DRUG AND DIETARY MODIFICATION OF PLATELET FUNCTION

Nanthakumaran Chetty

A thesis submitted to the Faculty of Medicine
University of the Witwatersrand, Johannesburg,
for the Degree of Doctor of Philosophy

JOHANNESBURG 1985.

ABSTRACT

A study of platelet aggregation and MDA production after an oral dose of 300mg aspirin indicated that partial recovery of platelet function occurred when approximately one third of the circulating platelets had been replaced by new (uninhibited) platelets. *In vitro* studies on mixtures of normal and aspirin inhibited platelets indicated partial restoration of platelet aggregation and thromboxane B_2 production with as little as 10% of normal platelets in some subjects. Restoration of full function required a higher proportion of normal platelets. There was considerable variation between subjects. These data suggest that complete suppression of platelet functions in all normal subjects requires daily administration of the drug.

Despite the hyperaggregability of platelets in Familial Hypercholesterolaemia, 150 mg aspirin was shown to inhibit platelet aggregation to the same extent as occurs in normals.

Theoretical considerations suggest that an ideal dose of aspirin would be one that inhibits platelet aggregation without affecting prostacyclin production by vascular endothelium and thus causing a shift in the

TXA_2:PGI_2 balance. A dose of 40mg may be too high since daily doses may be cumulative and suppress both PGI_2 and TXA_2. Although daily doses of 20mg aspirin inhibits thromboxane production with no effect on vascular prostacyclin this dose does not adequately inhibit platelet aggregation. Therefore when monitoring threshold doses for low dose aspirin, platelet aggregation tests seem to be mandatory.

The concept of a low dose of aspirin that would discriminate between the inhibitory effects on platelets and on the endothelium, although attractive, may well be illusory.

Dietary manipulation of platelet fatty acids, which is an attractive alternative to pharmacological suppression of platelet function, revealed the following: After daily ingestion of 300-400g sardines, pilchards, herring and/or kabeljou which supplied 1-4g EPA and elimination of AA containing foods - the ratio of eicosapentaenoic EPA to arachidonic acid (AA) in the platelet increased. This was accompanied by a decrease in platelet aggregation to ADP, epinephrine, collagen and a decrease in thromboxane B_2 production. Serum and HDL cholesterol levels fell significantly but LDL and triglyceride values did not change. Elimination of dietary AA without addition of

EPA did not inhibit platelet aggregation or thromboxane production. Changes in platelet function were therefore attributed to the increase in dietary EPA.

Dietary supplementation with 1,8g MaxEPA showed no reduction in platelet aggregation responses despite an increase in platelet EPA. Bleeding times were prolonged in the absence of any change in platelet function. Serum HDL cholesterol was increased and triglycerides reduced. These findings suggest that the mechanism by which EPA-rich fish oil influences primary haemostasis requires re-evaluation. This study remains compatible with the proposition that dietary fish or fish oil delays primary haemostasis and influences serum lipids, and may decrease the incidence of arterial thrombosis in the human population. The mechanism of this effect has not yet been determined.

EPA did not inhibit platelet aggregation or thromboxane production. Changes in platelet function were therefore attributed to the increase in dietary EPA.

Dietary supplementation with 1,8g MaxEPA showed no reduction in platelet aggregation responses despite an increase in platelet EPA. Bleeding times were prolonged in the absence of any change in platelet function. Serum HDL cholesterol was increased and triglycerides reduced. These findings suggest that the mechanism by which EPA-rich fish oil influences primary haemostasis requires re-evaluation. This study remains compatible with the proposition that dietary fish or fish oil delays primary haemostasis and influences serum lipids, and may decrease the incidence of arterial thrombosis in the human population. The mechanism of this effect has not yet been determined.

This is to certify that the thesis

"DRUG AND DIETARY MODIFICATION OF PLATELET FUNCTION"

presented for the degree of Doctor of Philosophy to the University of the Witwatersrand is my own work, and has not been presented for a degree at any other University.

N.I. Chetty

Nanthakumaran Chetty

M.Sc. (Witwatersrand).

November, 1985.

Portions of the work included in this dissertation have been published in the following papers:-

BRADLOW, B.A. and CHETTY, N. Dosage frequency for the suppression of platelet function by low dose aspirin therapy. Thromb. Res. 27: 99-110, 1982.

SHIRES, R., CHETTY, N., BAKER, S.G. and BRADLOW, B.A. The effect of low-dose aspirin on plateelt aggregation in familial hypercholesterolaemia. S. Afr. Med. J. 64: 49-52, 1983.

BRADLOW, B.A., CHETTY, N., VAN DER WESTUYZEN, J., MENDELSOHN, D. and GIBSON, J.E. The effects of a mixed fish diet on platelet function, fatty acids and serum lipids. Thromb. Res. 29: 561-568, 1983.

CHETTY, N. and BRADLOW, B.A. The effects of a vegetarian diet on platelet function and fatty acids. Thromb. Res. 30: 619-624 1983.

VAN DER WESTHUYZEN, J., CHETTY, N., GIBSON, J.E. and BRADLOW, B.A. Fatty acid composition of a fish diet. S. Afr. J. Sci. 80: 330-331, 1984.

CHETTY, N., ATKINSON, P.M. and BRADLOW, B.A. Long term ultra low dose aspirin therapy and platelet function. S. Afr. Med. J. 68: 307-309, 1985.

ACKNOWLEDGEMENTS

I wish to thank Professor J. Metz, Director of the South African Institute for Medical Research, Johannesburg, for provision of the facilities for the studies included in this dissertation. To Professor Basil Bradlow, who introduced me to the "world of platelets" and who guided me through the major part of the work in this dissertation my sincerest gratitude. To my supervisors, Professors Barry Mendelow and Dennis Mendelsohn, my sincerest appreciation, not only for the many hours they spent in reviewing this dissertation, but also for the encouragement and advice they so freely and willingly gave me at all times. To Dr Peter Atkinson for his advice and useful discussion in the preparation of this manuscript.

To Dr Lee Zar and Mrs Reverie Kuschke in the Department of Chemical Pathology for all the help with the serum lipid assays.

To Dr Justin van der Westhuizen for his assistance with the fatty acid analysis. To Jane Gibson for her technical assistance during the fish study.

To Dr Roy Shires of the lipid disorders clinic for providing the pacients used in the hyper-cholesterolaemic study.

To all my colleagues and friends who so willingly volunteered as subjects in the study.

To Dr S.G. Reinach of the Institute of Bio-Statistics, M.R.C. for his help with the statistical analysis.

To Mrs Lilly Battaglia and her staff of the S.A.I.M.R. library for their help in tracing references.

To Mr Mel Anderson and his staff for the excellent photographic work and to Ms S. Hall, Mrs J. Gallant and Mrs A. Erasmus for their help in doing some of the artwork.

In the preparation of this manuscript I am indebted to Mrs Debbie Sror and Ms. Gayle Spring for their typing and to Mrs L Moore for printing and Mr H.C. Peyper for its duplication.

To my mother, Pathmavathie, my brothers and sisters who gave me the opportunity to proceed this far. Finally I would like to express my appreciation to my wife, Sharala and son, Kinesh for their seemingly endless encouragement and patience during the preparation of this manuscript.

To my father, Soobramany Naransamy whose memory nurtured me throughout this study.

CONTENTS

	Page
CHAPTER 1. GENERAL INTRODUCTION ----------	2
1.1. The role of platelets in haemostasis and thrombosis ----------	2
1.1.1. Platelet adhesion and shape change----------	4
1.1.2. Platelet aggregation -------	5
1.1.2.1. ADP induced aggregation ------	5
1.1.2.2. Thrombin induced aggregation ------	7
1.1.2.3. Collagen induced aggregation ------	8
1.1.2.4. Epinephrine induced aggregation ------	9
1.1.2.5. Arachidonic acid induced aggregation ----------	10
1.1.3. Platelet secretion or release ----------	11
1.1.4. Platelets and prostaglandins ----------	14
1.1.5. The regulation of platelet function ----------	18

1.1.6. Platelets, the vessel wall and thrombosis ---------- 21

1.1.7. Strategies for the manipulation of the TXA_2 : PGI_2 ratio ---------- 25

CHAPTER 2. AIMS OF THE PRESENT STUDY---------- 35

CHAPTER 3. MATERIALS AND METHODS ---------- 38

3.1. Materials ---------- 38

3.1.1. Chemicals and reagents ------ 38

3.2. Methods ---------- 40

3.2.1. Sample collection ---------- 40

3.3. Platelet aggregation ---------- 41

3.3.1. The variability of platelet aggregation ---------- 42

3.3.1.1. The effect of anticoagulants and sodium citrate concentrations --- 42

3.3.1.2. Centrifugation and cellular contamination ----- 43

3.3.1.3. Platelet concentration ----- 44

3.3.1.4. Effect of time --- 45

3.3.1.5. Effect of pH ----- 45

3.3.1.6. Effect of temperature------- 46

3.3.1.7. Effect of stirring and stir bar size ---------- 47
3.3.2 The quantitation of platelet aggregation ---------- 48
3.4. Malondialdehyde (MDA) estimations -- 49
3.5. Thromboxane B_2 assay ---------- 51
3.5.1. Sample collection and storage ---------- 51
3.5.2. Assay procedure ---------- 51
3.5.3. Calculation of unknowns ---- 54
3.6 Beta-thromboglobulin (B-TG) -------- 56
3.6.1. Preparation and storage of plasma samples ---------- 56
3.6.2. Assay procedure ---------- 58
3.6.3. Calculation of unknowns ---- 60
3.7. Circulating platelet aggregates ---- 61
3.8. Platelet survival studies ---------- 61
3.8.1. Platelet labelling ---------- 62
3.8.2. Recovery studies ---------- 63
3.8.3. Platelet survival studies -- 63
3.9. Lipid extraction procedure ---------- 64
3.9.1. Fatty acid analysis -------- 65
3.9.2. Gas liquid chromatography -- 66
3.10. Serum cholesterol ---------- 66
3.11. Statistical analysis ---------- 67

CHAPTER 4. DOSE FREQUENCY FOR SUPPRESSION OF PLATELET FUNCTION BY LOW DOSE ASPIRIN THERAPY ---------- 69

4.1. Introduction ---------- 69

4.1.1. Clinical evaluation of aspirin as an antithrombotic ---------- 70

4.1.1.1. Aspirin and coronary heart disease --------- 70

4.1.1.1.(a) Clinical studies ---------- 71

4.1.1.2. Aspirin and cerebrovascular disease ---------- 77

4.1.1.2(a) Clinical studies -------- 77

4.1.1.3. Aspirin and thromboembolism -- 80

4.1.1.3.(a) Clinical trials ---------- 81

4.1.1.4. Use of aspirin in other clinical thromboembolic conditions -------- 83

4.2. Methods ---------- 89

4.2.1. Ex vivo studies ---------- 89

4.2.1.1 Platelet aggregation ----- 89
4.2.1.2. Malonyldialdehyde (MDA) production ---------- 90
4.2.1.3. Platelet survival studies ---------- 90
4.2.2. In vitro studies ---------- 90
4.2.2.1. Platelet aggregation------- 91
4.2.2.2. Thromboxane B_2 production ------- 92
4.2.3. Effects of 20mg aspirin ---- 92
4.2.3.1. Platelet aggregation ------- 93
4.2.3.2. TXB_2 production-- 93
4.2.3.3. Bleeding times ---- 93
4.3. Results ---------- 94
4.3.1. Ex vivo studies ---------- 94
4.3.1.1. Platelet aggregation and MDA production -------- 94
4.3.1.2. Platelet survival studies ---------- 102
4.3.2. In vitro studies ---------- 103
4.3.2.1.(a) Platelet aggregation ---- 103

