R., Q.J. Exp. Physiol, 30:121-145, 1940.

54. Brocklehurst W.E., Ciba Symposium on Histamine, Chruchill, London, 1956.

55. Brocklehurst W.E., Prog. Allergy, 6:539-558, 1962.

56. Orange R.P. and Austen K.F., Adv. Immun., 10:105-144, 1969.

57. Orange R.P. and Austen K.F., Progr. in Immunol., 173-186, 1971.

58. Augstein J., Farmer J.B., Lee T.B., Sheard P. and Tattersall M.L., Nature (New Biol.) 245:215-217, 1973.

59. Orange R.P., Murphy R.C., Karnovsky M.L. and Austen K.F., J. Immunol., 110:760-770, 1973.

60. Orange R.P., Murphy R.C. and Austen K.F., J. Immunol., 113:316-322, 1974.

61. Walker J.L., Adv. Biosci., 9:235-240, 1973.

62. Jakschik B.A., Falkenhein S., and Parker C.W., Proc. Natl. Acad. Sci., U.S.A., 74:4577-4581, 1977.

63. Morris H.R., Taylor G.W., Piper P.J., Sirois P. and Tippins J.R., FEBS Letters, 87:203-206, 1978.

64. Sirois P., Prostaglandins, 17:395-404, 1979.

65. Bach M.K. and Brashler J.R., Life Sci. 23:2119-2126, 1978.

66. Murphy R.C., Hammarstrom S., and Samuelsson B., Proc. Natl. Acad. Sci, U.S.A., 76:4275-4279, 1979.

67. Hammarstrom S., Murphy R.C., Samuelsson B., Clark, D.A., Mioskowski C. and Corey E., Biochem. Biophys. Res. Commun., 91:1266-1272, 1979.

68. Morris H.R., Taylor G.W., Piper P. J., Samboun M.N. and Tippins J.R., Prostaglandins, 19:185-201, 1980.

69. Samuelsson B., Trends Pharmacol, Sci., 1:227-230, 1980.

70. Dahlen S.E., Hedgvist P., Hammarstrom S., and Samuelsson B., Nature, 288:484-486, 1980.

71. Samuelsson B., Proc. V International Conference on Prostaglandins, Raven Press, New York, p1, 1982.

72. Christ E.J. and Van Dorp D.A., Biochem. Biophys. Acta., 270:537-545, 1972.

73. Pace-Asciak C.R. and Rangaraj G., Biochem. Biophys. Acta, 486, 579-582, 1977.

74. Sun F.F., Chapman J.P. and McGuire J.C., Prostaglandins, 14:1055-1074, 1977.

75. Borgeat P. and Sirois P., J. Med. Chem., 24:121-126, 1981.

76. Coleman R.A. et al, Br. J. Pharmacol., 69:265P-267P, 1980.

77. Schafer A.I., Cooper B., O'Hara D. and Handin R.I., J. Biol. Chem., 254:2914-2917, 1979.

78. Jouvenaz G.N., Nugteren D.N., Berrthuis, R.K., and Van Dorp D.A., Biochem. Biophys. Acta., 202:231-234, 1970.

79. Wolfe L.S., in Basic Neurochemistry, (Siegel G.J., Albers R.W., Agramoff B., and Katzman R., eds) 3rd ed., Little, Brown and Co., Chapt. 16, 1981.

80. McGiff J.C., Ann. Rev. Pharmacol Toxicol., 21:479-509, 1981.

81. Lands W.E.M., Ann Rev. Physiol., 41:633-652, 1979.

82. Bell R.L., Kennerly D.A., Stanford N., and Majerns P.W., Proc. Natl. Acad. Sci. U.S.A., 76:3238-3241, 1979.

83. Rousseau A. and Gatt S., J. Biol. Chem., 254:7741-7745, 1979.

84. Kuehl F.A. Jr., Prostaglandins, 5:325-340, 1974.

85. Hirata F. and Axelrod J., Science, 209:1082-1090, 1980.

86. Blackwell G.J., Flower R.J., Nijkamp F.P., and Vane J.R., Br. J. Pharmacol., 62:79-89, 1978.

87. Hong S.C. and Levine L., Proc. Natl., Acad. Sci., U.S.A., 73:1730-1734, 1976.

88. Flower R.J. and Blackwell G.J., Nature 278:456-458, 1979.

89. Samuelsson B., Goldyne M., Granstrom E., Hamberg, M., Hammarstrom S. and Malmsten C., Ann. Rev., Biochem., 47:997-1029. 1978.

