PROSTAGLANDINS AND ACUTE INFLAMMATORY REACTIONS

STUDIES ON RAT PLATELETS AND CARRAGEENIN-INDUCED PAW EDEMA

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PROMOTOR : PROF. DR I.L. BONTA

CO-REFERENTEN : PROF. DR O. VOS
DR IR D.H. NUGTEREN

DIT PROEFSCHRIFT KWAM TOT STAND OP DE AFDELING FARMACOLOGIE VAN DE ERASMUS UNIVERSITEIT ROTTERDAM EN WERD GESUBSIDIEERD DOOR DE STICHTING VOOR MEDISCH WETENSCHAPPELIJK ONDERZOEK FUNGO.

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I.L.Bonta, H.Bult, L.L.M. van de Ven & J.Noordhoek. Essential fatty acid deficiency: a condition to discriminate prostaglandin and non-prostaglandin mediated components of inflammation. Agents and Actions, 6(1976)154-158.

I.L.Bonta, H.Bult & J.Noordhoek. Effect of arachidonic acid and indomethacin on carrageenin inflammation of essential fatty acid deficient (EFAD) rats (Abstr.). In: Adv. Prostaglandin and Thromboxane Research, Vol. II, p 889; Eds.: B.Samuelsson and R.Paoletti, Raven Press, New York (1976).

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I.L.Bonta, H.Bult, J.E.Vincent & F.J.Zijlstra.Acute anti-inflammatory effects of aspirin and dexamethasone in rats deprived of endogenous prostaglandin precursors. J.Pharm.Pharmac., 29(1977)1-7.

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CONTENTS page

ABBREVATIONS

AA	arachidonic acid
ADP	adenosine 5' diphosphate
ANOVA	analysis of variance
ATP	adenosine 5' triphosphate
ASA	acetylsalicylic acid, aspirin
Bk	bradykinin
BPP	bradykinin potentiating peptide
C	curie
cAMP	3'5'-cyclic-adenosine monophosphate
CAR	carrageenin
dpm	desintegrations per minute
EFA	essential fatty acid
EFAD	essential fatty acid deficient
9,11 EM	(15S)-OH-9α,11α-(epoxymethano)-prosta-5,13-dienoic acid
G	gravity
GSH	glutathione
H	histamine
h	hour
HETE	12L-hydroxy-5,8,10,14-eicosatetraenoic acid
HHT	12L-hydroxy-5,8,10-heptadecatrienoic acid
5HT	5-hydroxytryptamine , serotonin
IC_{50}	inhibitor concentration at which the inhibition is 50%
IM	indomethacin
i.v.	intraveneous
MDA	malondialdehyde
NA	noradrenaline
NSAID	non-steroidal anti-inflammatory drug
p	probability
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PG	prostaglandin
PGL	prostaglandin-like activity
PI	phosphatidyl inositol
PLA_2	phospholipase A_2
PPP	platelet poor plasma
PRP	platelet rich plasma
PS	phosphatidyl serine
PUFA	polyunsaturated fatty acid
RAS	rabbit aorta strip
RC	rat colon
RCA	rabbit coeliac artery strip
RCS	rabbit aorta contracting substance
RCS-RF	RCS-releasing factor
RSS	rat stomach strip
s	second
s.c.	subcutaneous
s.p.	subplantar
Sph	sphingomyelin
TBA	2-thiobarbituric acid
TLC	thin layer chromatography
TX	thromboxane
TYA	eicosatetraynoic acid

1. INTRODUCTION

Despite the vast amount of accumulated data on inflammation, there are still large gaps in our knowledge about the pathways involved in the development of an inflammatory response (see Ebert & Grant, 1974). Thus, effective therapy of several chronic inflammatory diseases is, at this moment, beyond medical capabilities. For the control of the inconvenient and painful symptoms of diseases like rheumatoid arthritis, physicians still have to rely on drugs which have largely been developed empirically and are used in an empirical way (eg. Kaye & Pemberton, 1976). The mode of action of several of these "anti-inflammatory drugs", which often only relieve anoying symptoms of the disease, has for a long time been shrouded in mystery. Only recently, Vane and co-workers (1971) discovered the main biochemical action of a group of these drugs, known as non-steroidal anti-inflammatory drugs (NSAIDs), of which aspirin (acetylsalicylic acid) is the most familiar representative. It appeared that aspirin-like drugs suppress prostaglandin (PG) biosynthesis, both in vitro and in vivo (82,233,254). This led to the concept that inhibition of PG release explains the anti-inflammatory effect of NSAIDs.

Several experimental models are used in the development of new anti-inflammatory drugs. For example, both non-steroidal and steroidal anti-inflammatory agents suppress certain inflammatory reactions evoked in animals. These models are not only useful for the development of new drugs, but may lead to some understanding of their modes of action. Moreover, since such models apparently mimick certain features of human diseases, they may provide information about endogenous factors which are of importance for the development -or persistence- of the disease or its symptoms. The main purpose of the experiments described in this thesis was to obtain more insight into the involvement of PGs in an in vivo model of acute inflammation. The process is evoked by the injection of carrageenin, a material extracted from seaweed and containing sulphate polysacharides, into the foot of a rat. This results in a short-lasting reaction (CAR edema), that exhibits macroscopical features of inflammation, such as swelling, a local increase in temperature, redness and an decreased pain threshold. CAR edema, as well as other inflammatory reactions, is partially governed by mediators. These endogenous substances are formed or released at the inflammatory site, and are able to induce one or more signs of inflammation. Whenever their release is depressed by a drug or depletion, the inflammatory reaction should be reduced (see 267,274). On the basis of the

sensitivity of CAR edema to aspirin-like drugs, it is generally assumed that PGs are involved as mediators in this inflammatory model. However, unequivocal meaurements of local PG levels have never been published using CAR edema, one of the most used models of acute inflammation. Thus, it was tempting to see if the PGs fulfill the first criterion of a mediator, namely enhanced local concentrations during the inflammatory reaction in the paw. Secondly, the pro- or anti-inflammtory activities of PGs were examined on CAR edema. These studies were partly carried out with essential fatty acid deficient (EFAD) rats. EFAD rats lack the precursors from which PGs are formed. With the aid of these rats, it was further investigated if the anti-inflammatory activity of two aspirin-like drugs, was indeed explained by inhibition of PG biosynthesis.

Since PGs are formed easily as a result of only minor tissue damage, the assessment of PG levels in vivo is a procedure full of pitfalls. Therefore, another in vitro model was used, in which biosynthesis of PGs and related products by aggregating rat platelets was studied. In this model the NSAIDs are active as inhibitors of aggregation and PG biosynthesis. Certain PG products are necessary for aggregation of human blood platelets. In fact, the prolonged bleeding-time, which is sometimes observed after ingestion of aspirin, can now be explained as a consequence of its inhibition of PG biosynthesis. Thus, apart from PG biosynthesis per se, the significance of this formation for rat platelet behaviour has also been studied. As with the in vivo model, the activity of certain drugs and the influence of EFA deficiency on both platelet PG release and aggregation were investigated.

Short reviews of recent literature on several aspects of the studies are given in chapter 2. Not all selected data are of direct relevance to the experimental section (Chapters 3-7). However, the reader, who is interested, may find more information on certain basal aspects, which are of importance for a better understanding of the processes studied.

In order to facilitate the understanding of some biological aspects, the experiments with the in vitro model will be described first (Chapter 4), and will be followed by description of the in vivo studies (Chapter 5-7). Most results have already been published, or will be published elswhere. In this thesis, the results have been rearranged, sometimes within a broader frame than that offered by editors of journals.

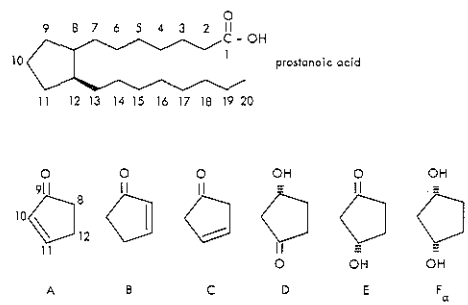
2. LITERATURE

2.1. Prostaglandin (PG) biosynthesis.

2.1.1. Summary.

The term prostaglandins, first used by Von Euler (1937), is now the generic name for a number of related lipids. The basic structure of these fatty acids (prostanoic acid), was proposed by Bergström et al. (1963; see fig 2.1).

Fig. 2.1. Prostanoic acid and different types of classical PGs.



The naturally occurring, "classical" PGs are classified according to functional groups attached to C_9 and C_{11}. Numerical subscripts (e.g. PGE_2) indicate the number of double bonds in the aliphatic chain of the PGs. The "α" and "β" subscripts of PGF designate whether the C_9 hydroxylgroup points forward or down (α) or backwards (β).

The first reports of PG biosynthesis (7,250,251) showed, that certain n-6 unsaturated fatty acids were precursors of PGs. Thus, PGE_1, PGE_2 and PGE_3 can be formed by incubating more-or-less purified preparations of PG endoperoxide synthetase (also called cyclo-oxygenase) with dihomo-γ-linolenic acid (20:3, n-6), arachidonic acid (AA, 20:4, n-6) and eicosapentaneoic acid (20:5, n-3) respectively*. In the decade following the discovery of their structures, the activities of PGs have been intensively studied because of their various effects on physiological (eg. reproductive, gastrointestinal, cardiovascular and renal system) and pathological processes (eg. inflamma-

* For fatty acid nomenclature and notation; see 109,146 and fig. 2.3.

tory reactions and blood platelet aggregation).
They may be considered as local hormones or autacoids. It was assumed (eg. 221) that the conversion of AA into stable PGs proceeded as follows:

arachidonic acid $\longrightarrow$ PG endoperoxides $\longrightarrow$ PGD_2, PGE_2, $PGF_{2\alpha}$

The isolation of the unstable endoperoxide intermediates in PG biosynthesis was described by Nugteren & Hazelhof and Hamberg & Samuelsson in 1973. Since these unstable PG endoperoxides (PGG_2, PGH_2) are much more potent than the classical PGs in certain aspects (eg. platelet aggregation), their isolation has opened new areas of PG research.