4.3.2.1.(b) Thromboxane B_2 production ----- 111

4.3.3. Control experiments ---------- 116

4.3.4. Effects of 20mg aspirin ----- 121

4.3.4.1. Platelet aggregation ------- 121

4.3.4.2. Bleeding times ---- 121

4.3.4.3. Thromboxane B_2 production -------- 122

4.4. Discussion ---------- 125

4.5. Summary ---------- 132

CHAPTER 5. THE EFFECT OF LOW DOSE ASPIRIN ON PLATELET AGGREGATION IN FAMILIAL HYPERCHOLESTEROLAEMIA ---------- 134

5.1. Introduction ---------- 134

5.2. Materials and methods ---------- 137

5.2.1. Subjects ---------- 137

5.2.2. Experimental design ---------- 139

5.2.3. Methods ---------- 140

5.2.3.1. Platelet aggregation ------ 140

5.2.3.2. Beta thromboglobulin and circulating platelet aggregates ------- 142

5.3.2.3. Thromboxane B_2-- 142

5.3. Results ---------- 142
5.3.1. Platelet aggregation ------- 150
5.3.2. Circulating platelet aggregates ---------- 150
5.3.3. Beta thromboglobulin ------- 150
5.3.4. Thromboxane B_2 ---------- 150
5.4. Discussion ---------- 155
5.5. Summary ---------- 159
CHAPTER 6. THE EFFECTS OF A MIXED FISH DIET ON PLATELET FUNCTION, PLATELET FATTY ACIDS AND SERUM LIPIDS ---------- 161
6.1. Introduction ---------- 161
6.2. Materials and methods ---------- 169
6.2.1. Fatty acid composition of fish ---------- 169
6.2.2. Subjects ---------- 169
6.2.3. The experimental diet ----- 170
6.2.4. Platelet aggregation ------ 171
6.2.5. Thromboxane B_2 production---------- 171
6.2.6. Platelet indices --------- 172
6.2.7. Serum lipids ---------- 172
6.2.8. Fatty acid analysis ------- 172
6.2.9. Statistical analysis ------ 173
6.3. Results ---------- 173
6.3.1. Fish analysis ---------- 173
6.3.2. Platelet aggregation ------ 176

6.3.3. Thromboxane B_2 production ---------- 186
6.3.4. Platelet indices ---------- 191
6.3.5. Serum lipids ---------- 193
6.3.6. Fatty acids ---------- 198
6.4. Discussion ---------- 205
CHAPTER 7. THE EFFECTS OF A VEGETARIAN DIET ON PLATELET FUNCTION, PLATELET FATTY ACIDS AND SERUM LIPIDS ---------- 213
7.1. Introduction ---------- 213
7.2. Materials and methods ---------- 214
7.2.1. Volunteer subjects ------ 214
7.3. Results ---------- 215
7.3.1. Platelet aggregation ---- 215
7.3.2. Thromboxane B_2 production ---------- 226
7.3.3. Platelet indices ------- 232
7.3.4. Platelet fatty acid ----- 234
7.3.5. Serum lipids ---------- 236
7.4. Discussion ---------- 242
CHAPTER 8. THE EFFECTS OF A DIETARY SUPPLEMENTATION WITH AN EXTRACT OF FISH OIL (MAXEPA) ON PLATELET AGGREGATION, PLATELET FATTY ACID AND SERUM LIPIDS ---------- 249
8.1. Introduction ---------- 249
8.2. Materials and methods ---------- 253
8.2.1. Subjects ---------- 253

8.2.2. Experimental design ----- 254
8.2.3. Methods ----- 254
8.3. Results ----- 255
8.3.1. Fatty acid composition of MaxEPA ----- 255
8.3.2. Platelet aggregation ---- 256
8.3.3. Bleeding times ---------- 256
8.3.4. Platelet indices -------- 260
8.3.5. Serum lipids ---------- 260
8.3.6. Platelet fatty acids ---- 265
8.4. Discussion ----- 268
CHAPTER 9. GENERAL DISCUSSION AND CONCLUSION- 273
9.1. Aspirin ----- 273
9.2. Dietary fish oil ----- 274
9.2.1. Side effects ---------- 275
9.2.2. Conclusion ----------- 278
CHAPTER 10. REFERENCES ----- 281
CHAPTER 11. APPENDIX ----- 343

LIST OF FIGURES

Page

CHAPTER 1.

1.1. The biochemistry of arachidonic acid in platelets ---------- 17

1.2. A scheme for the regulation of platelet function ---------- 20

CHAPTER 4.

4.1. Effects of 300mg aspirin on aggregation response to ADP in seven normal subjects ---------- 95

4.2. Effects of 300mg aspirin on aggregation response to collagen in seven normal subjects ---------- 96

4.3. Effects of 300mg aspirin on aggregation response to arachidonic acid (AA) in seven normal subjects ---------- 97

4.4. Effects of 300mg aspirin on aggregation response epinephrine in seven normal subjects. --------- 98

4.5. Suppression and recovery of MDA production after a single dose of 300mg of aspirin ---------- 99

4.6. Arachidonic acid induced aggregation reduced to zero after aspirin ingestion ---------- 100

4.7. Dissapearance of the second wave of ADP stimulated aggregation after aspirin ingestion ---------- 101

4.8. Aggregation responses to ADP in mixtures of normal and aspirin inhibited platelets from one normal subject ---------- 105

4.9. Aggregation response to adrenalin in mixtures of normal and aspirin inhibited platelets from one normal subject---------- 106

4.10. Aggregation response to collagen in mixtures of normal and aspirin inhibited platelets from one normal subject ---------- 107

4.11 Aggregation responses to arachidonic acid in mixtures of normal and aspirin inhibited platelets from normal subjects --- 108

4.12. Percentage of normal platelets required to restore platelet function in mixtures of normal and aspirin inhibited platelets in 22 normal subjects ---------- 109

4.13. Aggregation responses and TXB_2 production in mixtures of normal and aspirin inhibited platelets in response to ADP---------- 112

4.14. Aggregation responses and TXB_2 production in mixtures of normal and aspirin inhibited platelets in response to adrenalin ---------- 113

4.15. Aggregation responses and TXB_2 production in mixtures of normal and aspirin inhibited platelets in response to collagen ---------- 114

4.16. Aggregation responses and TXB_2 production in mixtures of normal and aspirin ihibited platelets in response to arachidonic acid ------ 115

4.17 A representative experiment showing the absence of circulating plasma aspirin After one hour aspirinized PPP has no effect on normal (pre- aspirin) platelets when stimulated with ADP ---------- 117

4.18. A representative experiment showing the absence of circulating plasma aspirin. After one hour aspirinized PPP has no effect on normal (pre-aspirin) platelets when stimulated with adrenalin ---------- 118

4.19. A representative experiment showing the absence of circulating plasma aspirin. After 1 hour aspirinized PPP has no effect on normal (pre-aspirin) platelets when stimulted with collagen ---------- 119

4.20 A representative experiment showing the absence of circulating plasma aspirin. After 1 hour aspirinized PPP has no effect on normal (Pre aspirin) platelets when stimulated with arachidonic acid ---------- 120

CHAPTER 5.

5.1. Percentage of pre-aspirin platelets necessary to restore full aggregation to mixtures of pre-aspirin and post-aspirin platelets in normal subjects (N) and F.H. patients (P)---------- 144

5.2. Percentage of pre-asprin pltelets necessary to restore partial aggregation to mixtures of pre-aspirin and post-asprin platelets in normal subjects (N) and F.H. patients (P) ---------- 145

5.3. Number of day after administration of a single 150mg dose of aspirin before full aggregation (75%) was restored in normal subjects (N) and F.H. patients (P) ---------- 146

5.4. Percentage of the initial aggregation for each agonist at the end of 7 days of daily ingestion of 162mg aspirin ---------- 147

5.5. Percentage of the initial aggregation for each agonist at the end of 28 days of daily ingestion of 162mg aspirin ---------- 148

CHAPTER 6.

6.1. A representative experiment showing platelet aggregation of one subject in response to ADP before (B) and after (A) the fish diet ---------- 177

6.2. A representative experiment showing platelet aggregation of one subject in response to collagen before (B) and after a fish diet ---------- 178

6.3. A representative experiment showing platelet aggregation of one subject in response to epinephrine before (B) and after (A) the fish diet ---------- 179

6.4. A re-presentative experiment showing platelet aggregation of one subject in response to arachidonic acid ---------- 180

6.5 Aggregation responses of all 8 subjects in response to ADP before (B) and after (A) the fish diet ---------- 181

6.6 Aggregation responses of all 8 subjects in response to epinephrine before (B) and after (A) the fish diet ---------- 182

6.7 Aggregation responses of all 8 subjects in response to collagen before (B) and after (A) the fish diet ---------- 183

6.8 Aggregation responses of all 8 subjects in response to arachidonic acid before (B) and after (A) the fish diet ---------- 184

6.9. Thromboxane B_2 production in all 8 subjectø in response to ADP stimulation before (B) and after (A) the fish diet ---------- 187

6.10. Thromboxane B_2 production in all 8 subjects in response to epinephrine stimulation before (B) and after (A) the fish diet ---------- 188

6.11. Thromboxane B_2 production in all 8 subjects in response to collagen stimulation before (B) and after (A) the fish diet ---------- 189

6.12. Thromboxane B_2 production in all 8 subjects in response to arachidonic acid stimulation before (B) and after (A) the fish diet ---------- 190

6.13. Total cholesterol of all 8 subjects before (B) and after (A) the fish diet ---------- 194

6.14 Triglycerides of all 8 subjects before (B) and after (A) the fish diet ---------- 195

6.15 LDL cholesterol of all 8 subjects before (B) and after (A) the fish diet ---------- 196

6.16 HDL cholesterol of all 8 subjects before (B) and after (A) the fish diet ---------- 197

6.17. Platelets and plasma linoleic acid in all 8 subjects before (B) and after (A) the fish diet ---------- 200

6.18. Platelets and plasma linolenic acid in all 8 subjects before (B) and after (A) the fish diet ---------- 201

6.19. Platelets and plasma arachidonic acid in all 8 subjects before (B) and after (A) the fish diet ---------- 202

6.20. Platelets and plasma sicosapentaenoic acid in all 8 subjects before (B) and after (A) the fish diet ---------- 203

6.21. Ratio of 20:5/20:4 in platelets and plasma of all 8 subjects before (B) and after (A) the fish diet ---------- 204

CHAPTER 7.