90. Lysz T.W. and Needleman P., J. Neurochem., 38:1111-1117, 1982.

91. Miyamoto T., Ogino N., Yamamotos S, and Hayaishi O., J. Biol. Chem., 251:2629-2636, 1976.

92. Hemler M., Lands W.W.M., and Smith W.L., J. Biol. Chem., 251: 5575-5579, 1976.

93. Lands W.E.M., Ann, Rev. Physiol., 41:633-652, 1979.

94. Pace-Asciak C. and Wolfe L.S., Biochem. Biophys. Acta., 152: 784-787, 1968.

95. Gryglewski R.J., in Prostaglandin Synthetase Inhibitors, (Robinson H. J. and Vane J.R., eds), Raven Press, New York, p. 33, 1974.

96. Roth G.J. and Majerus P.W., J. Clin. Invest., 56:624-626, 1975.

97. Livio M., Villa S., and deGaetano G., Lancet, 1307, 1978.

98. Hensby C.M., Biochem. Biophys. Acta., 409:225-324, 1975.

99. Dunn M.J. and Hodd V.L., Am. J. Physiol., 233:F169-F184, 1977.

100. Pace-Asciak C., J. Biol. Chem., 250:2795-2800, 1975.

101. Hammarstrom S. and Falardeau P., Proc. Natl. Acad. Sci., U.S.A., 74:3691-3695, 1977.

102. Yoshimoto T., Yamamoto S., Okuma M., and Kayaishi O., J. Biol. Chem., 252:5871-5874, 1977.

103. Pace-Asciak C.R. and Rangaraj G., Biochem. Biophys. Acta., 486-579-582, 1977.

104. Olley P.M. and Goceani F., Ann. Rev. Med., 32:375-385, 1981.

105. Vane J.R. and Bergstrom S. (eds.), Prostacyclins, Raven Press, New York, 1979.

106. Chang W.C., Nurota S.I., Nakao J. and Orimo H., Biochem. Biophys. Acta., 620:159-166, 1980.

107. Chang W.C. et al, Biochem. Biophys. Acta, 620:472-482, 1980.

108. Pace-Asciak C.R. and Nashat M., Biochem. Biophys. Acta., 487:495-507, 1977.

109. Huang F.C., Zmijewski M., Girdaukas G. and Sih C.J., Bioorg. Chem., 6:311-322, 1977.

110. Egan E.A., Egan R.W., Soderman D., Gale P.H. and Kuehl F.A., J. Biol. Chem., 254:2191-2194, 1979.

111. McGuire J.C. and Sun, F.F., Arch. Biochem. Biophys., 189:92-96, 1978.

112. Wong P.Y.K., McGiff J.C., Cagen L., Malik K.U. and Sun F.F., J. Biol. Chem., 254:12-14, 1979.

113. Forsterman U., Neufang B. and Hertting G., Prostaglandins, 22:915-932, 1981.

114. Pace-Asciak C.R., Carrara M.C. and Domazet Z., Biochem. Biophys. Res., Commun., 78:115-121, 1977.

115. Rosenkraz B., Fischer C., Reimann I., Weimer K.E., Beck G. and Frolich J.C., Biochem. Biophys. Acta., 619:207-213, 1980.

116. Salmon J.A., Smith D.R. and Cotee F., Prostaglandins, 17:747-752, 1979.

117. Wong P.Y.K., Sun F.F. and McGiff J.C., J. Biol. Chem., 253: 555-5557, 1978.

118. Abdel-Halim M.S., Lundén I., Cseh G. and Anggard E., Prostaglandins, 19:249-257, 1980.

119. Wong P.Y.K., Lee W.H., Quilley C.P. and McGiff J.E., Fed. Proc., 40:2001-2004, 1981.

120. Vane J.R., in Advances in Prostaglandin and Thromboxane Research, (Coceani F. and Olley P.M., eds.), Vol. 4, pp. 27-44, Raven Press, New York, 1978.

121. Hong S.L., Carty T. and Deykin D., J. Biol. Chem., 255:9538-9540, 1980.

122. Lewis P.J. and O'Grady J., eds., Clinical Pharmacology of Prostacyclin, Raven Press, New York 1981, 258 pp.

123. Robbins S.L. and Cotran R.S., Pathologic Basis of Disease, W.B. Saunders Co., Toronto, 1979.

124. Hamberg N. and Samuelsson B., J. Biol. Chem., 242:5329-5335, 1967.

125. Sun F.F., McGuire J.C., Wallach D.P. and Brown V.R., In Advances in Prostaglandin and Thromboxane Research, (Samuelsson B., Ramwell P.W. and Paoletti R., eds.) Vol. 6, p. 111; Raven Press, N.Y., 1980.