Recently two new enzymatic conversions of these endoperoxides have been discovered (see fig. 2.2). The first pathway, initially found in platelets (120,179) leads to the formation of the non-prostanoic thromboxanes. Thromboxane A_2 (TXA_2) is highly unstable in aqueous solution and is even more potent than the endoperoxides in producing platelet aggregation and in contracting an isolated rabbit aorta strip. TXA_2 is proabbly identical to the elusive rabbit aorta contracting substance (RCS), first described by Piper & Vane (1969). It has now become apparent that in several cells (eg. platelets, lung and spleen cells) the classical PGs are only formed in minute amounts when compared to the amounts of thromboxanes.

The second new pathway leads to prostacyclin (PGX, PGI_2; 170) and was first discovered in rat stomach and cells of arterial walls. Interestingly the effects of prostacyclin are directly opposed to those of TXA_2: it relaxes arterial smooth muscle and is a very potent inhibitor of platelet aggregation.

Since the PG endoperoxides possess important biological actions, and can be almost completely transformed to and released as highly active non-prostanoic products, the importance of PGs in physiological and pathological processes should be reappraised. Some of the more important factors for PG biosynthesis will be described in the following paragraphs (Key to other literature: Samuelsson et al., 1975).

2.1.2. Cyclo-oxygenase substrates.

A large number of polyunsaturated fatty acids (PUFA's) with 18 to 22 C atoms, and at least 3 double bonds in a skipped position, can act as PG-pre-

cursors (143,236,265). The methylene-interrupted all cis double bond system is normally at n-6. Location at n-5 or n-7 still allows conversion into PGs, but a larger shift of the double bond system, towards the carboxylgroup (from (n-8), prevents any conversion at all (17,236,251). Thus, the 20:3(n-9) acid, which accumulates during essential fatty acid (EFA) deficiency is not a PG precursor, nor is it converted to an hydroperoxy fatty acid by the cyclo-oxygenase (see 236). The main naturally occurring substrates are dihomo-γ-linolenic acid and AA. AA is by far the most common in different species and tissues (eg. 146,180), except for the vesicular gland where dihomo-γ-linolenic acid is predominantly present (143,265).

2.1.3. Phospholipase A_2: the rate limiting factor in PG release?

In most cells the bulk of PUFA's, including EFA's is esterified at the 2 position of phospholipids (109). Phosphatidylethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI) are generally the richest sources of EFA's. These phospholipids are mainly present on the inside of the lipid bilayer in mammalian plasma membranes (eg. platelets (54), erythrocytes (285), liver cells (75)) and phosphatidylcholine (PC) and spingomyelin (Sph), which contain very little AA, are predominantly found in the outer half of the lipid bilayer.

Thus, in most mammalian cells a phospholipase A_2 (PLA_2) is likely to play a role in the liberation of PG-precursors and its activation or its access to the substrate has been postulated as a regulating factor in endogenous PG biosynthesis (Kunze & Vogt, 1971). The importance of endogenous PLA_2 in controlling PG release has recently been established in spleen and lung tissue, and platelets (see 2.4). In spleen slices, preincubated with ^{14}C-AA, the substrate is indeed released from the 2 position in phospholipids, and not from neutral lipids. This release increases during gentle tissue damage (produced by vibration), and anaphylactic shock, and is antagonized by the anti-malarial drug mepacrine (88). In guinea pig perfused lungs an increased PLA_2 activity coincided with an enhanced PG production (26).

Apart from the possibility that activation of PLA_2 is rate limiting for release of AA, the increased release of PG precursors may be explained without recourse to changes in basal endogenous PLA_2 activity. Some alternatives are: 1. Changes in membrane structure, such as in interfacial pressure between the phospholipids in the bilayer (68,286), and/or changes in fluidity,

can lead to enhance degradation of phospholipids. Both below and above the transition temperature, the ordered lipid molecules in liposomes are not accesible to pancreatic PLA_2, but during phase-transition hydrolysis takes place. Some venom phospholipasesare able to degrade their substrate in the liquid phase (228). The transition of phospholipidbilayers, from the solid to the liquid-crystalline form is influenced by the unsaturation and/or length of the acyl chains, the properties of the polar head groups, the incorporation of cholesterol into the bilayer, and the presence of divalent cations (eg. 67,199,247). 2. A reduced reacylation (eg. 213) of liberated AA. 3. PLA_2 may liberate other PUFA's than AA, eg. linoleic acid, which are competitive inhibitors of PG synthetase (198). A decreased liberation of these fatty acids in favour of an increased AA-release will result in an enhanced PG formation.

Some of the stimuli that increase endogenous PG production, are given in table 2.2. It is obvious that one of the explanations for the increased PG output is an enhanced availability of substrate, but only in the perfused guinea pig lung has a possible increase in PLA_2 activity been demonstrated to be responsible for the enhanced hydrolysis of fatty acids (Blackwell et al., 1977). They injected sn 2 (1-^{14}C) oleoyl PC into the pulmonary artery and observed a small basal release of hydrolyzed label. Histamine (H), RCS-releasing factor (RCS-RF) and bradykinin (Bk) stimulated this release 3 to 5 times. These results indicate that H, Bk and RCS-RF stimulate PLA_2 activity, provided that the flow of the label through the lungs is unaltered by H, Bk and RCS-RF, and that reacylation of free fatty acids remains unchanged.

Lipase. In contrast to most tissues, PG release in adipose tissue might also be dependent on the activities of lipases. Data on the origin of PG precursors in adipose tissue are however, lacking at this moment. The concomitant release of substrate analogues, such as oleic and linoleic acid (see 2.2.1) which inhibit PG biosynthesis, might be considerable if triglycerides serve as source of AA.

2.1.4. Fatty acid cyclo-oxygenase (E.C. 1.14.99.1.).

PG endoperoxide synthetase (= cyclo-oxygenase), which forms PGG_2 from AA, is present in many vertebrate and non-vertebrate tissues (57). Rich sources include: seminal vesicles, kidney medulla, gastro-intestinal tract,

spleen, lung and platelets. The enzym is membrane bound and has been solubilized and purified to a high extent (121,166,167,219,246). Estimates of its molecular weight range between 69,000 and 85,000 (121,219,246). The initial step in PG biosynthesis from AA is removal of the 13-L-hydrogen, followed by introduction of one molecule of oxygen at C 11 in a lipoxygenase-like reaction (see 221 and fig. 2.2). The peroxyfatty acid is subsequently transformed by a complex reaction to PGG_2, with the introdution of second molecule of oxygen. With certain PUFA's (eg. 20:2,n-6; 22:3,n-8) the initial reaction occurs without PG formation and the corresponding hydroxyfatty acids are formed. Eicosatrienoic acid (20:3,n-9) does not lead to formation of an hydroxyfatty acid (188,236).

Purified synthetases of bovine (166) and ram seminal vesicles (246) displayed both cyclo-oxygenase and peroxidase activity. The cyclo-oxygenase reaction (eg. AA→PGG_2) requires haemin, free or protein bound. Haemin acts as prosthetic group and is possibly lost during purification. Haemin and some non-haem iron were found to be present in the preparation described by Hemler et al. (1976). A suitable hydrogen donor, such as tryptophane (166) or hydroquinone (189,246), is necessary for the reduction of the 15-hydroperoxy group by the peroxidase activity. Thus, in the presence of haem compounds and a hydrogen donor the major endproduct of the purified enzyme is PGH_1, if 20:3 (n-6) is used as substrate (166,246). Without a hydrogen donor a very rapid in activation occurs.

The rather complex kinetics of the enzyme have been studied by the group of Lands, who used microsomal preparations from ram and bovine seminal vesicles. The enzyme shows a positive feedback, in being activated allosterically by its own products, and a negative feedback in catalyzing its own destruction (eg. 142). The initial lag-phase, which is a feature of the possitive feedback was not observed in a solubilized cyclo-oxygenase from ram seminal vesicles, and the delayed start in a microsomal preparation might be the result of a slow access of subtrate and/or oxygen to the active site (233).

The in vitro self-destruction of the enzyme has been established for different microsomal preparations (eg. 142,209,280) and a purified cyclo-oxygenase (166). The self-catalyzed breakdown of enzymatic activity takes place within a very short time at high substrate concentrations. If this self-destruction also occurs in intact cells, it might be a limiting factor in PG biosynthesis. Due to the limiting substrate concentrations, it seems unlikely that autocatalytic breakdown of enzymatic activity, does normally

occur in vivo.

2.5.1. The metabolism of PG endoperoxides.

Non-enzymatic conversion. The half life ($t_{\frac{1}{2}}$) of PGH_2 in buffer (pH 7.4, 37°C) is 5 min. PGH_1 and PGH_2 decompose non-enzymatically to form PGE, PGF_α, PGD and a 12-hydroxy-C 17 fatty acid with concomitant production of MDA. Without reducing agents and haem the yield of PGE and PGD is 85-90%, PGE being the main product (189). Certain serum albumins (cow, sheep and pig) facilitate the decay of PG endoperoxides, probably via a fatty acid binding site, with an enhanced yield of PGD (58,113).