7.1. Aggregation response of all 9 subjects in response to ADP before (B) and after (A) a vegetarian diet ---------- 217

7.2. Aggregation response of all 9 subjects in response to collagen before (B) and after (A) a vegetarian diet ---------- 218

7.3. Aggregation response of all 9 subjects in response to epinephrine before (B) and after (A) a vegetarian diet ---------- 219

7.4 Aggregation response of all 9 subjects in response to arachidonic acid before (B) and after (A) a vegetarian diet ---------- 220

7.5. A representative experiment showing an increase in height of aggregation in response to epinephrine after a vegetarian diet ---------- 221

7.6. A representative experiment showing an increase in the height of aggregation, increase in the reaction rate and decrease in the lag phase in response to arachidonic acid after a vegetarian diet ---------- 222

7.7 Thromboxane B_2 production by 9 subjects in response to ADP stimulation before (B) and after (A) a vegetarian diet ---------- 227

7.8 Thromboxane B_2 production by 9 subjects in response to epinephrine stimulation before

(B) and after (A) a vegetarian diet ---------- 228

7.9 TXB_2 production by 9 subjects in in response to arachidonic acid stimulation before (B) and after (A) a vegetarian diet ---------- 229

7.10. TXB_2 production by 9 subjects in in response to collagen stimulation before (B) and after (A) a vegetarian diet ---------- 230

7.11. Total cholesterol of 9 subjects before (B) and (A) the vegetarian diet ---------- 237

7.12. HDL cholesterol of 9 subjects before (B) and after (A) the vegetarian diet ---------- 238

7.13. LDL cholesterol of 9 subjects before (B) and after (A) the vegetarian diet ---------- 239

7.14. Triglycerides of 9 subjects before (B) and after (A) the vegetarian diet ---------- 240

7.15. Metabolic transformations of three major unsaturated fatty acid families by desaturation and elongation ---------- 246

LIST OF TABLES

Page

CHAPTER 1.

1.1. Summary of the metabolic end products of arachidonic acid and eicosapentaenoic acid and their effects on platelets and the vessel wall ---------- 33

CHAPTER 3.

3.1. Source of chemicals and reagents------ 38

3.2. Table showing sample and appropriate blank ---------- 56

CHAPTER 4.

4.1. A comparison of mean platelet lifespan determined by the Indium ^{111} labelled platelet method and the time required for aggregation responses to return to normal after aspirin (300mg) ingestion in four normals ---------- 102

4.2. Percentage of normal platelets required to restore full and partial aggregation responses to mixtures of normal and aspirin inhibited platelets ---------- 110

4.3. Thromboxane B_2 production after stimulation by the 4 agonists -------- 111

4.4. Aggregation response after 20mg aspirin/day ---------- 122

4.5. Aggregation response to daily doses of 20mg and 40mg aspirin in two subjects expressed as a percentage of the initial response ---------- 123

4.6. Thromboxane B_2 production before and after 20mg aspirin ---------- 124

CHAPTER 5.

5.1. Clinical and bio-chemical data of F.H. Type lla patients when first diagnosed ---------- 138

5.2. Platelet aggregation responses after aspirin 162mg/d for 1 and 4 weeks in F.H. patients and control subjects -- 149

5.3. Circulating platelet aggregation ratios before and after aspirin ----- 151

5.4. Beta thromboglobulin before and after aspirin therapy ---------- 152

5.5. Thromboxane B_2 production of normal subjects before and after aspirin (ASA) therapy ---------- 153

5.6. Thromboxane B_2 production of F.H. patients before and after aspirin (ASA) therapy ---------- 154

CHAPTER 6.

6.1. Fatty acid composition of fish with high 20:5/20:4 ratios ---------- 174

6.2. Fatty acid composition of fish with low 20:5/20:4 ratios ---------- 175

6.3. Platelet aggregation before and after a "fish diet" ---------- 185

6.4. Platelet counts before (B) and after (A) a "fish diet" ---------- 191

6.5. Plateletcrit before (B) and after (A) a "fish diet" ---------- 191

6.6. Mean platelet volume before (B) and after (A) a "fish diet" ---------- 192

6.7. Platelet distribution width before (B) and after (A) a "fish diet" -------- 192

6.8. Serum lipids before (B) and after after (A) a fish diet ---------- 193

6.9 Fatty acid composition of platelets before (B) and after (A) ingestion of a fish diet ---------- 199

CHAPTER 7.

7.1. Platelet aggregation before and after a vegetarian diet ---------- 223

7.2. The minimal concentration of arachidonic acid (mM) required to produce maximal aggregation response before and after a vegetarian diet ---------- 224

7.3. Reaction time (cms per sec.) and lag phase (s) in response to arachidonic acid after a vegetarian diet ---------- 225

7.4. TXB_2 production by platelets before and after a vegetarian diet ---------- 231

7.5. Platelet count of 9 subjects before and after vegetarian diet ---------- 232

7.6. Plateletcrit (%) of nine subjects before and after a vegetarian diet ---- 233

7.7. Mean platelet volume of 9 subjects before and after a vegetarian diet ---- 233

7.8. Platelet distribution width of 9 subjects before and after a vegetarian diet ---------- 234

7.9. Fatty acid composition of platelets before and after a vegetarian diet ---- 235

7.10. Serum lipids before and after a vegetarian diet ---------- 241

7.11. Summary of the two diets - rich in EPA (fish) and poor in arachidonic acid (vegetarian) ---------- 247

CHAPTER 8

8.1. Fatty acid composition of the MaxEPA fed to the subjects ---------- 255

8.2. Platelet aggregation in response to ADP stimulation after 4 weeks of MaxEPA ingestion. Values shown are the height of the aggregation response in cms ---------- 257

8.3. Platelet aggregation in response to epinephrine stimulation after 4 weeks of MaxEPA ingestion. Values

shown are the heights of the aggregation responses in cms ---------- 257

8.4 Platelet aggregation in response to collagen stimulation after 4 weeks of MaxEPA ingestion. Values shown are tne height of the aggregation response in cms ---------- 258

8.5. Platelet aggregation in response to arachidonic acid stimulation after 4 weeks of MaxEPA ingestion. Values shown are the height of the aggregation response in cms ---------- 258

8.6. Bleeding times in 8 subjects before and after 4 weeks MaxEPA ingestion -------- 259

8.7. Platelet counts before, during and after 4 weeks of MaxEPA ingestion ----- 261

8.8. Plateletcrit before, during and after 4 weeks of MaxEPA ingestion ---------- 261

8.9. Mean platelet volume before, during and after 4 weeks of MaxEPA ingestion- 262

8.10. Platelet distribution width before, during and after 4 weeks MaxEPA ingestion ---------- 262

8.11. Total serum cholesterol before, during and after 4 weeks of MaxEPA ingestion-- 263

8.12. Serum triglycerides before, during and after 4 weeks of MaxEPA ingestion ---- 263

8.13. Serum LDL cholesterol before, during and after 4 weeks of MaxEpa ingestion ---------- 264

8.14. Serum HDL cholesterol before, during and after 4 weeks of MaxEPA ingestion-- 264

8.15. Platelet fatty acid composition before and after MaxEPA ingestion ---- 266

8.16. Platelet arachidonic acid (20:4) and eicosapentaenoic acid (20:5) composition in 8 subjects before,during and after 4 weeks MaxEPA ingestion --- 267

LIST OF ABBREVIATIONS

SAIMR	South African Institute for Medical Research
FH	Familial Hypercholesterolaemia or type ll hyperlipoproteinaemia
ADP	Adenosine-5-diphosphoric acid trilithium salt
AMP	Adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
ATP	Adenosine triphosphate
GTP	Guanidine thymidine phosphate
	Alpha
	Beta
PRP	Platelet Rich Plasma
PPR	Platelet Poor Plasma
EDTA	Ethylenediamine tetra acetic acid
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
TXA_2	Thromboxane A_2
PGI_2	Prostacyclin or Prostaglandin I_2
HPETE	12L-hydroperoxy-5,8,10,14 eicosatetraenoic acid
HETE	12L-hydroxy-5,8,10,14, eicosatetraenoic acid

HHT	12L-5,8,10 hepta deca trienoic acid
PF_3	Platelet Factor 3
VLDL	Very low density lipoprotein
LDL	Low density lipoprotein
HDL	High density lipoprotein
MDA	Malondialdehyde
TRG	Triglyceride
ECG	Electro cardiograph
BTG	Beta thromboglobulin
rpm	revolutions per minute
g	relative centrifugal force
PBS	Phosphate Buffered saline
P	Partial
F	Full

CHAPTER 1

1. GENERAL INTRODUCTION

1.1. The role of platelets in haemostasis and thrombosis

Under normal circumstances, the platelet circulates in the blood for approximately 10 days as a disc shaped cell that is non-adherent to other platelets, cellular elements, or the vascular endothelium (Triplett, 1978; White, 1983). Johnson et al.,(1967), defined haemostasis as the arrest of bleeding from a breach in the wall cf a blood vessel, and it required simultaneously the presence of adequate numbers of normal, circulating platelets and a normally functioning coagulation system. The reaction of the platelet to a damaged vessel wall is similar whether the damage is traumatic or a result of the atherosclerotic process (Philp, 1981). When there is endothelial damage, platelets rapidly adhere to the exposed subendothelial surface (Preston and Greaves, 1985). In most of the studies in normal animals, there has been little evidence of thrombus formation on the subendothelium of large arteries in areas of undisturbed flow (Mustard et al., 1985). Instead, the subendothelium becomes covered with a carpet of platelets with some platelet aggregates in a few areas such as at vessel orifices and branches (Groves et al., 1979). Adhesion of platelets to the subendothelium appears to be largely independent of blood coagulation since the formation of the platelet carpet is not significantly inhibited by heparin at concentrations

that inhibit the coagulation process (Groves et al.,1982).

When the endothelium is removed from areas where blood flow is disturbed platelet aggregates and platelet-fibrin thrombi form on the subendothelium of apparently normal blood vessels (Mustard et al., 1985). The extent of thrombus formation is probably the result of a balance between the factors causing thrombus formation and those causing dissolution. The patterns of blood flow obviously affect platelet accumulation: for example, platelets accumulate in vortices. The force of blood flow may also disrupt thrombi (Mustard et al., 1985). There have been very few detailed studies of the interaction of the various factors that lead to the formation of thrombi. Occlusive thrombi rarely form unless blood flow is considerably disturbed and the vessel is narrowed, as at a stenosis (Folts et al., 1982).

The roles of plasma proteins in platelet adherence to the subendothelium are of considerable interest. The observations concerning fibronectin are confusing. From some studies it has been suggested to be the receptor for collagen on platelets (Bensusan et al., 1978) or to promote platelet interaction with collagen (Grinell et al., 1979). In other studies, fibronectin has not been found necessary (Sochynsky et al., 1980) and in some experiments, preincubtion of fibronectin with collagen has been reported to inhibit platelet interaction with

collagen (Bensusan et al., 1978). Following adhesion, the platelets undergo a secretory process, during which substances found in the alpha granules and dense bodies (ADP, serotonin, beta-thromboglobulin, platelet factor 4) are secreted from the cell. This is known as the release reaction (Grette, 1962). Thus, exposure of sub-endothelial structures results in adhesion of platelets, release of their granular constituents, aggregation, and the interweaving of the platelet haemostatic plug with fibrin filaments and the arrest of bleeding (Philp, 1981)

1.1.1. Platelet adhesion and shape change

Upon adherence to the sub-endothelium, dramatic morphological changes occur in platelets, and these are similar to platelet responses which can be induced in vitro by connective tissue particles, tumor cells, bacteria, or purified collagen. Dendritic pseudopodia are thrown out by the adhering platelets, multivesicular membranous sacs appear in the terminal bulb of the pseudopodia, and these become swollen, eventually rupturing and releasing their contents to the surrounding medium It has been postulated that this represents the physical manifestation of the release reaction (Warren and Vales, 1972). The initial adhesion is followed quickly by a change from the circulating, discoid shape of the platelet to a spiny spherical configuration and the

centralization of granular constituents (Scarborough et al., 1969).