126. Siegel M.I., McConnell R.T., Porter N.A., Cuatrecasas P., Proc. Natl. Acad. Sci., U.S.A., 77:308-312, 1980.

127. Jaschik B.A., Sams A.R., Sprecher H., Needleman P., Prostaglandins, 20:401-409, 1980.

128. Morris Hr. et al, in SRS-A and Leukotrienes, (Piper P.J., ed.) p. 19, John Willey and Sons, New York, 1981.

129. DeBault L.E. and Cancilla P.A., Science, 207:653-655, 1980.

130. Albert A., Orlowski M., Rzucidlo A. and Orlowska J., Acta. Histochem., 25:312-320, 1966.

131. Griffith O.W., Bridges R.J. and Meister A., Proc. Natl. Acad. Sci., U.S.A., 76:6319-6322, 1979.

132. Swinnen J., Clin. Chem. Acta, 17:255-260 , 1967.

133. Szasz G., Clin. Chem., 15:124-130, 1969.

134. Hughey R.P. and Curthoys N.P., J. Biol.Chem., 251:7863-7870, 1976.

135. Orning L. and Hammarstrom S., J. Biol. Chem., 255, 8023-8026, 1980.

136. Wolfe L.S., in Advances in Neurochemistry, (Agranoff B.W. and Aprison M.H., eds.), Vol. 1, p.1-49, New York, Plenum, 1975.

137. Wolfe L.S., see Ref. 120 , pp. 215-220, 1978.

138. Coceani F., in Brain Dysfunction in Infantile Febrile Convulsions, (Brazier M.A.B. and Coceani F., eds.) Raven Press, New York, pp. 55-67.

139. Coceani F., Arch. Int. Med., 133:119-129, 1974.

140. Wolfe L.S. and Coceani F., Ann. Rev. Physiol., 41:669-684, 1979.

141. Samuelsson B., Biochem. Biophys. Acta., 84:218-219, 1964.

142. Horton E.W. and Main I.H.M., Br. J. Pharmacol., 30:582-602, 1967.

143. Holmes S.W. and Horton E.W., J. Physiol. (London) 195:731-741, 1968.

144. Wolfe L.S., Rostworowski K. and Marion J., Biochem. Biophys. Res. Commun., 70:907-913, 1976.

145. Abdel-Halim M.S., Lunden I, Cseh G. and Anggard E., Prostaglandins, 19:249-257, 1980.

146. Abdel-Halim M.S. and Anggard E., Prostaglandins, 17:411-418, 1979.

147. Abdel-Halim M.S., Hamberg M., Sjoguist B. and Anggard E., Prostaglandins, 14:633-643, 1977.

148. Cseh G., Szabo I.K., Lang T. and Palkovits M., Brain Res. Bull. 3:293-297, 1978.

149. Kataoka K., Ramwell P.W. and Jessup S., Science 157:1187-1189, 1967.

150. Wolfe L.S., Pappius H.M. and Marion J., In Advances in Prostaglandin and Thromboxane Research, (Samuelsson B. and Paoletti R., eds.), Vol. 1, pp. 345-355, Raven Press, New York, 1976.

151. Marion J. and Wolfe L.S., Biochem. Biophys. Acta., 574:25-32, 1979.

152. Nakano J., Prancan A.V. and Moore S.E., Brain Res., 39:545-584, 1972.

153. Tse J. and Coceani F., Prostaglandins, 17:71-77, 1979.

154. Lee S.C. and Levine L., J. Biol. Chem. 250:548-552, 1975.

155. Anggard E., Larsson C., and Samuelsson B., Acta. Phys. Scand., 81:396-404, 1971.

156. Lee S.C. and Levine L., J. Biol. Chem., 249:1369-1375, 1974.

157. Wolfe L.S., Rostworowski K. and Pappius H.M., Can. J. Biochem., 54:629-640, 1976.

158. Bito L.Z. and Davson H.J., J. Physiol. (London), 236:39P-40P, 1974.

159. Bito Z., Davson H. and Hollingsworth J.R., J. Physiol. (London), 256:273-285, 1976.

160. Hedqvist P., in The Prostaglandins, (Ramwell P.W., ed.), Vol. 1: 101-132, Plenum Press, New York, 1973.

161. Mills D.C.B. and MacFarlane D.E., Thromb. Res., 5:401-412, 1974.

162. Kebabian J.W., In Advances in Cyclic Nudeotide Research, (Greengard P. and Robinson G.A., eds.), Vol. 8, pp. 421-508, Raven Press, New York, 1977.