Enzymatic conversion. Presumably, the metabolism of PG endoperoxides into PGE, TXA_2 and prostacyclin (PGI_2) takes place at, or very near the site of the cyclo-oxygenase activity. In the situations studied, the endoperoxides are rapidly metabolized by the specific enzyme system present, and very little endoperoxides can accumulate. Examples of these preferred pathways in certain tissues are: seminal vesicles (20:3, n-6 → PGE_1), renal medulla (AA → PGE_2), platelets (AA → TXA_2), heart and vessel wall (AA → PGI_2). Some of these enzymes have only recently been described and have only been subjected to preliminary characterization.

PGH → PGD isomerase. This reaction occurs in homogenates of several rat tissues such as spleen, lung, small intestine and skin. The enzyme is not membrane bound (189).

PGH → PGE isomerase. This enzyme is localized in membranes of bovine (167) and ram (189) seminal vesicles and rat renal papilla (196) and has been solubilized (167). In all cases GSH is needed for the reaction.

PGH → PGF reductase. The reductive cleavage of the 9,11-endoperoxide ring of PGH_2, which yields $PGF_{2\alpha}$, is performed by rat liver glutathione-S-transferases (58). According to some authors PGF formation from PGH seems to be a non-enzymatic process (eg. 209).

Thromboxane synthetase (PGH_2 → TXA_2 isomerase). Biosynthesis of thromboxane A_2 (TXA_2) takes place in washed human platelets (120), and in lungs and spleen of guinea pig (112,118) and rat (197). TXA_2 is very labile ($t_{\frac{1}{2}}$ = ± 30 sec, at 37°C) and decays either into TXB_2 (112,120) or into MDA and a C-17-hydroxy fatty acid (HHT; 84,101,239). A recent report indicates that

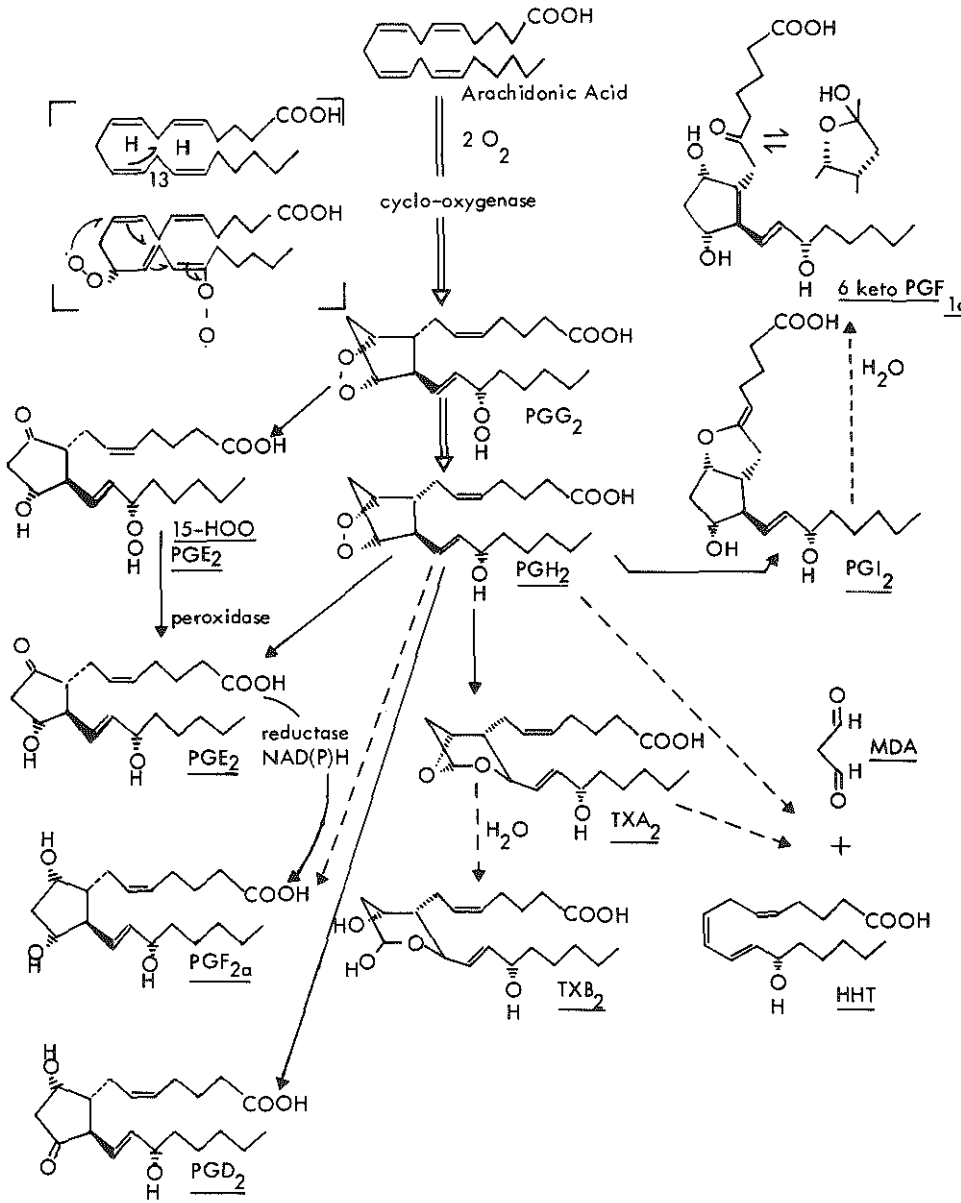


Fig. 2.2. Pathways in PG biosynthesis. The cyclo-oxygenase may act as catalyst in the conversion of free (see 2.1.2 & 2.1.3) arachidonic acid into PG-endoperoxides (PGG_2 and PGH_2; see 2.1.4). The latter may be enzymatically (—▶) or non-enzymatically (--▶) transformed into HHT and MDA, stable PGs, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) (see 2.1.5).

TXA_2 is more stable in human plasma (232). Since TXA_2 is a much more potent constrictor of rabbit aorta strips than the PG endoperoxides, PGG_2 and PGH_2 (178,179) it is now assumed (120) that TXA_2 is the main constituent of rabbit aorta contracting substance (RCS), which is released from the guinea pig lung during anaphylactic shock (Piper and Vane, 1969).

The platelet enzyme is membrane bound and can be obtained in a microsomal fraction. PGG_2, PGG_3, PGH_2 and PGH_3, but not PGH_1, serve as substrate for the microsomal preparation from platelets (178,179,187). The enzyme of human platelets showed little pH dependence between pH 5-8.5, was not inhibited by NSAIDs, and not affected by azide, GSH and AA metabolites PGE_2, PGD_2, $PGF_{2\alpha}$, HETE or TXB_2. However, this preparation was strongly inhibited by stable substrate analogues such as 9,11 epoxymethano prostanoic acid and 9,11 EM (239).

Other inhibitors of thrombxane synthetase have been described. Benzydamine is somewhat more effective as inhibitor of TXA_2 synthetase, than as cyclo-oxygenase inhibitor (172). A non-acidic anti-inflammatory agent, L8027, has been claimed to be a selective inhibitor of TXA synthetase (104). Imidazole selectively inhibits platelet TXA_2 synthesis (IC_{50}= 22µg/ml) when compared with its effect on cyclo-oxygenase (IC_{50}= 800µg/ml; 168). Future research may reveal whether imidazole exerts its analgesic, anti-pyretic and anti-inflammatory activities in carrageenin edema and adjuvant arthritis in rats through interference with TXA_2 biosynthesis, and/or through one of its other activities (208).

Prostacyclin (PGI_2) synthetase ($PGH_2 \rightarrow PGI_2$ isomerase). This enzyme is present in the endothelium of arteries and veins of several species and can be obtained in a microsomal fraction (48,170). Prostacyclin (originally designated as PGX, and now designated as PGI_2; 9) is unstable at pH 7.6 ($t_{\frac{1}{2}}$ = 10 min, 20°C), when it disintegrates into 6-keto-$PGF_{1\alpha}$, and stable at pH 8.4 and above. In most solvent systems the chromatographic properties of 6-keto-$PGF_{1\alpha}$ are very similar to PGE_2, but are not changed during treatment with mild alkali (eg. 130,195). The discovery of 6-keto-$PGF_{1\alpha}$ followed earlier observations that homogenates from rat tissues synthetize 2 cyclic enolethers from AA (194). Thus, it is now evident that PGI_2 synthesis occurs also in tissues other than vascular endothelium. A comparison of rat tissues indicated a significant formation in the stomach (especially fundus) and lung (197), and PGI_2 is the main metabolite of AA in the isolated perfused rabbit heart (66,130).

PGI_2, but not its degradation product 6-keto-$PGF_{1\alpha}$, is a very potent inhibitor of platelet aggregation (48,170). Moreover, it causes profound relaxation of isolated strips of mesenteric, coeliac and coronary artery (48,72). PGI_2 synthetase is not inhibited by indomethacin, but 15-hydroperoxy arachidonic acid inhibits the conversion of PG endoperoxides into PGI_2 (48,72). It has been postulated that PGI_2 is important for prevention of deposition of platelets on the vessel wall and that the inhibition of PGI_2 generation by hydroperoxy arachidonic acid is a factor in the development of diseases where increased lipid peroxidation occurs, such as atherosclerosis (171).

2.1.6. Prostaglandin transport and metabolism.

Most of the catabolism of the "classical" PGs occurs intra-cellularly (see below). Thus, if they are released into circulation, they must penetrate cell membranes before degradation can take place. Free diffusion through a biomembrane generally does not occur (eg. 22). This implies that PGs may be long-acting substances when present in extracellular fluid (eg. inflammatory exudate), provided that metabolizing enzymes are not present in the same compartment, and that metabolism requires an initial step of transport across a membrane.