1.1.2. Platelet aggregation

Platelet to platelet aggregation follows platelet adhesion. Platelet aggregation can be measured turbidometrically with a platelet aggregometer (Born, 1962). The aggregometer is basically a photo optical instrument that is connected to a strip recording chart. A cuvette of platelet rich plasma is placed in the path of a beam of light and is stirred, so that the diffuse cloud of tiny platelets scatters the light and prevents much of it from reaching a photocell on the other side of the transparent tube. The transmittance of light relative to a platelet poor blank, is recorded. When an aggregating agent is added, the platelets clump together, allowing more light to pass through the suspension. A tracing of the amount of light reaching the photocell provides a continuous record of the course of platelet aggregation. Aggregation agents such as ADP, thrombin, collagen, epinephrine and arachidonic acid induce platelet aggregation.

1.1.2.1. ADP induced aggregation

ADP may be derived from endothelial cells (Born, 1985) erythrocytes (Gaarder et al., 1961) or platelets (Harms, 1978). In 1962, Born reported that of 22 nucleotides

tested, only ADP and deoxyadenosine diphosphate induced the aggregation of platelets, and that adenosine monophosphate (AMP), ATP and adenosine were capable of inhibiting ADP-induced aggregation. The aggregation of platelets by ADP was, moreover, concentration dependent.

A biphasic aggregation response, that is, a primary and secondary wave of aggregation, is induced by low concentrations of ADP. At very low levels only a primary wave will be observed, followed by disaggregation. At high concentrations a single broad wave will occur (Born, 1962). In 1964, Cross et al., suggested that ADP might be involved in platelet aggregation induced by a variety of stimuli. They showed that adenosine inhibited platelet aggregation induced not only by ADP but also by adrenaline, noradrenaline, serotonin, and thrombin. Gaarder and Laland (1964), demonstrated that ADP was not taken up into cell cytoplasm but appeared rather to be associated loosely with the platelet membrane, and suggested the existence of a membrane receptor for ADP. Hampton and Mitchell (1966) using ADP induced changes in the electrophoretic mobility of platelets, estimated binding sites at $0{,}85 \times 10^5$ per platelet. Lips and Sixma (1977) studied the uptake of ^{14}C ADP by isolated human platelet membranes and found both high and low affinity binding sites. The former was calcium dependent but not temperature dependent, and had an optimum pH of

tested, only ADP and deoxyadenosine diphosphate induced the aggregation of platelets, and that adenosine monophosphate (AMP), ATP and adenosine were capable of inhibiting ADP-induced aggregation. The aggregation of platelets by ADP was, moreover, concentration dependent.

A biphasic aggregation response, that is, a primary and secondary wave of aggregation, is induced by low concentrations of ADP. At very low levels only a primary wave will be observed, followed by disaggregation. At high concentrations a single broad wave will occur (Born, 1962). In 1964, Cross et al., suggested that ADP might be involved in platelet aggregation induced by a variety of stimuli. They showed that adenosine inhibited platelet aggregation induced not only by ADP but also by adrenaline, noradrenaline, serotonin, and thrombin. Gaarder and Laland (1964), demonstrated that ADP was not taken up into cell cytoplasm but appeared rather to be associated loosely with the platelet membrane, and suggested the existence of a membrane receptor for ADP. Hampton and Mitchell (1966) using ADP induced changes in the electrophoretic mobility of platelets, estimated binding sites at $0,85 \times 10^5$ per platelet. Lips and Sixma (1977) studied the uptake of ^{14}C ADP by isolated human platelet membranes and found both high and low affinity binding sites. The former was calcium dependent but not temperature dependent, and had an optimum pH of

7.3. Born and Cross (1964) have reported a requirement for extra-cellular Ca^{++} or Mg^{++} and the presence of such divalent cations has been claimed as an essential requirement for human platelet aggregation (Ruscher,1985).

1.1.2.2 Thrombin induced aggregation

Thrombin-induced platelet aggregation and secretion involves the participation of specific binding sites on the platelet membrane,(Phillips and Agin, 1974). The process appears to be pH dependent but energy independent (Hattey and Binder, 1979) and inducers a permanent alteration in membrane configuration (Subbarao and Kakkar, 1979). The arachidonic acid metabolic pathway does not appear to play an essential role (Leband and Simons, 1979). Kinlough-Rathbone et al., (1977) suggested that thrombin was capable of inducing aggregation by different mechanisms, with low concentrations involving ADP release and the participation of the arachidonic acid pathway and higher concentrations utilizing another system, possibly involving internal shifts of ionic calcium. Fujimura et al., (1980) speculated that thrombin binds to the platelet surface membrane glycoprotein (GP)1 and hydrolyzes GPV specifically, and that the hydrolyzed GPV might act as a signal to induce the platelet release reaction.

1.1.2.3. Collagen induced aggregation

Platelet activation and release induced by collagen is a complex process which appears to involve more than one mechanism. It has been suggested by Huzoor-Akbar and Ardlie (1976) that one mechanism involves a direct effect on platelets and the other is mediated via thrombin. Kinlough-Rathbone et al., (1977), showed that collagen could aggregate platelets which had been previously degranulated by thrombin, and in a medium which contained creatine phosphate/creatine phosphokinase which converts available ADP to ATP. This aggregation was inhibited by aspirin and indomethacin, indicating that in the absence of extracellular ADP, collagen aggregation can be mediated through the arachidonic acid pathway. Recently using suspended platelets pretreated with pyridoxal 5'-phosphate (biologically active coenzyme form of pyridoxine or vitamin B_6 which reacts with free amino groups) and $NaBH_4$, Krishnamurthi and Kakkar (1985), have suggested that collagen induced aggregation may also be via the same mechanism/receptor as that used by ADP, but platelet adhesion seems to involve a distinct mechanism/receptor that is not affected by Pyridoxal 5'-phosphate. Thus, free amino groups on the platelet membrane may be involved in the induction of platelet aggregation by collagen, but not platelet adhesion to collagen. Collagen induces only a single wave of aggregation, which corresponds to the release reaction of platelets. This is characterized by a

lag period before aggregation begins (Wilner et al.,1968).

1.1.2.4. Epinephrine induced aggregation

Epinephrine produces a biphasic pattern of aggregation (MacMillan, 1966; O'Brien, 1966), with the release of granular constituents. Epinephrine has been reported by some investigators to induce the uptake of calcium by platelets (Owen et al., 1980; Owen and LeBreton 1980). In contrast, others have been unable to demonstrate an effect of epinephrine on calcium uptake (Brass and Shattil, 1982; Clare and Scrutton, 1984; Lalau Keraly et al., 1985) except in circumstances in which the release reaction occurs (Clare and Scrutton, 1984). The uptake of epinephrine by the platelet appears to involve an adrenergic receptor since the effect on platelets can be blocked by α-adrenergic blocking drugs such as phentolamine (Oppenheim and Youdin 1979; Lalau Keraly et al., 1985). However inhibition of the β-receptor potentiates the action of epinephrine in inducing aggregation of platelets that are refractory to ADP (Kerry and Scrutton, 1983; Lalau Keraly et al ., 1985) and supports the view that the effect of ephinephrine is probably dependent upon the balance of α and β receptor stimulation (Kerry and Scrutton 1983). In view of the actions of epinephrine on the adenylate cyclase system (α receptor agonist inhibit adenylate cyclase; β receptor agonists stimulate it, Scrutton and Wallis, 1981) it may

be that at least part of the action of epinephrine is mediated through adenylate cyclase. The report by Figures et al., (1984) that epinephrine increases the binding of ADP to platelets, indicates that this has to be considered as another pathway by which epinephrine may exert its effect on platelets.

1.1.2.5. Arachidonic acid induced aggregation

Arachidonic acid (AA) is a free fatty acid that induces both platelet aggregation and prostaglandin synthesis. AA is one of the major fatty acids in human platelets. It is present in both intracellular granules and in membrane compartments, mainly esterified to the phospholipid fraction, and is the precursor of thromboxanes and hydroxy fatty acids (Marcus, 1982). Arachidonate cannot be transformed unless it is hydrolyzed from phospholipid and made available in free form (Marcus, 1978). Recent evidence indicates that AA is liberated from platelet phospholipids by a dual mechanism. The first involves the combined action of a phosphatidyl inositol-specific phospholipase C (Rittenhouse-Simmons, 1979), followed by a diglyceride lipase (Bell et al., 1979), thereby yielding free AA. In addition there appears to be a phospholipase A_2 type of activity liberating AA from phosphatidyl ethanolamine (Broekman et al., 1980). Recent evidence indicates that phosphatidyl inositol may be the major source of AA in the platelet (Rittenhouse-Simmons, 1979;

Guichardant and Lagarde, 1980; Broekman et al., 1980). When arachidonic acid is added to platelet rich plasma the phospholipase step is by passed and cyclooxygenase is activated directly. A normal shape change and full aggregation response ensue. The arachidonate is transformed to Thromboxane A_2 (TXA_2), which may directly mobilize calcium which then inhibits adenylate cyclase and platelet cyclic AMP levels fall. In addition ADP and serotonin are released from platelet dense granules. TXA_2 is also released and together with ADP serves to 'recruit' other platelets, ie. the released ADP and TXA_2, aggregate other platelets in the surrounding medium (Silver et al., 1973; Marcus 1979).

1.1.3. Platelet secretion or release

The secretory process or release reaction was first described by Grette (1962) and is the selective extrusion of stored platelet constituents. It is essentially a process of exocytosis and resembles the secretory mechanisms of endocrine cells (Hovig, 1962). Day and Holmsen (1971) termed the release of the constituents of dense granules Release I, and that of both dense and α granules Release II The release of α-granules does not occur in the absence of dense body release.

Dense granules or dense bodies have been shown to contain adenine nucleotides (ADP, ATP and GTP) (Day and Holmsen,

1971), serotonin and calcium (Salganicoff et al., 1975). Dense granule release (Release 1) is induced by high concentrations of ADP and epinephrine and low concentrations of thrombin and collagen, and leaves ∝-granules intact (Day and Holmsen, 1971). It proceeds quicker than ∝-granule secretion. Platelet factor 4 has been reported to be located in dense granules (Youssef and Barkhan, 1968). The release of ∝-granule contents (Release II) is induced by thrombin and collagen (Holmsen et al., 1977).

Packham et al., (1977) classified a large number of aggregating agents according to their mechanism of action, their ability to induce release, and their dependence upon released ADP and prostaglandin endoperoxide formation.