163. Tateson J.E., Moncada S., and Vane J.R., Prostaglandins, 13:389-395, 1977.

164. Potts W.J., East P.F. and Mueller R.A., Prostaglandins, 3:145-167, 1977.

165. Terragno D.A., Crowshaw K., Terragno N.A. and McGiff J.C., Circ. Res., 36, 37:Suppl. 1, 76-80, 1975.

166. Pickard J.D., Vinall P.E. and Simeone F.A., Surg. Forum, 26:496-498, 1975.

167. White R.P., Terragno D.A., Terragno N.A., Hagen A.A. and Robertson J.T., Stroke 8:135-139, 1977.

168. Hagen A.A., White R.P., Terragno N.A. and Robertson J.T., Fed. Proc., 37:384-391, 1978.

169. Hagen A.A., White, R.P., and Robertson J.T., Stroke 10:306-309, 1979.

170. Abdel-Halim M.S., Von Holst H., Meyerson B., Sachs C. and Anggard E., J. Neurochem., 34:1331-1333, 1980.

171. Rosenblum W.I., Stroke, 6:293-297, 1975.

172. Yamamoto Y.L., Feindel W., Wolfe, L.S., Ketoh H. and Hodge C.P., J. Neurosurg. 37:385-397, 1972.

173. Yamamoto Y.L., Feindel W., Wolfe L.S. and Hodge C.P., Adv. in Biosci., 9:359-367, 1973.

174. Ellis E.F., Wei E.P. and Kontos H.A., Am. J. Physiol. 237:H381-H385, 1979.

175. White R.P., Hagen A. and Robertson J.T., J. Neurosurg. 44:45-49, 1976.

176. Ellis E.F., Nies A.S. and Oates J.A., Stroke 8:480-483, 1977.

177. Boullin D.J., Bunting S., Blaso W.P., Hunt T.M. and Moncada S., Br. J. Pharmacol., 7:139-147, 1979.

178. Chaplean C.E. and White R.P., Prostaglandins, 17:573-580, 1979.

179. Chapleau C.E., White R.P. and Robertson J.T., J. Neurosurg., 53: 188-192, 1980.

180. Sasaki T., Wakai S., Asano T., Watanabe T., Kirino T. and Sano K., J. Neurosurg., 54:357-365, 1981.

181. Pickard J.D., MacDonell L.A., MacKenzie E.T. and Harper A.M., Eur. J. Pharmacol., 43:343-351, 1977.

182. Bruyn G.W., in Clinical Neuropharmacology, (Klawans H.L., ed.), Vol. 1, pp. 185-213, Raven Press, New York, 1976.

183. Raskin N.H., Ann. Rev. Pharmacol. Toxicol, 21:463-478, 1981.

184. Pickard J., Tamura A., Stewart M., McGeorge A. and Fitch W., Brain Res., 197:425-431, 1980.

185. Kontos H.A., Ann. Rev. Physiol., 43:397-407, 1981.

186. Larsen B. and Lassen N.A., in Adv. in Neurol., (Goldstein M. et al. eds.), Vol. 25, pp. 7-22, Raven Press, New York, 1979.

187. Scheinberg P., in The Peripheral Circulations, (Zelis R., ed.) pp. 151-162, Grune and Stratton, New York, 1975.

188. Brozek J. and Simonson E., eds., The Physiological Mechanisms of Cerebral Blood Circulation, Charles C. Thomas, Ill., U.S.A., 1970, 115 pp.