PG transport. Energy dependent, uni-directional transport of PGE_1 and $PGF_{2\alpha}$ has been demonstrated *in vitro* (rabbit vagina, lung and kidney cortex) and *in vivo* (24). It is a rapid, saturable, temperature dependent process that can establish a relatively high concentration gradient and is inhibited by metabolic inhibitors, $PGF_{2\beta}$, probenecid, bromcresol green and indomethacin (IC_{50}: 10-50 μM; 23).

PG metabolism. In several tissues, i.e. brain, liver, spleen, heart, lung and erythrocytes, PGE can be stereospecifically reduced to PGF_α by a PG-9-keto reductase (115; see fig. 2.2). Its *in vivo* activity may be regulated by the balance between oxidized and reduced coenzymes (145), namely NADH for the cytoplasmatic and NADPH for the microsomal enzymes (eg. 122, 145,147). PG metabolizing enzymes were found to be absent in plasma, except for PGA isomerase in sheep plasma (133).

When injected intravenously PGs are rapidly metabolized in the lung (>97% within 1.5 min) by 15-hydroxy-PG dehydrogenase (E.C. 1.1.1.141) to 15-keto-PGs (177) which are substrates for a reduction at C13 by Δ-13 re-

ductase. 15-keto-PGs were shown to be considerably less active in several biological systems (7,61,203). Both cytoplasmatic enzymes are present in several tissues, especially in lung, spleen and kidney cortex (8). The metabolism of PGs proceeds further in mitochondria with β-oxidation (one or two steps) of the carboxyl end. Moreover, ω-oxidation, yielding ω-hydroxy compounds and eventually dicarboxylic acids may take place. In some species, β-oxidation of the ω-end of the dioic acids leads to formation of C_{14} metabolites. Pathways, which are rather complex, have been elucidated, for instance for man (eg. 100), and rat (238).

2.1.7. Other fatty acid oxygenating pathways.

Non-enzymatic lipid peroxidation of PUFA's, either as free acids or when esterified in phospholipids proceeds easily in the presence of oxidized haem compounds and iron sulphur compounds (136). The membrane fatty acids are protected against this destructive oxidation by α-tocopherol (vitamin E). Once an autocatalytic lipid peroxidation has started, cell damage can be reduced by glutathione peroxidase, that converts hydroperoxides to the less damaging alcohols (eg. 126). During auto-oxidation of PUFA's a small percentage of PGs can be formed (190,207).

In platelets a lipoxygenase transforms AA in 12-L-hydroperoxy eicosetraenoic acid, which is finally converted into the corresponding hydroxyfatty acid (HETE) (fig. 2.3). It is a soluble enzyme, for which free arachidonic is the "best" substrate, but other eicosapolyenoic acids, possessing at least two cis double bonds at n-9 and n-12 (eg. 20:3 (n-9)) are also metabolized into L-12 hydroxyfatty acids (Nugteren, 1975). In contrast to the "explosive" burst in cyclo-oxygenase activity in platelets, the lipoxygenase activity is relatively long lasting in vitro (Nugteren, 1977). Rabbit neutrophils contain a lipoxygenase that transforms AA into 5-hydroxy-eicosatetraenoic acid (38). The physiological role of both lipoxygenases is unclear, but HETE is claimed to be chemotactic in vitro for human polymorphonuclear leukocytes (244). The plant lipoxygenase product 15-hydroperoxy AA is an inhibitor of PGI_2 biosynthesis (see 2.1.5).

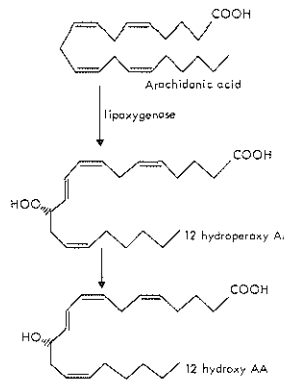


Fig. 2.3. Lipoxygenase pathway in blood platelets.

2.1.8. Prostaglandin assay methods.

For the determination of nanogram (ng) quantities of PGs several methods are available: gaschromatography with electron capture detectors, gaschromatography- mass spectrometry, radioimmunoassay (see review 222), and bioassay (eg. 98,204). With bioassay the unknown sample is tested on an isolated tissue immersed in, or superfused with, a physiological salt solution, and the contraction (or relaxation) of the tissue is calibrated with standard doses of the agonists (eg. PGs). For practical reasons, the cascade superfusion technique (Vane, 1964), which permits simultaneous parallel bioassay with several tissues, was used (see chapter 3). Both radioimmunoassay and bioassay are rather non-specific, since compounds other than those which are supposed to be detected, may interfere with the assay. The selectivity can be improved by extraction and purification of biological samples. Moreover, the physiological salt solution, that superfused the tisseus, contained a mixture of antagonists to rule out as many interfering substances as possible (98). Bioassay has one advantage over the other methods in that it enables a relative assessment of unstable, biologically active compounds, such as TXA_2 (eg. 179), to be carried out. The selectivity of some assay tissues is shown in table 2.1.

Suspension of the superfused tissue in mineral oil raises the sensitivity and permits assay of autacoids in the picogram (pg) range (Ferreira & De Sou-

Table 2.1. Different biological activities of PG endoperoxides, TXA_2, PGI_2, PGE_2 and $PGF_{2\alpha}$.

Tissue	Agonists					References
	TXA_2	PGG_2 PGH_2	PGE_2	$PGF_{2\alpha}$	PGI_2	
RAS	+++	++	+	+	0	48, 114, 179, 204
RCA*	+++	±	±	0	rel.	42, 47, 48
RSS	?	++	+++	++	+	98, 114, 204
RC	0	+	++	++	±	98, 48, 104
BCA	+	+	+	?	rel.	72

+, ++, +++: increasing contractile potency. ±: without clear-cut activity or conflicting data. 0: without activity. rel.: causes relaxation. ?: biological activity not yet reported. *: the rabbit mesenteric artery has a similar selectivity. RAS: Rabbit aorta strip. RCA: Rabbit coeliac artery strip. RSS: Rat stomach strip. RC: Rat colon. BCA: Bovine coronary artery strip.

za Costa, 1976). An adaption of this laminar flow technique permitted superfusion of several tissues (Bult et al., 1977; chapter 3).

2.2. Compounds which interfere with prostaglandin biosynthesis.

2.2.1. Substrate analogues (see review by Flower, 1974)

PUFA's. Some naturally-occurring all cis unsaturated fatty acids, such as oleic (18:1, n-9), linoleic (18:2, n-6), linolenic (18:3, n-3), and eicosatrienoic (20:3, n-9) acid are competitive inhibitors of cyclo-oxygenase from seminal vesicles, rat stomach (198,282), and human skin (280). Oxygen uptake was undetectable with 18:1(n-9), 20:3(n-9) and 20:3(n-3), which again indicated that eicosatrienoic acid (n-9) was not a substrate for the cyclo-oxygenase (282). This competition of naturally-occurring PUFA's with AA may be a regulating factor in PG biosynthesis (Pace-Asciak & Wolfe, 1968).

Acetylenic analogues. Eicosatetraynoic acid (TYA, or 20:4, n-6), an analogue of AA with acetylenic bonds, is a potent inhibitor of cyclo-oxygenase, soybean lipoxygenase (3) , and platelet AA lipoxygenase (117). TYA inhibits PG biosynthesis in microsomal enzyme preparations (245), intact platelets (275), and in vivo (8).

Cis-trans conjugated unsaturated PUFA's. The 8-cis, 12-trans, 14-cis analogues of 20:3(n-6) and AA are competitive inhibitors of PG biosynthesis and could be recovered at the end of the reaction (185,248).

2.2.2. Non steroidal anti-inflammatory drugs (NSAIDs).

The drugs which comprise this group have diverse chemical structures, but they all share (to varying degrees) the antipyretic, analgesic and anti-inflammatory aspects of aspirin (acetylsalicylic acid; see for reviews: Flower (1974); Ferreira & Vane (1974)). Indomethacin, phenylbutazone, paracetamol and salicylate, together with aspirin, are the more familiar NSAIDs. In 1971 3 important reports appeared together, showing that NSAIDs inhibit PG synthesis (82,233,254), a finding that has been confirmed in numerous assay-systems (see Flower, 1974). The rank order of potency of the NSAIDs is independent of the enzyme preparation tested, and, in vitro, the rank order or decreasing potency is: indomethacin > phenylbutazone > aspirin (see 83,86). Salicylate does not inhibit cyclo-oxygenase in vitro (eg. Vane, 1971). Although the above rank order of potency is generally consistent with most papers, the IC_{50} values of the different drugs may vary by 100 fold for different enzyme sources (see 86).

In assessing IC_{50} values, the following factors may be of importance: 1. The substrate concentrations influence the apparent IC_{50} data (62). The potency of indomethacin was identical in several rabbit tissues, if similar experimental conditions were employed (205). 2. The time of preincubation of the enzyme preparation with the drug may affect the nature of inhibition (see below). 3. The presence of albumin in the assay-system may lead to an underestimate of the IC_{50} value. For instance, the binding of indomethacin to plasma proteins may range from 90% to 99% (see 86,159) and is influenced by other substances that bind to albumin (eg. 63,160).

In general, the in vivo anti-inflammatory potency correlates well with actions against PG production in vitro (see 83,86), with the exception of salicylate, which has a much weaker activity than aspirin in vitro (82,89, 254). It has been suggested that salicylate requires metabolic transformation for activity (86,90). Gentisic acid, one of the minor metabolites of salicylate, has indeed, been found to possess greater efficacy than salicylate in inhibiting subcellular PG synthesis, but was still less potent than aspirin (90). Therefore, salicylate, which is as active as aspirin as anti-

inflammatory drug, probably acts independently of cyclo-oxygenase inhibition (230).