According to their scheme, group I agents aggregate platelets without inducing the release reaction or endoperoxide formation, even when present in high concentrations. These would include serotonin in platelet rich plasma (PRP), sodium periodate in a medium without glucose, and ADP in a medium containing physiological concentrations of calcium ions and protein. Under these circumstances only the initial wave of aggregation would be observed.

Group 2A agents cause the initial wave of aggregation, which is independent of released ADP or endoperoxide formation but, in sufficient concentration and in a suitable medium, will induce a second wave of aggregation with endoperoxide formation and the release of ADP. These would include ADP in citrated PRP or in a low Ca^{++} medium; adrenaline and noradrenaline in PRP and vasopressin in heparinized PRP or in an artificial medium with calcium and fibrinogen, free radicals of O_2 and OH in an artificial medium. Group 2B agents are agglutinating substances. The term agglutination as distinct from aggregation, is reserved for those agents which form bridging complexes between platelets. This group includes ristocetin, bovine factor VIII and polylysine in citrated PRP, lectins and concanavalin A in an artificial medium. A second wave of aggregation with release of ADP and endoperoxide formation occurs which is blocked by EDTA.

Group 3 agents do not induce primary aggregation but induce release, with secondary aggregation occuring as a consequence of the release of ADP and endoperoxide formation. The aggregation is inhibited by EDTA. This group includes collagen, arachidonic acid, antigen-antibody complexes, and foreign surfaces such as latex particles. Group 4 agents are similar except that they involve further mechanisms which can cause aggregation and release independent of the release of ADP and endoperoxide

formation. These include proteolytic enzymes such as thrombin and trypsin, calcium ionophore A23,187, and Newcastle Disease Virus.

1.1.4. Platelets and Prostaglandins

The demonstration that intact platelets or platelet homogenates were capable of synthesizing PGH_2 + PGG_2 from exogenous arachidonic acid (Glenn et al., 1972) and that arachidonic acid induced platelet aggregation when added either to washed platelets or platelets in plasma (Silver et al., 1973; Marcus 1979), whereas other unsaturated fatty acids did not, provided evidence for an important role for the arachidonic acid pathway in the regulation of platelet function.

In 1974, Hamberg and Samuelsson demonstrated that two endoperoxide intermediates, PGG_2 and PGH_2, were produced during prostaglandin synthesis in platelets and were potent initiators of platelet aggregation. In 1975, Hamberg, Svensson and Samuelsson, identified a substance, thromboxane A_2 (TXA_2), which was an intermediate between PGG_2 and the inactive end product thromboxane B_2 and which was even more potent than the endoperoxides in inducing platelet aggregation.

The biochemical pathways involved in the formation of prostaglandins and thromboxanes have been thoroughly

reviewed by numerous authors (Needleman et al., 1976; Moncada and Vane, 1978; Marcus 1978; Philp, 1981; Holmsen and Karpatkin, 1983). Characteristically, in platelets as in other cells, prostaglandins are not stored but are synthesized rapidly in response to an appropriate stimulus. Initially, the enzymes phospholipase C and phospholipase A release arachidonic acid from membrane phospholipids (principally phosphatidylinositol and phosphatidylethanolamine respectively), (Rittenhouse-Simmons, 1979; Broekman et al., 1980). Thrombin, platelet activating factor, collagen and arachidonic acid induce the activation of phospholipase C during shape change of human platelets (Siess et al., 1983a; Siess et al., 1983b; Lapetina and Siegel 1983; Siess et al., 1984). Phospholipase C activation generates 1,2 diacylglyerol, which stimulates protein kinase C, and leads to formation of phosphatidic acid myo-inositol 1,4,5,-triphosphate, which has been implicated in intracellular Ca^{++} mobilization (Streb et al., 1983). The arachidonic acid made available by the phospholipase may enter either the lipoxygenase pathway or the cyclooxygenase pathway.

Lipoxygenase pathway: under the action of the lipoxygenase enzyme, arachidonic acid may be converted to the non prostanoic structures 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE) and thence by a peroxidase,

reduced to 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE). HPETE can also be transformed into the leukotrienes.

Cyclooxygenase pathway: the enzyme cyclooxygenase converts arachidonic acid to the endoperoxides PGG_2 which, by peroxidase converts to PGH_2. The endoperoxides may either be converted by thromboxane synthetase to thromboxane A_2 which, being unstable, is hydrolyzed to thromboxane B_2, converted to the stable prostaglandins PGE_2, PGD_2 (either enzymatically by an isomerase or non-enzymatically) and PGF_2 (enzymatically by a reductase), or converted to 12L,5,8,10--heptadecatrienoic acid (HHT) and malondialdehyde (MDA). A summary of the biochemistry of the AA metabolic system is shown schematically in fig (1.1).

In the vascular endothelium, PGH_2 is converted to prostacyclin (PGI_2) an antiaggregatory agent and vasodilator (Moncada et al., 1976). PGI_2 was shown to be many times more potent at inhibiting aggregation than either PGE_1 or PGD_2. PGI_2 is thought to cause the general inhibition of platelet aggregation, adhesion (Fr[illegible] and Barton, 1977) and the release reaction (Gorman et al., 1977) by activating platelet membrane adenylate cyclase (Gorman et al., 1977; Tateson et al., 1977) and thus causing an increase in the intracellular level of cyclic AMP (Best et al., 1977).

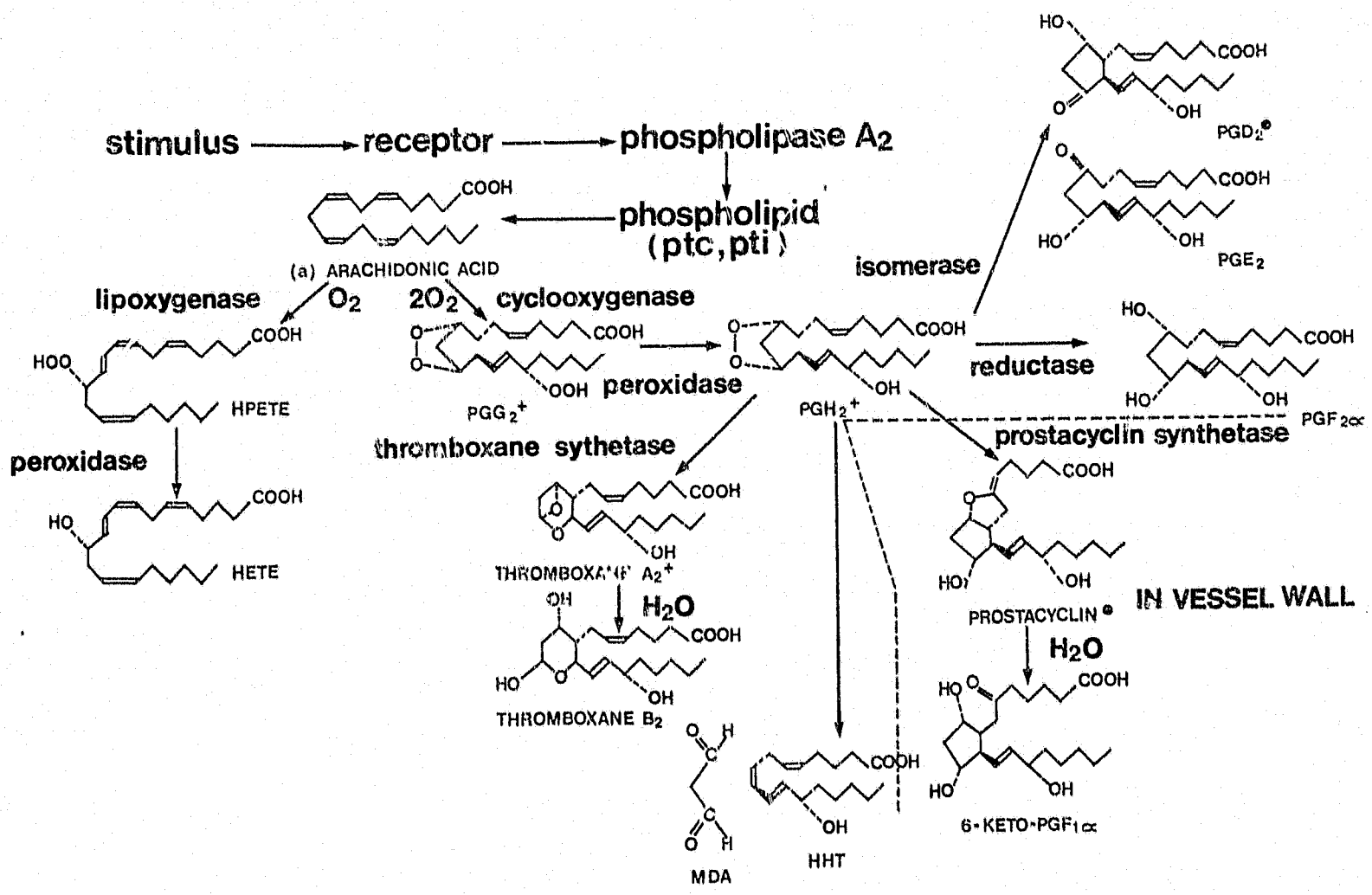

Fig. 1.1. The biochemistry of arachidonic acid in platelets (adapted from Philp 1981; Holmsen and Karpatkin, 1983)

1.1.5. The regulation of Platelet Function

A number of schemes for the regulation of platelet function with minor variations have been formulated (Gerrard and White, 1978; Gorman et al., 1979; Philp 1981; Luscher 1985; Siess et al., 1985). One such scheme is shown in fig 1.2. According to current concepts, proaggregatory agents such as thrombin, collagen, ADP, epinephrine etc. interact at the surface of the platelet membrane with a receptor, which is probably a phosphorylated protein in most instances and which may or may not be a calcium binding protein. Stimulus receptor interaction dephosphorylates the protein and releases bound calcium, which stimulates the formation of diacylglycerol and phosphatidic acid, indicating the activation of phopholipase C. This sets in motion the arachidonic acid (AA) cascade. The formation of TXA_2 provides the physiological calcium ionophore to mobilize additional Ca^{++} from various storage sites (Philp, 1981). TXA_2 then releases Ca^{++}, either through conversion to TXB_2 or by exchange with Mg^{++}, and Ca^{++} inhibits adenylate cyclase (AC) (Luscher, 1985), lowering cyclic adenosine monophosphate (cAMP) levels and removing any inhibitory effect of cAMP on phospholipase C and cyclooxygenase. Thus a positive feedback mechanism is set in motion to accelerate the AA cascade. The free calcium also activates excitation - contraction - secretion coupling, resulting in centralization and the

Subsequent extrusion of dense granules. An antiaggregatory stimulus (S-) such as PGI_2, PGD_2 or adenosine, acting upon specific receptors on the membrane surface, activates adenylate cyclase (AC) which forms cAMP from ATP. cAMP activates a protein kinase which phosphorylates a protein believed to promote Ca^{++} re-uptake and hence maintain a low level of free Ca^{++}. cAMP also ihibits phospholipases and cyclo-oxygenase, thus inhibiting TXA_2 formation. The action of various inhibitors of platelet aggregation can now be viewed in the light of their ability to prevent the mobilization of intracellular Ca++. Thus inhibitors of cyclooxygenase or thromboxane synthesis would limit the ability of the calcium ionophore TXA_2. Stimulators of AC or inhibitors of phosphodiesterase promote the accumulation of cAMP which inhibits phospholipase A_2 and cyclooxygenase and which may promote the compartmentalization of Ca++.