189. McHenry Jr., L.C., Cerebral Circulation and Stroke, W.H. Green, St. Louis, U.S.A., 1978, 269 pp.

190. Pickard J.D. and MacKenzie E.T., Nature (New Biol.), 245:187-188, 1973.

191. Sakabe T. and Siesjo B., Acta. Physiol. Scand., 107:283-284, 1979.

192. Kontos H.A., Wei E.P., Povlishock J.T., Dietrich W.D., Magiera C.J. and Ellis E.F., Science, 209:1242-1245, 1980.

193. Rebollo M.A. and Casas M., Acta. Neurol. Latinoamer., 6:331-339, 1960.

194. Davson H., Physiology of the Cerebrospinal Fluid, Churchill, 1967.

195. Wright E.M., Rev. Physiol. Biochem. Pharmacol., 83:1-34, 1978.

196. Ramwell P.W., J. Physiol. (London), 170:21-38, 1964.

197. Wolfe L.S. and Mamer O.A., Prostaglandins, 9:183-192, 1975.

198. Aizawa Y. and Yamada K., Prostaglandins, 11:43-50, 1976.

199. Egg D., Herold M., Rumple E. and Gunther R., J. Neurol., 222: 239-248, 1980.

200. Abdel-Halim M.S., Ekstedt J. and Anggard E., Prostaglandin, 17:409, 1979.

201. Mathé A.A., Wiesel F.A., Sedvall G., and Nyback H., Lancet 1: 16-17, 1980.

202. Bernheim H.A., Gilbert T.M. and Stitt J.J., J. Physiol., 301: 69-78, 1980.

203. Cranston W.I. et al, J. Physiol. (London), 259:239-249, 1976.

204. Hagen A.A., Gerber J.N., Swelley C.C., White R.P. and Robertson J.T., Stroke 8: 672-675,1977.

205. Flower R.J., Pharmacol. Rev., 26:33-67, 1974.

206. Pace-Asciak C.R., Carrara M.C., Rangaraj G. and Nicolaou K.C., Prostaglandins, 15:1005-1009, 1978,

207. Botha J.H., Leary W.P. and Asmal A.C., Prostaglandins, 19:285-289, 1980.

208. Okuma M., Yamori Y., Ohta K. and Uchino H., Prostaglandins 17: 1-7, 1979.

209. Weeks J.R. and Compton L.D., Prostaglandins, 17:501-513, 1979.

210. Needleman P. et al., J. Clin. Invest., 61:839-849, 1978.

211. Marcus A.J., J. Lipid Res. 19:793-826, 1978.

212. Mustard J.F., Kinlough-Rathbone R.L., and Packham M.A., Ann. Rev. Med. 31:89-96, 1980.

213. Moncada S. and Vane J.R., N.E.J.M., 300:1142-1147, 1979.

214. Moncada S. and Vane J.R., Br. Med. Bull., 34:129-135, 1978.

215. Altura B.M., ed., Vascular Endothelium and Basement Membranes, Adv. in Microcirculation, Vol. 9, S. Karger, Basel, 1980, 338 pp.

216. Gimbrone Jr. M.A. and Alexander R.W., Science, 189:219-220, 1975.

217. Weksler B.B., Marcus A.J. and Jaffe E.A., Proc. Natl. Acad. Sci., 74:3922-3926, 1977.

218. Macintyre D.E., Pearson J.D. and Gordon J.L., Nature, 271: 549-551, 1978.

219. Marcus A.J., Weksler B.B. and Jaffe E.A., J. Biol. Chem., 253:7138-7141, 1978.

220. Weksler B.B., Ley C.W. and Jaffe E.A., J. Clin. Invest., 62:923-930, 1978.

221. Marcus A.J., Weksler B.B. and Jaffe E.A., in Ref. 105, pp. 65-76.

222. Jaffe E.A. and Weksler B.B., J. Clin. Invest., 63:532-535, 1979.

223. Saba S.R., Zucker W.H. and Mason R.G., Ser. Haemat. 6:456-468, 1973.

224. Buonassisi V. and Vanter J.C., Proc. Natl. Acad. Sci. 73:1612-1616, 1976.

225. Eisenberg H.M. and Suddith R.L. eds., The Cerebral Microvasculature, Adv. in Exp. Med. Biol., Vol. 131, Plenum Press, New York, 1980, 337 pp.

226. Matheson D.F., Oei R. and Roots B.I., Neurochem. Res., 5:43-59, 1980.

227. Williams S.K., Gillis J.F., Mattews M.A., Wagner R.C. and Bitensky M.W., J. Neurochem., 35:374-381, 1980.

228. Karnushina I.L., Palacios J.M., Barbin G., Dux E., Joo F. and Schwartz J.C., Neurochem., 34:1201-1208, 1980.

229. Siakotos A.N., Rouser G., and Fleischer S., Lipids 4:235-239, 1969.

230. Joo F. and Karnushina I., Cytobios, 8:41-48, 1973.

231. Brendel K., Meezan E., and Carlson E., Science 185:953-955, 1974.

232. Goldstein G.W., Wolinsky J.S., Csetey J. and Diamond I., J. Neurochem., 25:715-717, 1975.

233. Mrsulja B.B., Mrsulja B.J., Fujimoto T., Klatzo I. and Spatz M., Brain Res., 110:361-365, 1976.

234. Selvionchick D.P. and Boots B.I., Lipids 12:165-169, 1977.

235. Hjelle J.T., Baird-Lambert J., Cardinale G., Spector S. and Udenfriend S., Proc. Natl. Acad. Sci., U.S.A. 75:4544-4548, 1978.