The mode of action of NSAIDs at a molecular level. Due to the complex kinetics of the PG synthetase, there are several possibilities for inhibition. NSAIDs, except for paracetamol, act at the substrate binding site (142). The binding of the drug is initially reversible, but then a time dependent, irreversible inhibition occurs, for which the free carboxylic groups of NSAIDs are necessary since their methylesters and their primary alcohols lack the destructive properties of the parent NSAIDs (205,210,216). It has been conclusively shown that the acetyl group of aspirin selectively acetylates purified oxygenase in proportion to the amount of native, undenaturated enzyme (216,217). Indomethacin and AA interfere with this acetylation by aspirin (121,219). The irreversible blockade seems an important feature of aspirin since the prolonged presence of low concentrations, which interact with only 10% of the total enzyme, may result in a progressive loss of all enzymatic activity (Flower, 1974).

Effects of NSAIDs which are not directly related to PG biosynthesis. It is now generally accepted that NSAIDs, with the exception of salicylate, act preferently via inhibition of PG biosynthesis. However, no drug has a single mechanism of action and a number of side effects have been summarized by Flower (1974). The only effects occurring at, or just above, the concentrations that suppress PG biosynthesis, are inhibitory actions on phosphodiesterase, PG metabolism and inhibition of neutrophil mobilization *in vivo*.

Phosphodiesterase-inhibition (152,235). The concentrations needed for 50% inhibition are relatively high, but aspirin was only 3 times less active in inhibiting phosphodiesterase from guinea pig gastric mucosa than as inhibitor of PG biosynthesis (152).

Interference with PG transport (23) and metabolism (86). Lower concentrations of NSAIDs stimulate, while higher concentrations suppress PG transport. The IC_{50} of indomethacin in some preparations was between 10 and 12 μM, which was higher than the IC_{50} in subcellular cyclo-oxygenase preparations (23), but it must be realized that higher concentrations of indomethacin are needed to block PG biosynthesis in tissue slices (eg. 210). For inhibition of 15-hydroxy PG-dehydrogenase the reader is referred to Flower (1974).

Inhibition of leukocyte migration. Migration of rat neutrophils *in vivo* is inhibited by high doses of NSAIDs (262). This inhibition is not due to a

reduction in PGs, since 80% inhibition of PG biosynthesis by TYA did not alter leukocyte migration (93,230).

Some miscellaneous actions of NSAIDs may possibly be related to their anti-inflammatory activity. NSAIDs increase membrane permeability in cells, cell-organells and liposomes and uncouple mitochondrial ATP formation (eg. 76). NSAIDs have metal chelating properties (161), which led to the suggestion that salicylates, and especially their copper complexes, enhances dismustation of superoxide anion, generated from phagocytizing leukocytes and thereby protect phagocytizing cells and synovial cells from destruction by hydroxyl radicals (212). Salicylate and aspirin, in contrast to other NSAIDs, increase urinary excretion of imidazole acetic acid, a metabolite of histamine and L-histidine, which is reported to have analgesic activity (12).

2.2.3. Corticosteroids (review: see Gryglewski, 1976).

Recent reports (table 2.2) indicate that hydrocortisone and synthetic corticosteroids inhibit the release of PGs from intact cells, tissues and organs, but do not inhibit the formation of PGs from AA. A reasonable explanation for these observations is that corticosteroids reduce availability of endogenous PG precursors. This is supported by data on inhibition of AA release by corticosteroids from guinea pig lung and transformed mouse fibroblasts. The spontaneous, RCS-RF-induced and H-induced, but not the Bk-induced release of AA from guinea pig lungs is suppressed by corticosteroids (26, 182). The liberation of AA in transformed mouse fibroblasts, stimulated by serum, bradykinin or thrombin (128) is also inhibited by corticosteroids. The inhibition of PG-release by different steroids correlates well with their potencies as anti-inflammatory drugs (182). There is some specificity with respect to different types of steroids since neither aldosterone (85, 103) nor progesterone (85) are able to reduce AA release, but an oestrogen showed some inhibitory activity (182). The in vitro inhibitory action in guinea pig lungs reached a maximum only after 60 min (26), and the route of administration may influence the efficiency of corticoids in vitro (102).

Corticoids fail to inhibit AA and/or PG release from disrupted cells (26, 82,254). This in contrast to the weak anti-PLA_2 drugs mepacrine (257) and procaine, which inhibited PLA_2 activity in homogenates of guinea pig lung (26). Thereby a direct action on PLA_2 seems to be ruled out. As stated in 2.1.3 an apparent increase in PLA_2 activity may result from:

Table 2.2. Inhibition of PG and AA release by corticosteroids.

tissue	species	stimulus	inhibition of release of AA	inhibition of release of PGs	ref.
IN VIVO					
inflammatory exudate	rat	CAR	?	+	80,105[a]
hindleg	dog	exercise	?	+	123
IN VITRO					
perfused spleen	cat	NA	?	+	102
fat pad / adipocytes	rabbit	ACTH	?	+	51,150
mesenteric artery	rabbit	NA	?	+	106
lung	guinea pig	none	+	+	26,182
		histamine	+	+	
		RCS-RF	+	+	
		Bk	-	-	
sensitized lung	guinea pig	antigen	+	+	26,182
transformed fibroblasts	mouse	culturing	+	+	128,241
		serum	+	+	
		Bk	+	+	
		thrombin	+	+	
rheumatoid synovia	human	culturing	?	+	135
synovial fibroblasts	human	Bk	+	+	181
synovia	rat	arthritis	?	+	85

+ : inhibited, - : not inhibited, ? : not measured. a : the inhibition was possibly due to interference with neutrophil migration.
ACTH: adrenocorticotropic hormone.

1. Activation of PLA_2. 2. Increase in zymogen pro-PLA_2 from lysosomal sources. 3. Alterations in the fluidity of the phospholipid bilayer in biomembranes, allowing a PLA_2 attack (68,228,286). 4. Reduced reacylation of liberated AA.

These factors may be influenced by steroids. The introduction of cholesterol into a phospholipid membrane markedly influences the properties of the bilayer (eg. 67,199). Cholesterol in monotectic mixtures interacts non-randomly with different phospholipids in membranes (67). Data on the effects of corticoids on membrane behaviour with respect to fluidity, packing, per-

meability and vulnerability towards attack by different phospholipases are lacking. Such data might provide a basic understanding of their pharmacological actions, and for the suggestion that their stabilization of membranes results in a reduced PG release (103). Lewis & Piper (1975) suggested an alternative mode of action for corticosteroids, based on their experiments with adipocytes. They suggested that, during lipolysis, PGs are transported (or leak) from the inside to the outside of the adipocyte and that corticoids inhibit this transport.

In conclusion, the mechanisms of corticosteroid inhibition of PG release in different situations are still a subject for controversial dispute. More biochemical and cellular research is needed to clarify the various biochemical and pharmacological actions of these compounds.

2.3. Essential fatty acid deficiency (EFA deficiency)

2.3.1. Description

EFA deficiency was first described by Burr & Burr (1929). Some of the symptoms that may occur are listed in table 2.3. (for reviews, see 1,4).

Table 2.3. Some symptoms of EFA deficiency in the rat

1. weight	decreased
2. skin	increased permeability to water, epithelial hyperplasia (scaly lesions), tail necrosis. The latter symptoms are favoured by low humidity
3. kidney	enlarged, intertubular haemorrhage
4. heart	enlarged
5. adrenals	weight decreased in females and increased in males
6. reproduction	females: irregular oestrus, impaired reproduction males: degeneration of seminiferous tubules

The biochemical basis of EFA deficiency is well known (see 109,146). In higher organisms certain saturated fatty acids (eg. 18:0) may be transformed into monoenoic acids (eg. 18:1, n-9) by a desaturase reaction in the endoplasmatic reticulum. Additional double bonds are introduced between the first double bond and the carboxyl group in animals (fig. 2.3), and towards the methyl end in plants. Thus linoleic acid (18:2, n-6) may be formed from

oleic acid in plants. It is the first member of the n-6 family, which are necessary to maintain animals in a healthy state. The inability of animal tissues to desaturate oleic acid towards the methyl end of the chain implies that linoleic acid must be supplied in the diet. Linoleic acid and related n-6 PUFA's are therefore, called essential fatty acids. A simplified scheme of the pathway leading to AA (n-6 family) and the pathway that predominates if linoleic acid is omitted from the food, is shown in fig. 2.3 (see also 18,109,234).

During elongation and desaturation competitive inhibition occurs among different series of PUFA's which normally maintains the balance between fatty acyl residues of different biomembranes (eg. 18,234). It is possible to induce EFA deficiency by feeding with several different diets, as long as unsaturated fats are lacking. In all cases, the synthesis of 5,8,11 eicosatrienoic acid increases (eg. 95) and it replaces AA in several tissues.

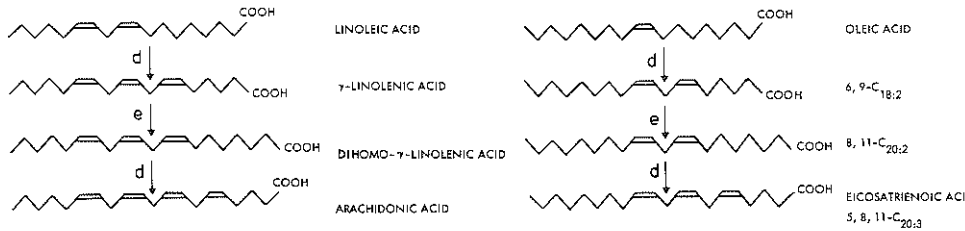


Fig. 2.3. Pathways of fatty acid biosynthesis in animals (adapted from 109,146). The normal pathway starts from linoleic acid and leads to formation of arachidonic acid (shown on the left). When an EFAD diet, lacking linoleic acid, is given to the animals, the pathway from oleic acid to 5,8,11 eicosatrienoic acid becomes more important (shown on the right). d=desaturation, e=elongation.