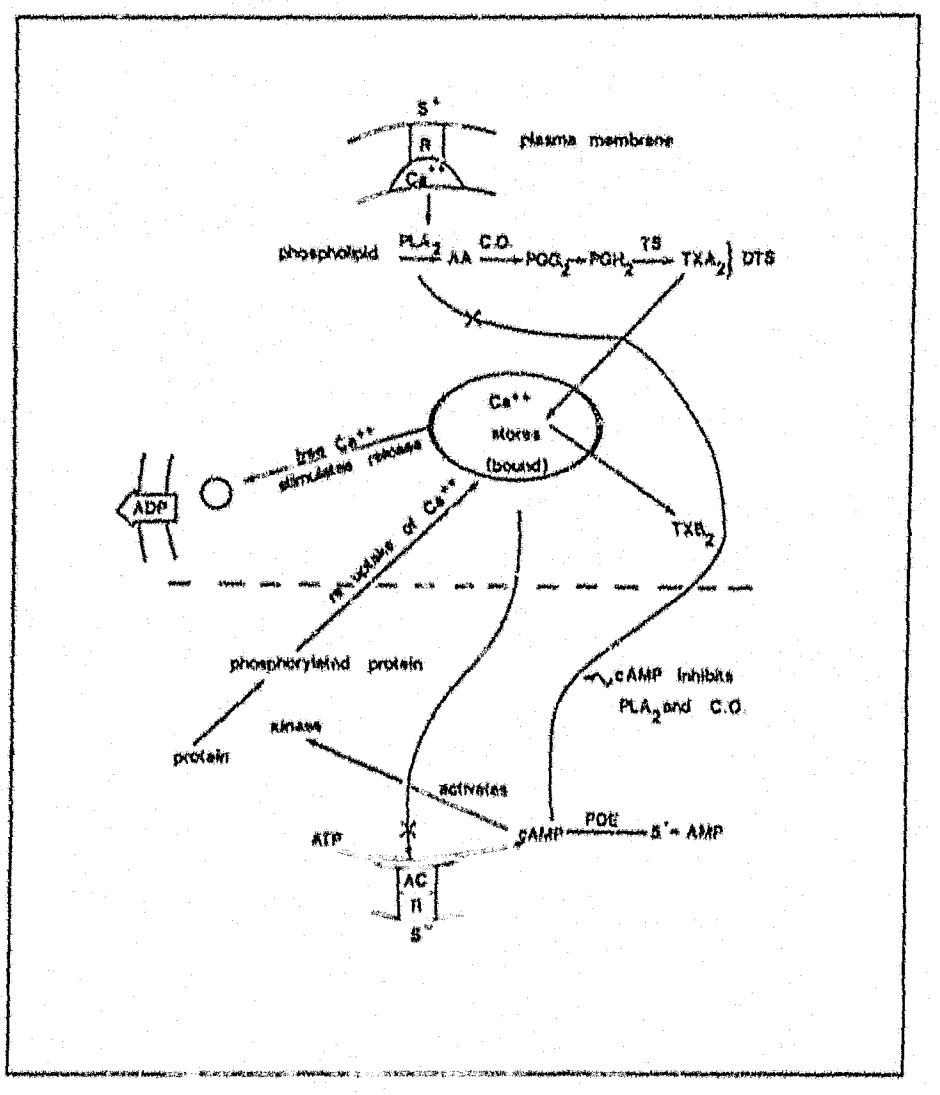

Fig. 1.2. A scheme for the regulation of platelet function (Adapted from Philp, 1981).

1.1.6. Platelets, the vessel wall and thrombosis

Experimental studies suggest that it is likely that platelets are important in the initiation and progression of arterial thromboembolic disease (Lowe, 1981; Preston and Greaves, 1985). Activation of platelets is associated with physico-chemical changes which, under some conditions, encourag atelet-platelet and platelet-vessel wall interactions. These changes may be associated with activation of other haemostatic mechanisms which may ultimately give rise to platelet-fibrin thrombi (Preston and Greaves, 1985).

Within the circulation, the association between platelets and arteries is encouraged by the rheological properties of arterial blood, the flow of which tends to direct the platelets towards the vessel wall, particularly at arterial bifurcations and right angled junctions (Murphy et al., 1962). Endothelial cell damage at these sites, occasioned by haemodynamic stresses, causes the subendothelial elements to be exposed. Local arterial thrombus formation is thus encouraged, since platelets avidly adhere to these elements (Preston and Greaves, 1985).

In addition, platelets may make an important contribution to the development of atherosclerosis, as first proposed in 1842 by Rokitansky (cited by Born, 1985). There are at

least three mechanisms by which platelets may participate in atherogenesis. First, through damaging arterial endothelial cells by releasing injurious agents, presumably where circulating platelets adhere (Mustard et al., 1977). Secondly through the release in such situations of a factor responsible for smooth muscle proliferation in the arterial wall (Ross and Glomset, 1973). Thirdly, through the formation of persistent mural thrombi, which are organised into intimal thickenings (Walton, 1975). Underlying all three claims is the assumption that some normal circulating platelets settle on arterial walls for long enough to release some of their contents (Born, 1985). These considerations have led to attempts to influence the course of thrombotic and atherosclerotic disease by the use of drugs which reduce platelet reactivity. Arguably, the most important of these is aspirin (Preston and Greaves, 1985).

In recent years the effects of prostaglandin endoperoxides on the interaction between platelets and the vessel wall have received much attention (Moncada and Vane, 1978; Bunting et al.,1983; Moncada, 1985). Prostaglandin endoperoxides are at the cross roads of AA metabolism, for they are the precursors of substances with opposing biological activities. On the one hand, TXA_2 produced by the platelets is a strong vasoconstrictor, induces the release of ADP from dense granules and decreases cyclic

adenosine monophosphate (cAMP) and hence stimulates platelet aggregation. On the other hand, prostacyclin produced by the vessel wall is a strong vasodilator and increases cAMP levels and hence inhibits platelet aggregation. A balance between pro-aggregatory TXA_2 and anti-aggregatory PGI_2 has been proposed by Moncada and Vane (1978) and Moncada, (1985). This balance could determine the intensity of local thrombosis and vascular tone. Upset in this balance favouring platelet aggregation is postulated as predisposing to thrombosis (Moncada and Vane 1978; Moncada, 1985)

Several diseases have now been related to an imbalance in the TXA_2 - PGI_2 system. Platelets from patients with arterial thrombosis, deep vein thrombosis, or recurrent venous thrombosis produce more PG endoperoxides and TXA_2 than normal and have a shortened survival time (Lagarde and Dechavanne, 1977). Platelets from rabbits made atherosclerotic by dietary manipulation (Shimamoto et al., 1978) and from patients who have survived myocardial infarction (Szczeklik et al., 1978) are abnormally sensitive to aggregation agents and produce more TXA_2 than controls. Elevated TXB_2 levels have been demonstrated in the blood of patients with Prinzmetals angina (Lewy et al., 1979). Hirsch et al., (1981) also reported increased TXB_2 levels in coronary sinus blood of patients with unstable angina. Platelets from rats

made diabetic display an increased release of TXA_2, whereas their blood-vessels show a reduced production of prostacyclin (Harrison et al., 1978). Platelets from diabetic patients are known to be more sensitive to several aggregating agents (Mustard and Packham, 1977; Colwell et al., 1979) and to produce more TXA_2 than normals (Halushka et al., 1981; DiMinno, 1985). Prostacyclin production by blood-vessels from patients with diabetes are lower than normals (Johnson et al., 1979), and circulating levels of 6-keto-PGF, are reduced in diabetic patients with proliferative retinopathy (Dollery et al., 1979).

Thrombotic thrombocytopenic purpura (TTP), like diabetes, is associated with formation of microvascular thrombo-emboli, and a deficiency in prostacyclin production may contribute to the increased platelet consumption that occurs in TTP (Remuzzi et al., 1978; Cocchetto et al., 1981). This deficiency is postulated to be secondary to a lack of a "plasma factor" which normally stimulates prostacyclin production (MacIntyre et al., 1978). An increased prostacyclin production, resulting from an accumulation of the "plasma factor" that stimulates prostacyclin synthesis, has been suggested to explain the haemostatic defect in uraemic patients (Remuzzi et al., 1977). Patients with Bartter's syndrome excrete about four times as much 6-keto-PGF, as controls in the urine

(Gullner et al., 1979). This has led to the suggestion that increased production of prostacyclin mediates both the hyperreninaemia and the hyporesp siveness to pressor agents observed in these patients (Gullner et al., 1979).

In general, it seems that, in diseases where there is a tendency for thrombosis to develop, TXA_2 production is increased, or prostacyclin production reduced, or both, whilst the opposite is found in some diseases associated with an increased bleeding tendency.

1.1.7. Strategies for the manipulation of the TXA_2: PGI_2 ratio

TXA_2 and PGI_2 represent, in biological terms, the opposite poles of the same general homeostatic mechanism for regulation of platelet aggregability *in vivo*. Manipulation of this control mechanism will affect thrombus and haemostatic plug formation (Moncada, 1985). Several pharmacological strategies have been advocated to manipulate the ratio of these two substances. One such drug, aspirin, wa used as an analgesic, an anti-inflammatory agent, and an antipyretic agent for many years before its effect on haemostasis was recognised. Frick (1956) reported that aspirin prolonged the bleeding time in patients with haemorrhagic disorders. Within the past decade, several investigators have demonstrated that aspirin influences prostaglandin synthesis by inhibiting

the initial enzyme of the biosynthetic pathway (Ferreira et al., 1971; Smith and Lands, 1971; Smith and Willis, 1971; Hamberg and Samuelsson, 1974; Roth et al., 1975; Roth and Majerus, 1975).

The therapeutic use of aspirin with regard to its platelet anti-aggregatory activity has been based on the hypothesis that increased platelet-vessel and platelet-platelet interactions are causative factors in the development of arterial and venous thrombi, as well as thromboembolic phenomena (Marcus, 1983). Aspirin may serve to prevent these thromboembolic events, since it induces a functional defect in platelets, demonstrable clinically as a prolongation of the bleeding time.

Biochemically this is thought to be due to inactivation through acetylation of the enzyme cyclooxygenase (Roth and Majerus, 1975) thereby eliminating the synthesis of thromboxane A_2. Aspirin also affects the synthesis of PGI_2, within the blood vessel wall (Villa and de Gaetano, 1977; Moncada, 1985). When it was shown that aspirin prevented the formation of aggregatory TXA_2 in platelets and the anti-aggregatory prostacyclin I_2 in vascular cells - the "aspirin dilemma" emerged (Marcus 1977; de Gaetano et al., 1982). Inhibition of vascular prostacyclin production by aspirin was seen as potentially undesirable. Platelet cyclooxygenase was found to be

extremely sensitive to aspirin inhibition (Baenziger et al., 1977) and the endothelial cell enzyme was found to be considerably less sensitive to aspirin inhibition than the platelet enzyme (Baenziger et al., 1977; Masotti et al., 1979; Jaffe and Weksler, 1979). This is presumably because the cyclooxygenase enzyme can be resynthesized by endothelium but not by platelets, which cannot synthesize protein (Patrono and Patrignani, 1984). Because endothelial cell prostacyclin production can be resumed between doses of aspirin (de Gaetano et al., 1982; Marcus, 1983), the aspirin dilemma could have been easily resolved by administering low doses of the drug. This would tilt the balance in favour of prostacyclin by inhibiting platelet thromboxane synthesis and sparing vascular prostacyclin production. Different aspirin trials to test this hypothesis are discussed in chapter 4.