236. Djuricic B.M., Rogac Lj., Spatz M., Rakic Lj.M. and Mrsulja B.B., In Advances in Neurology, (Cervos-Navarro J. et al eds.), Vol. 20:197-205, Raven, 1978.

237. Betz A.L. and Goldstein G.W., Science, 202:225-227, 1978.

238. Betz A.A., Firth J.A. and Goldstein G.W., Brain Res., 192: 17-28, 1980.

239. Frank H.T.L. and Pardridge W.M., Diabetes, 30:757-761, 1981.

240. Brecher P., Tercyak M.A. and Chobanian A.V., Hypertension, 3: 198-204, 1981.

241. Wu P.H. and Phillis J.W., J. Neurochem., 38:687-690, 1982.

242. Hwang M.S., Weiss S. and Segal S., J. Neurochem., 35:417-424, 1980.

243. Kolber A.R., Bagnell R.C., Krigman M.R., Hayward J. and Morell P., J. Neurochem., 33:419-432, 1979.

244. Goldstein G.W., J. Physiol. 286:185-195, 1979.

245. Eisenberg H.M. and Suddith R.L., Science, 206:1083-1085, 1979.

246. Kowaloff H., Gavras H. and Brecher P., Am. J. Physiol., 238: H384-H387, 1980.

247. Lai F.M., Udenfriend S. and Spector S., Proc. Natl. Acad. Sci., U.S.A., 72:4622-4625, 1975.

248. Hardebo J.E. and Owen C., Acta. Physiol. Scand., 108:223-230, 1980.

249. Reinhard Jr., J.F., Liebman J.E., Schlosberg A.J., Morkowitz M.A., Science, 206:85-87, 1979.

250. Birkle D.L., Wright K.F., Ellis C.K. and Ellis E.F., Prostaglandins, 21:865-877, 1981.

251. Gerritsen M.E., and Printz M.P., Prostaglandins, 22:553-566. 1981.

252. Gerritsen M.E., Parks T.P. and Printz M.P., Biochem. Biophys. Acta., 619:196-206, 1980.

253. Maurer P., Moskowitz M.A., Levine L., and Melamed E., Prostaglandins and Medicine 4:153-161, 1980.

254. Goehlert U.G., Ng Ying Kin N.M.K. and Wolfe L.S., J. Neurochem., 36:1192-1201, 1981.

255. Sommer A.J., Am. J. Med. Tech., 20:244-253, 1954.

256. Naftalin L., Sexton M., Whitaker J.F., and Tracey D., Clin. Chem. Acta., 26:293-296, 1969.

257. Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J., J. Biol. Chem., 193:265-275, 1951.

258. Pace-Asciak C.R. and Nashat M., Biochem. Biophys. Acta., 388: 243-253, 1975.

259. Moncada S., Herman A.G., Higgs, E.A. and Vane J.R., Thromb. Res., 11:323-344, 1977.

260. Shimizu T., Mizuno N., Amano T., and Hayaishi O., Proc. Natl. Acad. Sci., U.S.A., 76:6231-6234, 1979.

261. Armstrong J.M., Lattimer N., Moncada S. and Vane J.R., Br. J. Pharmacol. 62:125-130, 1978.

262. Skidel R.A. and Printz M.P., Prostaglandins, 16:1-16, 1978.

263. Gryglweski R.J., Korbut R. and Ocettkiewicz A.C., Nature, 273: 765-767, 1978.

264. Gryglweski R.J., Biochem. Pharmacol., 28:3161-3166, 1979.

265. Sessa G., Orlowski M. and Green J.P., J. Neurobiol. 7:51-61, 1976.

266. Torack R.M. and Barnett R.J., J. Neuropath. Exp. Neurol. 23:46-59, 1964.

267. Hamberg M. and Fredholm B.B., Biochem. Biophys. Acta, 431:189-193, 1976.