Data on fatty acids are often reported using a shorthand notation, showing the number of carbon atoms, followed by a colon and then a figure denoting the double bonds. The position of the double bonds is defined by counting the carboxylgroup as 1, and indicated by the preceding numbers. Thus, linoleic acid is 9,12 $C_{18:2}$. The position of the methylene-interupted can also be defined by counting from the methylgroup (n). This facilitates the identification of fatty acids that are derived from one another by chain elongation. Thus, oleic acid can be referred to as 18:1(n-9).

Holman (1960) has suggested that the ratio of 20:3 to 20:4 might be used as an indication of the degree of EFA deficiency, and that a ratio of 0.4 or less indicates normal EFA status in rats. This 0.4 ratio is taken rather arbitrarily in the gradual transition from a normal status into an EFAD condition, and is dependent on lipid class and cell type. AA is removed less quickly from phosphilipids than from neutral fats, and AA is retained much longer in brain, in intestinal muscle and in mucosa cells, than in erythrocytes, liver and heart cells (see 4). Thus, conclusions on the basis of EFA deficiency as a model for AA (PG precursor) deficiency are only possible if a fatty acid analysis of the tissue under study is carried out. The Holman criterion does not give a quantitative indication (as it is only a ratio) of the absolute reduction of AA in a certain cell type.

The induction of EFA deficiency is accellerated by feeding the animals before birth with an EFAD diet, as otherwise linolec acid in the mothermilk can provide a pool of EFA's for the neonates (eg. 96). Moreover, EFA deficiency is more easily induced in male rats than in female rats, and is retarded when the animals have access to their faeces (see 1).

2.3.2. EFA deficiency and prostaglandins.

PGs are synthetized from EFA's (17,250,251). Van Dorp and colleagues tried to find evidence for the hypothesis that the EFA's are irreplaceable because no biologically active PGs can be formed from other PUFA's. Several PUFA's, which do not occur naturally in animal tissues were synthetized and their rates of conversion to PGs increased from 19:3 (n-5), 21:3 (n-7), 19:4 (n-5), 21:4 (n-7), 20:3 (n-6) to 20:4 (n-6) and, when measured by weight gain and the skin permeability test, their EFA activity increased in the same order. Moreover, the "artificial" PGs possessed several biological activities (14,236,248). The discovery of odd-numbered PUFA's with EFA activity lead to the revision of the hypothesis that a pair of n-6 and n-9 double bonds (present in linoleic acid) is a prerequisite for EFA activity (223). In all PUFA's that have an EFA activity, 3 methylene-interrupted cis double bonds are present at c8, c11, c14, and a parallelism does exist between EFA potency and the substrate requirements of the cyclo-oxygenase (223,249).

Direct evidence for a causal relationship between a decreased PG production and the variety of EFAD symptoms is relatively scarce. In rat adipose tissue does experimental evidence exist for the lack of PG precursors being

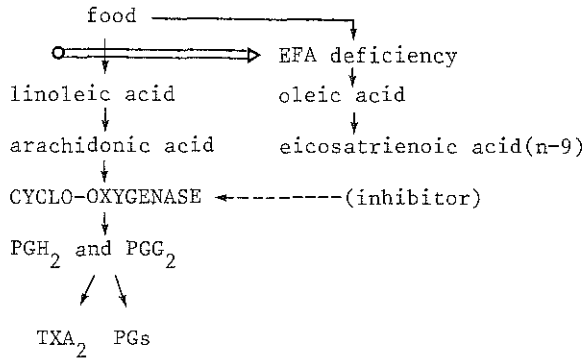


Fig. 2.4. Lack of linoleic acid in food leads to EFA deficiency. This is attended with shortage of PG endoperoxide precursors.

positively correlated with an enhanced lipolysis during EFA deficiency (53). EFA deficiency reduces the capacity of rat platelets to aggregate in response to threshold doses of collagen (42, chapter 4). This coincided with a reduced endogenous synthesis of TXA_2. Daily topical treatment with PGE_2 in high doses (50-100 µg/paw), reduced the scaly lesions, which might be associated with an inhibition of abnormal sterol esterification in EFAD rat skin (281). Decreased PG biosynthesis during EFA deficiency has been observed in rat epidermis and rabbit inner kidney medulla (248).

2.3.3. EFA deficiency influences properties of biological membranes

A shortage of EFA's is accompanied by morphological and biochemical changes (see 4), for example in mitochondria (enlarged, increased tendency to swell, uncoupling of oxidative phosphorylation) and erythrocytes (more liable to osmotic lysis), which are unlikely to be dependent on changes in PG metabolism. This is supported by the fact that daily PG-metabolite production in man is 10^4 times less than the daily intake of linoleic acid (eg. 249).

Thus, EFA's must be essential in other respects, for instance, as constituents of certain phospholipids, where their acylchains will contribute to fluidity and hydrophobicity of biomembranes. Several reports indicate that a reduction of double bonds in phospholipids during EFA deficiency influences membrane properties and leads to changes in activities of membrane-bound

enzymes. Some examples are: decreased basal and stimulated adenylate cyclase- and increased (Na^+, K^+, Mg^{2+}) ATPase activities in EFAD rat liver cells (40), increased (Na^+, K^+) ATPase activity in EFAD mouse brain cells (240), and decreased monoamine oxidase activity in rats (19). The Hill coefficients (indicating the number of allosteric binding sites) of several rat enzymes were correlated with the double bond index, and the allosteric behaviour of Na^+ and K^+ ATPase (erythrocytes), acetylcholine esterase (erythrocytes), Mg^{2+} ATPase (erythrocytes, heart, kidney and brain) was directly influenced by EFA deficiency. It is likely that similar membrane studies will provide deeper insights into several metabolic aspects of EFA deficiency, such as uncoupling of phosphorylation, increased heat production, increased loss of water and decreased capacity to excrete Na^+ and K^+ in response to loading (218).

Summary. The amounts of PG precursors are diminished during EFA deficiency. It is, however, doubtful if all EFA symptoms are controlled by PGs. A basic role for AA in controlling membrane behaviour might be equally important. When using EFA deficiency as a model for PG precursor (AA) deficiency, the measurement of AA levels is an obvious prerequisite for clear cut conclusions. Experimental data have to be interpreted with caution since EFA deficiency does alter membrane properties, eg. activities of adenylate cyclases and ATPases.

2.4. Blood platelet aggregation

When blood vessels are damaged, platelets stick at the site of injury, aggregate and stabilize the haemostatic clot that is formed in order to stop bleeding. Platelet aggregation can be studied in vitro. Recently the important role of PG metabolism in controlling platelet function has become more clear.

2.4.1. General aspects (for reviews see: 175,269).

The data in this chapter refer to human platelets, unless another species is indicated. They are disc-shaped (thickness: 0.5-1 µM, diameter: 2-5 µM), their number ranges from 200,000 to 400,000 per µl of blood, and they are produced in bone marrow by invagination of the megakaryocyte plasma membrane (15). Normally, thrombocytes circulate in the blood for 10 days (shorter than

red blood cells), being nonadherent to each other or to vascular endothelium. When a blood vessel is damaged their capacity to adhere to collagen, or other substances, becomes apparent (269). Adhesion initiates a secretory process during which substances, present in subcellular granules, are extruded from the cell, while the other cell constituents (eg. mitochondria, cytoplasm) are retained. A short, morphological description is given below.

Plasma membrane. Proteins (176) and phospholipids are asymmetrically distributed in the plasma membrane. PE and PS are mainly localized in the inner half of the lipid bilayer, whereas PC and Sph are present in the outer half (54,224). Some PE is detectable on the outside during thrombin treatment (224). Invaginations of the membrane, forming a sponge-like system of open channels which penetrate through the platelet cytoplasm, enlarge the surface of the platelet and facilitate both the uptake of substances from blood plasma and release of granule bound products (15).

Granules. Platelets have no nucleus or DNA, and contain few ribosomes, little RNA, and few mitochondria. At least 2 types of granules are distinguishable, α granules (the majority) and dense granules. The former contain lysomal enzymes and the dense granules are filled with ADP, ATP (the non-metabolic or storage pool), calcium and serotonin (eg. 269). A band of cylindrical structures (microtubules) line the peripheral edge of platelets and probably maintain their asymmetrical form. Microtubules and actin-like filaments seem to be involved in the clustering of α granules in the centre of the cytoplasm, while dense granules are forced to the periphery during the shape change (64).

Aggregation. Platelet aggregation can be studied in vitro in an aggregometer. For this purpose, platelet-rich plasma (PRP) is prepared from blood, to which anti-coagulants have been added. The choice of anti-coagulant may have implications for platelet behaviour (eg. 175). PRP is then obtained from blood by centrifugation at relatively low G forces. Platelets are extremely sensitive to manipulation and may stick to each other in response to haemolysis and changes in temperature, pH, G forces, etc., etc. (175,220). The merits of different methods for preparing PRP have been discussed (65). The PRP is placed in a cuvette, continuously stirred at a constant temperature, and the light transmission is monitored (39). During aggregation, an increase in transmitted light is observed (see fig. 4.1, page 59).