Theoretically, the most logical way to manipulate the TXA_2:PGI_2 ratio, would seem to be the induction of a selective pharmacological blockade of platelet thromboxane synthetase. These inhibitors are either derivatives of pyridine or imidazole. Perhaps the most studied is dazoxiben, an imidazole derivative which contains a carboxyalkyl substituent at the 1-position (Smith, 1985). Some investigators have observed that dazoxiben inhibits arachidonic acid or collagen induced platelet aggregation in in vitro tests with platelet rich plasma from some

donors (responders) but not from others (non-responders) (Heptinstall et al., 1980; Bertele et al., 1981; Dale et al., 1983; Bertele et al., 1984). In a study by Smith (1982) all volunteers on dazoxiben were found to be non responders.

Originally it was thought that these would be ideal antithrombotic drugs sparing PGI_2 production while blocking synthesis of TXA_2. However, this is not without consequence since a complex rearrangement of arachidonic acid metabolism occurs within the cell (Bertele et al., 1981; Tyler et al., 1981; Vermylen et al., 1981; Smith, 1985). Thromboxane synthetase (TS) inhibitors allow accumulation of cyclic endoperoxides in platelets. The endoperoxides are potent platelet aggregating agents (Majerus, 1983) and this limits the effectiveness of TS inhibitors as platelet suppressive agents (Preston and Greaves, 1985). In fact, these drugs may act primarily by stimulating production of platelet inhibiting eicosanoids (Gorman, 1983). Prostacyclin production is increased by endoperoxide steal and conversion of PGH_2 to prostaglandin D_2 (PGD_2) occurs in plasma (Fitzgerald et al., 1983). Both prostacyclin and PGD_2 stimulate cAMP production in platelets and thereby inhibit platelet function. However, the elevations of PGI_2 and PGD_2 are relatively modest as judged from the level of metabolites, and the drugs currently known have a very short duration of action (Fitzgerald et al., 1983). While

the final answer will come from clinical trials, it seems unlikely that a drug of this class will surpass aspirin as an antithrombotic agent (Majerus, 1983). The sulphonamide derivative BM 13,177 blocks the thromboxane receptor on which endoperoxides act, and thus may overcome the limitations of thromboxane synthethase inhibitors (Gresele et al., 1984). Clinical trials of these drugs have not yet been completed (Smith, 1985).

The search for new and more effective approaches to platelet suppressive therapy thus goes on. The use of infusions of prostaglandin I_2 (prostacyclin) has recently received considerable attention. Some success has been claimed in Raynaud's syndrome and in severe peripheral arterial disease (Belch et al., 1983a.b; Prentice et al., 1983) and some patients with variant angina may also respond (Chierchia et al., 1982). Also, prostacyclin infusion appears to diminish platelet loss during haemodialysis (Turney et al., 1980) and extracorporeal circulation procedures (Radegran et al., 1982; Aren et al., 1983). However, further controlled clinical trials are indicated in the broader area of vascular disease in order to identify the type of patient who may respond to such therapy and the optimal dosage which should be used. Stable analogues of prostaglandin I_2 have also been developed and are currently under trial. In addition, the search goes on for agents capable

of the stimulation of endogenous prostacyclin synthesis Vermylen, 1979).

A possible beneficial change in the balance between platelet and vascular synthesis of the oxygenation products of arachidonic acid can apparently be achieved by augmentation of the dietary intake of the polyunsaturated fatty acid eicosapentaenoic acid, an attractive approach to platelet suppressive therapy (Dyerberg and Bang, 1979a).

The therapeutic potential of EPA was suggested by the findings of Dyerberg et al., 1978), who found a functional substitution of EPA for AA in the diet of Greenland Eskimos. These Eskimos consume a diet largely composed of cold water fish, which are rich in EPA. It has also been reported that Greenland Eskimos have a low incidence of cardiovascular disease and myocardial infarction (Editorial, Lancet, 1983, Bang and Dyerberg, 1972; Dyerberg and Bang 1982; Sinclair, 1984). (Kromhout et al., 1985) reported an inverse relation between fish consumption and a 20 year mortality from coronary heart disease.

Needleman et al.,(1979) demonstrated that EPA, the fatty acid precursor of the 3 series prostaglandin (PG) family, was enzymatically converted by human platelets into

thromboxane A_3 or by blood vessel microsomes into 17-prostacyclin (PGI_3). The 3 series endoperoxide and thromboxane were less potent than PGI_2 (Needleman et al., 1977). Comparative studies of the actions of metabolites of AA and EPA on platelets in platelet rich plasma produced unexpected results. Arachidonate, PGH_2 and thromboxane A_2 were potent aggregators of human platelets but, in sharp contrast, the EPA, PGH_3 and TXA_3 did not cause platelet aggregation (Raz et al., 1977) (The actions of the end products of AA and EPA metabolism is summarized in Table 1.1.). This latter observation was given potential physiological perspective by the finding that Eskimos who have a bleeding tendency have elevated EPA and depressed AA in platelets (Dyerberg et al., 1978). It was suggested that endogenous PGI_3 synthesis from EPA by the vasculature contributed to the bleeding tendency (Dyerberg et al., 1978; Fisher and Weber, 1984). However, it was shown that exogenous AA could aggregate Eskimo platelets (Dyerberg and Bang, 1979b). Although this demonstrates that cyclooxygenase and thromboxane synthetase are still functional, it does not necessarily refute their assumption that elevated PGI_3 levels are responsible for the lack of aggregation. Increased PGI_3 levels would increase platelet cAMP levels, which would prevent aggregation regardless of the stimulus i.e. AA as well as ADP. Whitaker et al.,(1979) and Needleman et al., (1982)

observed that EPA inhibits platelet aggregation even when simultaneously added with the agonist (eg. arachidonate, ADP or collagen) or when added before stimulation of platelets (Gryglewski et al., 1979). Needleman et al.,(1979) have also demonstrated that ^{14}C-EPA-labelled platelets stimulated with thrombin and intact, prelabelled, perfused kidneys stimulated with bradykinin will readily deacylate the prelabelled lipid and release EPA. Thus, no apparent distinction between EPA or AA release from tissue lipids exists. Previous experiments in platelets (Bills et al., 1976) have demonstrated that agonist stimulation is selective in its release of fatty acids (including oleate, linoleate and palmitate). Since EPA acts as a competitive antagonist for AA, the simultaneous release of these fatty acids should effectively suppress AA conversion to its aggregatory metabolites. The lowering of endogenous AA and increase in EPA which must result from a diet of the type that the Greenland Eskimos ingest would be anticipated to markedly reduce the generation of PGH_2 and thromboxane A_2 which cause aggregation. (Needleman et al., 1982). Dietary trials to test the above hypothesis are discussed in Chapter 6.

Table 1.1. Summary of the metabolic end products of arachidonic acid and eicosapentaenoic acid and their effects on platelets and the vessel wall.

Pro aggregating	Platelets	Precursors	Vessel wall	Anti aggregating
Yes	TXA_2	Arachidonic Acid	PGI_2	Yes
No	TXA_3	Eicosapentaenoic Acid	PGI_3	Yes

CHAPTER 2

2. Aims of the present study

Wyndham (1978) found South African whites to have the highest death rate from coronary heart disease in the Western World. The reasons for this high death rate are not clear. One important factor could be an upset in the TXA_2 - PGI_2 balance favouring platelet aggregation and thus predisposing to thrombus formation.

In general the aim of this study was to explore selective manipulation of prostaglandin metabolism with a view to reducing the synthesis and/or formation of thromboxane A_2 with minimal effect on prostacyclin synthesis. To this end the following specific studies were undertaken:

a) Pharmacological

   i) The effects of normal platelets on aspirin inhibited platelets in human subjects and to assess the relevance of this effect to dosage frequency.

   ii) The effect of 20mg aspirin daily on platelet function and TXA_2 production.

   iii) The effect of low dose aspirin on platelet function in Familial Hypercholesterolaemia

b) Dietary

   i) The effects of a mixture of fish rich in EPA other than mackerel or salmon, on platelet function, platelet fatty acid composition and serum lipids.

ii) The effects of a vegetarian diet on platelet function and platelet fatty acid composition.

iii) The effects of a dietary supplementation with an extract of fish oil (MaxEPA) on platelet function platelet fatty acid composition and serum lipids.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1. Chemicals and Reagents

The source of chemicals and reagents employed in this study is listed in Table 3.1. All chemicals were of analytical reagent quality unless stated.

Table 3.1 Sources of Chemicals and reagents used

Chemicals and reagents	Source
Acetone	Merck
ADP	British Drug House (BDH)
Chloroform	Merck
Epinephrine	Maybaker (SA)
Arachidonic Acid	Sigma Chemical Company
Benzene	Merck
Beta-thromboglobulin Kit	Amersham International
Boron trifluoride-methanol	Supelco Inc.
Calcium chloride	BDH
Carbon tetrachloride	Merck
Collagen	Hormon-Chemie Munchen
Dextrose	Merck
Disodium hydrogen orthophosphate	Merck
EDTA	BDH
Ethanol	SAAR Chemicals

Table 3.1 continued

Chemicals and reagents	Source
Fatty acid methyl esters (standards)	Supelo Inc
Formalin	SAIMR (Commercial grade)
Hexane	Merck
HCL	SAAR Chemicals
Indomethacin	MSD Pharmaceuticals
Malondialdehyde tetraethyl-acetal	Merck
Magnesium chloride	GD Searle (SA)
Max EPA	British cod liver oils
Methanol	Merck
Monosodium Phosphate	BDH
Nitrogen	Afrox
Perchloric acid	Merck
Potassium chloride	BDH
Potassium dihydrogen Orthophosphate	BDH
Potassium hydroxide	BDH
Repelcote	SMM Chemicals
Silar 10c (10%) on chromosorb WD MCC (60-80) Mesh	Applied Science Labs
Sodium bicarbonate	GD Searle Ltd
Sodium carbonate	SAAR Chemicals
Sodium chloride	SMM Chemical

Table 3.1 continued

Chemicals and reagents	Source
SP222-PS on 100/120 support	Supelco Inc.
Thiobarbituric ac d	Merck
Thromboxane B_2 RIA kit	New England Nuclear
Trichloroacetic acid	Merck
Trisodium citrate	SMM Chemicals