ADP-induced aggregation. ADP (not ATP, or AMP) induces a rapid shape change, from discs to spiny spheres (a process that is not Ca^{2+} dependent), and

when Ca^{2+} and fibrinogen are present, the platelets reversibly aggregate. With higher ADP concentrations the contents of the dense bodies are released (Release I; release of ADP, Ca^{2+} and 5-HT; eg. 175,269). This reaction is energy-dependent and ATP from the metabolic compartment is used. It is suggested that the released ADP induces the second wave of aggregation (eg. 175,269). The secondary wave of aggregation is not visible if a higher initial dose of ADP is used, and is absent in several other species (eg. rat) and if heparinized instead of citrated PRP is used (175). The suggested sequence of events is depicted schematically below:

ADP → primary aggregation → release reaction I → 2nd phase of aggregation

Thrombin- and collagen-induced aggregation. Thrombin can aggregate platelets, either directly or by inducing release reaction I. During platelet aggregation phospholipids become available, and serve as a catalytic surface (platelet factor, 3) for the conversion of prothrombin into active thrombin by factor X, factor V and Ca^{2+}. Thus, both clot formation and platelet aggregation are interrelated and reinforce each other via thrombin (see 269).

Collagen: after adhesion to soluble collagen, aggregation and the release reaction can occur (269). High doses of both collagen and thrombin may induce a "second release reaction" (release II), during which the contents of α-granules are liberated in addition to the substances that are secreted during release I (269).

2.4.2. The role of cyclo-oxygenase products in platelet aggregation.

Zucker and Peterson (1968) discovered that aspirin abolished release I and the second phase of ADP-induced aggregation. The discovery that aspirin inhibits endogenous PG biosynthesis in platelets (233) made the role of PGs in platelets rather paradoxical: classical PGs had little activity in platelets, except for PGE_1, which inhibited aggregation (140). Vargaftig & Zirinis (1973) were the first to show that AA induces aggregation with the concomitant formation of RCS (TXA_2 + PG endoperoxides) and classical PGs, and that both reactions are prevented by NSAIDs. The generation of RCS provided the clue to the role of PGs in platelet behaviour. It appeared that purified PG endoperoxides (PGG_2, PGH_2) are potent platelet-aggregating agents (10-300 ng/ml; 116). Moreover, during aggregation of washed platelets with thrombin, the PG endoperox-

ides are released into the medium in similar concentrations (119). This has been confirmed in PRP, stimulated with AA, collagen and adrenaline (231).

Later on it was suggested that the PG endoperoxides require a conversion into TXA_2 in order to induce aggregation (120). The isomerization of PG endoperoxides into TXA_2 is indeed the major pathway in platelets. TXB_2, and at least a part of the released HHT and MDA, are derived from TXA_2 (84,101,239). The hypothesis that TXA_2 is essential for induction of aggregation is strengthened by the fact that the endoperoxide PGH_1 is not converted to TXA_1 (178, 187) and fails to induce aggregation (276). On the other hand, it has been proposed that the physiological function of TXA_2 is to produce marked localized vasoconstriction, that enhances haemostasis by sharply reducing the blood vessel lumen (178). Specific inhibitors of TXA_2 isomerase may clarify the role of thromboxanes in platelet aggregation.

Exposure of washed platelets to collagen and thrombin leads to increased liberation of AA from phospholipids, through the action of a PLA_2 (20,25,225). Upto 80% of the AA is released during collagen-induced aggregation and PE (58%), PC (25%) and PI (14%) are the major sources of PG precursors (25). PLA_2 activity can be initiated with the Ca^{2+} ionophore A 23187, and the mobilization of intracellular Ca^{2+} may regulate AA release (202). Platelet PLA_2, which is not present on the platelet surface, preferentially hydrolyzes PE, PI and PS (the internal phospholopids), which contain 72% of the AA of human platelets. Dibutyryl cAMP inhibits thrombin - but not A 23178 - induced AA release (202). Evidence for the inhibitory action of dibutyryl cAMP on PLA_2 activity has been provided by Minkes et al., (1977), and by Lapetina et al. (1977). Cyclic AMP had no effect on cyclo-oxygenase activity (144). Since cAMP failed to inhibit the ionophore-induced PLA_2 activity, its inhibition may result from a reduction of free cytoplasmic Ca^{2+} levels in platelets (see fig. 2.6). The phospholipid (and AA) distribution in platelet plasma membrane makes it unlikely that, in intact platelets, an exogenous PLA_2 can mimick the events, which occur during aggregation induced by thrombin and collagen without lysis.

Once the AA has been liberated, it is either incorporated into plasmalogen PE (213) or converted to a large extent into PG endoperoxides (eg. 20). An explosive burst in oxygen consymption by the platelets is observed (eg. 173), which is inhibited by aspirin (174). According to most authors, the endoperoxides exert their effects via induction of the release I reaction (eg. 156,231,269,276), as shown in fig. 2.5. Other reports indicate that PG

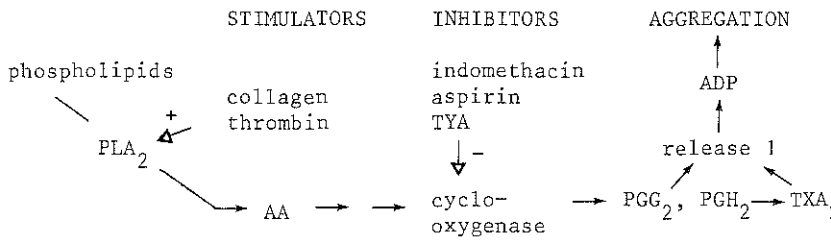


Fig. 2.5. Pathway leading to aggregation induced by AA, collagen and thrombin, and its inhibition by NSAIDs and TYA.

endoperoxides can induce aggregation without release reaction (139), or that the release reaction may start before PG endoperoxide formation (165,174).

The subcellular mechanisms of TXA_2 are unknown. PGG_2, PGH_2 and TXA_2 are inhibitors of PGE_1-stimulated cAMP accumulation in human platelets, but have no effect on basal levels of either cAMP or cGMP (163,164). Thus, it is unlikely that the pro-aggregatory effect of TXA_2 is directly connected to cyclic nucleotide metabolism.

Inhibitory effects of PGs on platelet behaviour. Recently, 2 prostanoic inhibitors of platelet aggregation, which are more potent than PGE_1 (140), have been discovered: PGD_2 (183) and PGI_2 (170). PGE_2 is less active as an inhibitor of platelet aggregation and may potentiate aggregation induced by other stimuli (eg. 154). The potencies of PGE_1 and PGD_2 are reduced when heparinized, instead of citrated PRP is used (154). The inhibition is species dependent, since PGD_2 fails to inhibit rat platelet aggregation (154,183).

The inhibition is mediated by platelet adenylate cyclase. PGI_2, the strongest inhibitor of platelet aggregation discovered so far, is also the most powerful activator of adenylate cyclase in intact platelets and isolated membranes (99,242). Ca^{2+} ions alone can inhibit PGE_1-induced stimulation of adenylate cyclase (215) and this may explain why PGE_1 and PGD_2 are less effective in heparinized PRP (see above).

How the increased intracellular levels of cAMP suppress platelet function is not clear yet. An interesting hypothetical scheme of the interrelations between PG production, cAMP metabolism, Ca^{2+} levels and platelet function has been given by Salzman (1976). An adaption of this scheme, in order to make it consistent with recent data, is shown in fig.2.6. It has many areas of uncer-

tainty, but it may help to understand some of the many interrelations which have been briefly summarized above. It proposes that platelet function is controlled by a balance between free, intracellular Ca^{2+} levels and cAMP concentrations. It is postulated that cAMP reduces Ca^{2+} levels and that TXA_2 and/or PG endoperoxides can elevate intracellular Ca^{2+} concentrations, either by an ionophore-like action or by an inhibitory effect on Ca^{2+} pumps. There is no evidence for the latter suggestions, and little evidence is available for some other features of the scheme, such as the location of the AA metabolizing enzymes.

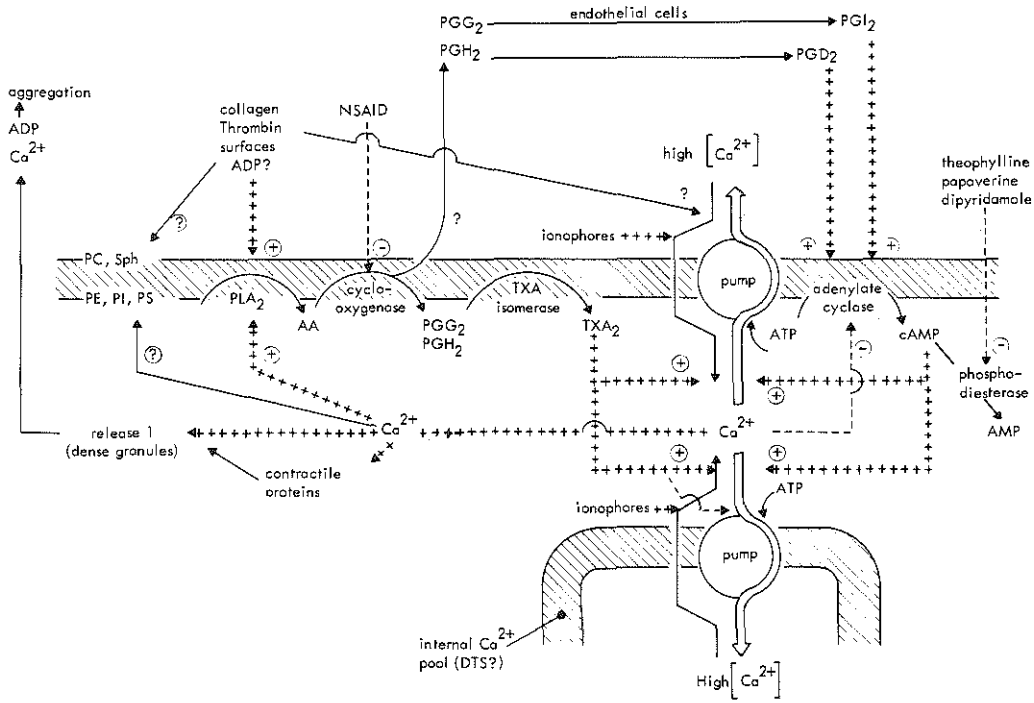


Fig. 2.6. Hypothetical interrelations between calcium transport, cyclic AMP metabolism, thromboxane A_2 production and platelet function. The hatched area's represent plasma membrane (horizontal area) and the membrane which lines an internal calcium pool (curved area). The enzymes (PLA_2, cyclo-oxygenase, TXA_2 isomerase and calcium pump) may be located in either one or both membranes. DTS: dense tubular system; +++▶: stimulation; ---▶: inhibition; $\xrightarrow{?}$: possible interaction not yet known (After Salzman, 1976).