3.2 Methods

3.2.1 Sample Collection and processing

Venous blood was collected into polyethylene tubes containing 1 ml 3.8% sodium citrate per 9 ml blood for platelet function studies and into plain glass tubes for the lipid estimations. Platelet rich plasma (PRP) for aggregation studies, was prepared by centrifuging the blood at 180g for 10 minutes(MSE Mistral 6L). Platelet poor plasma (PPP) was prepared by centrifuging the remaining red cell fraction and small amount of plasma (after the PRP had been removed) at 1800g for 15 minutes. Platelet counts were performed using a Thrombocounter C (Coulter Electronics). The platelet counts were adjusted with autologous PPP to 250 $\pm$ 10 x 10^9 per itre. Serum lipids measured included high

density lipoprotein (HDL) cholesterol, total cholesterol (TC), low density lipoprotein (LDL) cholesterol and total triglyceride (TRG)

3.3 Platelet Aggregation

Platelet aggregation was measured by the method of Born and Cross (1963) using a Payton Dual Channel aggregation module attached to an SP-H5V recorder and a Chronolog Dual Channel aggregometer attached to a Coulter Electronics omniscribe recorder. Aggregations were carried out within 2 hours of collection. Cuvettes were stired at 1000 rpm, and a constant temperature of 37 degrees C was maintained. During aggregations, platelet rich plasmas were kept in polyethylene tubes (15 mm diameter) that were capped at all times when not in use. The aggregating agents used were adenosine-5-diphosphoric acid trilithium salt (ADP) (0.5-10.0uM), epinephrine (EPI) (0.14-55uM), collagen (1-4 ug/ml) and arachidonic acid (AA) (0.2-1mM). Concentrations in parentheses refer to the final concentration in the cuvette. The lowest concentration of agonist required to produce maximum aggregation was determined for each agonist on each occasion that the patients were tested. All studies were carried out in glass cuvettes that were siliconized for 20 minutes in a solution of 10% Repelcote water repellant in 90% carbon tetrachloride.

3.3.1 The variability of platelet aggregation

Experience has shown that, as with any technical procedure, a certain amount of variability is inherent in the procedures associated with aggregation studies using the turbidometric method (Newhouse and Clark, 1978). The recognition of specific variables, their significance, and the means of eliminating or controlling them are vital if meaningful comparative data are to be obtained. In this section, an attempt was made to evaluate the known factors influencing platelet aggregation and the steps taken to eliminate or control them are discussed.

3.3.1.1 The effect of anticoagulants and sodium citrate concentrations

Anticoagulants most frequently utilized in coagulation studies include sodium citrate, heparin, ethylenediaminetetracetic acid (EDTA), and sodium oxalate (Newhouse and Clark,1978) Sodium oxalate and EDTA can initially be omitted from use in aggregation studies because sodium oxalate has previously been shown to increase the lability of the procoagulants present in the blood (Davidson and Henry, 1969),thus making it unsuitable for samples prepared for aggregation, and EDTA has been shown to have adverse effects upon platelets (Newhouse & Clark, 1978).

Anticoagulant activity is generally mediated through the interaction of free calcium ions in the blood, and calcium must be present in PRP for platelet aggregation to occur. Aggregation is enhanced as the concentration of Ca^{++} present in the plasma increases. Therefore anticoagulant solutions that bind calcium ions excessively must be avoided. Both EDTA and high concentrations of sodium citrate have been shown to inhibit aggregation by excessive binding of Ca++ (Newhouse and Clark, 1978). When heparin is utilized in the preparation of PRP, small platelet aggregates are often found unless special care is taken in mixing and handling the sample (Zucker, 1974).

In the present study, sodium citrate was used as the anticoagulant and the concentration of 3.8% was kept constant at all times.

3.3.1.2. Centrifugation and cellular contamination

Centrifugation should ensure that in PRP the maximum number of platelets is retained without any red or white cell contamination and that in PPP the plasma is as platelet free as possible. Obviously the speed and time of centrifugation from one sample to the next must be kept constant if reproducibility is to be maintained.

In the present study PRP was obtained by centrifuging the blood sample at 180g for 10 mir and PPP was obtained by centrifuging at 1800g for 15 minutes.

3.3.1.3. Platelet concentration

The initial optical density of the PRP is directly dependent upon the platelet concentration (Cronberg, 1970). As this concentration increases, the platelets in the cuvette become more closely packed together, increasing the possibility of collision and thus the rate of aggregation (Sixma,1972). Cronberg (1970) reported that although the increase in platelet concentration may produce qualitative differences,the actual stickiness of the platelets was not changed with variable concentrations. Differences in the appearance of the secondary phase of ADP induced aggregation, however, were noted in studies by Danta (1970). At low platelet counts the secondary phase, because of its diminished slope, tends to be obscured by the primary wave of aggregation and thus appears to be absent. Therefore, standardization of the platelet count by dilution of the PRP with autologous PPP is often performed.

In the present study all platelet counts were adjusted using autologous PPP to 250 $\pm$ 10 x 10^9 per litre.

3.3.1.4 Effect of time

The stability of PRP in relation to storage time after collection was found to vary from patient to patient, and from day to day in the same patient (Harrison et al.,1967).They reported that aggregation responses of some platelet rich plasmas tend to remain stable up to 3 hours after collection, while others become progressively less responsive as time progresses. Warlow et al., (1974), suggested that the interval between venepuncture and measurement of platelet aggregation should be fixed and should be stated with all data obtained.

In the present study, aggregations were commenced immediately after preparation of the PRP and PPP and completed within two hours of collection.

4.3.1.5. Effect of pH change

Changes in the pH are known to have profound effects upon the aggregating properties of the platelets. Generally, aggregation is not observed below a pH of 6.4 or above a pH of 10. Maximal aggregation is considered to occur at or near pH 8 (Skoza et al., 1967). Changes in the plasma pH are brought about as a result of preparation for aggregation studies, time being a vital factor. After centrifugation to separate PRP the resulting pH of the plasma is about 7.5. Longer

centrifugation to obtain PPP and the addition of this PPP to PRP to standardize the count yields a pH of about 7.6 for the beginning of aggregation studies providing that the plasma preparations have taken place within 2hrs. (Coller et al., 1976). Continued changes in pH with time are mediated by the rate at which CO_2 diffuses out of the plasma system into the ambient atmosphere. Factors affecting this rate of diffusion and subsequent rise in pH include: (1) the surface area to volume ratio of the plasma (2) whether the plasma is capped or uncapped while standing, and (3) how frequently the plasma is mixed (Kerby & Taylor, 1971).

In the present study the PRP and PPP were prepared immediately after blood collection and stored in polyethylene tubes (15mm diameter). The tubes were capped at all times when not in use and in between aggregations. The tubes were mixed by gentle inversion once every 15 minutes to prevent platelets from settling.

3.3.1.6. Effects of temperature

The temperature at which PRP is stored prior to aggregation studies and the temperature at which the actual aggregation is performed has a significant influence upon the rate and extent of aggregation

achieved by the plasma preparation. Platelets stored at room temperature may be relatively stable for the first two hours. However, platelets stored at 37 degrees C will, in this time, have lost their responsiveness (O'Brien, 1964). Lower temperatures, 0-4 degrees C, maintain aggregation ability for even longer periods of time. However, the major problem associated with the storage of plasma samples at progressively lower temperatures is the increased tendency towards spontaneous aggregation (Anstall and Hawkey, 1962).

In the present study, PRP was stored at room temperature prior to commencement of aggregation studies. All aggregation studies were performed at 37 degrees C.

3.3.1.7. Effects of stirring and stir bar size

Stirring the PRP with the selected aggregation agent is necessary for the subsequent aggregation to occur. This stirring process potentiates platelet collisions and thus aggregate formation (Born and Cross, 1963). The rate at which aggregation proceeds could be within a specific range of rpm, directly related to the rate of stirring, with the rate and extent of aggregation increasing as the stirring increases (Mustard and Packam, 1970). Significant changes in aggregation do not occur when stir speed is varied from 600 - 1200 rmp. When stir speed is reduced below 600 rpm,

a reduction is noted in the rate and extent of aggregation. When the stir speed is increased above 1200 rpm, increased disaggregation is evident (Sixma, 1972). Maximum aggregation is produced at an optimal stir speed of 1 000 rpm (O'Brien, 1962). Coller and Gralnick (1976) have shown that the width, length and roughness of the stir bars are associated with the ability of the stir bar to mix the PRP and the aggregating agent completely and efficiently.

In the present study, aggregations were performed with constant stirring at 1 000 rpm. Stir bars used for the aggregation studies were teflon coated and uniform in size.

3.3.2 The quantitation of platelet aggregation

In the present study, drug and dietary effects on platelet function were studied and major aggregation changes were sought. The clinical significance of slight changes is questionable. The parameters used to evaluate aggregation were: a) the lowest concentration of agonist required to produce a maximum aggregation response, b) the height of the aggregation curve, measured in centimeters.

Maximum aggregation response was defined as the maximal

change in light transmittance at an interval 5 minutes after the onset of aggregation. This method of assessing platelet aggregation was also used by Carvalho et al., (1974).

3.4. Malondialdehyde (MDA) Estimations

MDA production in platelets after aggregation was determined by a modification of the methods of Stuart et al., (1975) and McMillan et al.1977. Sodium arachidonate was used as the agonist to induce platelet aggregation.

Sodium arachidonate was prepared as described by Kinlough Rathbone et al. (1976).Arachidonic acid (AA) 50 mg was dissolved in 3.2 ml benzene to yield a 50 mM solution. The mixture was aliquotted into 0.3 ml quantities and stored at -20 degrees C. Before use the benzene was evaporated under nitrogen gas and replaced with 0.3 ml of 0.1 M $NaCO_3$.

MDA standards using malondialdehyde tetraethylacetal were prepared in the following way: a 0.01 molar solution was made (0.22g MDA-tetraethylacetal dissolved in 200 ml distilled water) and labelled as MDA stock solution. The stock solution was then diluted to yield standard concentrates of 2.0, 2.5, 5,8 and 10 nmoles/20 ul. A 0.53 % solution of 2-thiobarbituric acid was

prepared in 2.3% perchloric acid and stored at 4 degrees C. The solution was prepared by dissolving 800 mg of 2-thiobarbituric acid in 20 ml concentrated NaOH to which 50 ml of distilled water was then added. The pH of the solution was adjusted by the addition of concentrated perchloric acid and the volume then brought to 100 ml with distilled water. Fifty ml 7% perchloric acid was added to this 100 ml solution (Stuart et al., 1975). The MDA standards were then used to construct a standard curve in the following way: 20 ul of MDA standards(2, 2.5, 5, 8 and 10 nmoles/20 ul) were added to 200 ul PRP (PRP adjusted to 250 $\pm$ 10 x $10/^{9}$ per litre with autologous PPP (see section 4.2.2) Blanks without added MDA were also prepared using 200 ul PRP and 20 ul saline. Proteins were precipitated by adding an equal volume of 100 % trichloroacetic acid, and after centrifugation (1 200g for 5 minutes at room temperature) 200 ul volumes of the protein free supernatants were mixed with equal volumes 0.53% thiobarbituric acid. Samples were incubated for 30 minutes at 70 degrees C with a marble condenser on each tube, allowed to cool at room temperature, and mixed with 0.6 ml glass-distilled water. MDA was measured by recording absorbance at 532 nm in a Pye-Unicam SP2800 spectrophotometer. Cells of 1 cm path length were used.