2.5. Acute inflammatory reactions.

2.5.1. Introduction

Inflammation has been defined as a process which begins following sub-lethal injury to tissue and ends with complete healing or permanent destruction of the tissue (suggested by Ebert & Grant, 1974). It implies that it is a process, in which various homeostatic mechanisms influence one other, possibly in an attempt to preserve the viability of tissues. However, in certain cases, the inflammatory signals seem to be scattered and disarranged, leading as a consequence, to a chronic process, rather than proceeding stepwise, through a homeostatic cascade, to a non-imflammatory end-point (see 73,74,192).

2.5.2. Carrageenin-induced hind paw edema (CAR edema).

In vivo models have been developed in order to evaluate the anti-inflammatory activity of drugs (review: 105). In several acute models, the exudation in response to injury is measured. As a consequence, the drugs, which have been discovered with these models are capable of suppressing edema, but do not necessarily interfere with essential steps in the chain of events between injury and the end of the process. These models have also been employed for more basic studies. The carrageenin-induced reactions in rats are described as an example of acute, non-immunological inflammatory reactions. The seaweed extract carrageenin (carrageenan, CAR; 277) has become one of the most used irritants and a multi-step pathway has been proposed in CAR edema development (262).

Definition of the reactions. Vinegar et al. (1976) discriminate between edemagens (5HT, H), that lead to vascular leakage of plasma, without cell-mobilization, and inflammatory agents (such as CAR) that elicit edema and mobilization of inflammatory cells (see also 192). The action of edemagens is counteracted by their antagonists and not by anti-inflammatory drugs, whereas anti-inflammatory drugs suppress the edema evoked by inflammatory agents. The site of injection of the irritant is of importance. Thus, 5-HT is an edemagen in the rat hind paw, whereas the same dose is without effect in the pleural cavity.

Description of the process. After injection of CAR (0.5 - 1 mg) into the paw, a biphasic swelling is observed (262). The initial exudation (0 -30 min), which is absent in CAR-induced pleurisy, tapers off (30 - 60 min) and is fol-

lowed by a second, longer-lasting swelling. The maximal swelling is reached between 4 and 6 hours. After about 10h, the swelling decreases.

2.5.3. Cellular events in carrageenin-induced edema.

Neutrophils. The involvement of the following cell types seems to be established: mastcells, vascular endothelial cells and neutrophils. The neutrophil (also called polymorphonuclear leukocyte) is an active, motile, relatively short-living scavenger cell, which acts as the first line of defence against tissue damage (73). After injection of CAR (0.5 mg) into the pleural cavity the number of these cells increases from undetectable (0h) to about 10×10^6 cells (3h; 70,200,262). It has been suggested (262) that edema formation is a consequence of neutrophil infiltration, but experimentally induced leukopenia yielded varying results (13,70,262). In the proposed pathway of CAR edema the first steps are the generation and release of an hypothetical neutrophil-attracting factor, and the subsequent peri- and extravascular localization of neutrophils (262).

Monocytes. The numbers of these more persistent scavenger cells are less than 10% of all mobilized cells during acute CAR edema (200,262).

Platelets. Their possible involvement in CAR edema has been reviewed (227, 256). Positive evidence for their role in CAR edema has been obtained, since after i.v. injection of ^{51}Cr-labelled platelets, the label increased at the inflamed site (261). This was not due to an increased blood volume since erythrocyte accumulation was virtually absent (J.E. Vincent & F.J. Zijlstra personal communication). Whether an increased number of platelets stick to vessel walls at the inflammatory site or whether the label also appears outside the vessels is unknown as yet. Rabbit platelets release several putative mediators of inflammation during incubation with CAR (256,258), but several authors, using thrombocytopenic rats, failed to obtain an indication of involvement of platelets in CAR edema (see review by Vargaftig, 1977).

Lymphocytes. Leukopenia affected the absolute number of lymphocytes more than that of neutrophils and reduced both CAR edema and swelling induced by edemagens (211). Injection (i.v.) of lymphocytes, but not neutrophils, restored both processes substantially. In leukopenic, lymphocyte supplemented rats, a normal histological CAR-edema was seen, except for the striking absence of neutrophils (13,211).

2.5.4. Mediators of CAR edema (see 262,267,274).

The characteristics of a potential mediator have been given in the introduction (see 274). In most studies pharmacological methods have been employed to study the involvement of a pivotal mediator in CAR edema. Local levels of putative mediators are seldom reported. A short description of possible mediators is given below.

Histamine (H) and serotonin (5HT). Both H and 5HT are simultaneously released directly after application of CAR into the pleural cavity and the granuloma pouch model, possibly by mast cells (50,262,274). After an initial peak presumably also present if saline is used as irritant, prolonged high levels of H and 5HT are present for up to 48h. The levels of H are approximately 1000 times higher than those of 5HT . The latter is somewhat more potent as edemagen in the rat paw, but both are inactive in the pleural cavity (262). Both probably mediate the pedal CAR edema, from the start, by increasing vascular permeability. Pretreatment of the animals with compound 48/80, which depletes H and 5HT stores, or treatment with a combination of their antagonists (mepyrapine plus methysergide) gives a reduction of the pedal edema. The H_1 and 5HT blockers do not suppress the CAR edema when given separately (70,92,262).

Bradykinin (Bk). Bk may be formed from kininogen by the kallikrein enzyme, that is activated after prior activation of the Hageman factor. Bk is readily inactivated by kininase (see 74). Bk has been claimed to be present in CAR edema (10). Depletion of kininogen by elagic acid or cellulose sulphate results in a short- (70) or long- (184) lasting reduction of CAR edema. Exogenous Bk is an edemagen in the rat paw (59), but not in the pleural cavity. Bk activates PG release in numerous situations (see Vane 1976 and table 2.2).

Complement. In contrast to the suggestion by Di Rosa et al. (1971) no indication for a role of the complement system in Car edema has been found in recent reports (eg. 184,262).

2.5.5. Prostaglandins and CAR edema.

The role of PGs in inflammatory reactions has been reviewed several times (see 87,255,274,284), and only the role of PGs in CAR edema will be discussed here. The following evidence suggests the pro-inflammatory involvement of PGs in CAR edema:

1. NSAIDs inhibit the later phase of the edema (from 2-3h onwards). The main *in vitro* activity of NSAIDs (except for salicylic acid) is inhibition of PG biosynthesis (see 2.2.2).
2. In rats, immunized against PGE_1, CAR edema is reduced (255).
3. EFA deficient rats displayed a reduced CAR edema (34).
4. It is rather surprising that the report by Willis (1969) in which the presence of PGs is described in edema fluid that was squeezed from the paws in a rather traumatic way, if often cited as an indication of the presence of PGs in CAR edema. In this report, controls are lacking and the author admits that all PG in the fluid could be due to tissue damage.

Since the discovery of the main action of NSAIDs, several investigators have tried to establish pro-inflammatory effects of PGs.

Chemotactic activity. PGE_1 has been claimed to be chemotactic *in vitro* for rabbit peritoneal neutrophils (124). It is unlikely that this has physiological importance since the precursor of PGE_1 is relatively scarce (also in neutrophils, 279). Another report indicates that PGs are not chemotactic for *peripheral* neutrophils from rat, rabbit and man, and high doses of PGE_1 only attract neutrophils harvested from the rabbit peritoneal cavity (266). PGs possibly enhance overall mobility of neutrophils (226), a phenomenon observed with a variety of oxygenated PUFA's (see 268). It is, therefore, not surprising that TXB_2 (37) and HETE (244) display a moderate leucotactic activity. The *in vivo* significance of these observations is unclear yet.

PGs as edemagens. It has been claimed that PGE_1 and PGE_2 increase local vascular permeability in rat skin, as assessed by dye leakage (60,134). However, this method does not discriminate between increased blood flow and enhanced permeability, due to concomitant liberation of amines from mastcells (134). With more sophisticated techniques it appeared that PGs do not induce plasma leakage either in the rat paw (193) or in rabbit skin (271).

PGs and vasodilation. It is wellknown that PGs (A_2, E_1, E_2 and especially I_2) cause vasodilation, although species and tissue differences exist (see review by Messina et al., 1976). The marked increase in local blood flow induced by PGE_1 and PGE_2, but not by Bk, H or 5HT (132,193,271) is most probably the explanation for the "potentiation" by PGs of edema formation induced by H, 5HT and Bk (151,169,243,272). Thus, PGs can modulate the pro-inflammatory effects of edemagens that are normally stored (amines) or not formed (Bk). This may explain why NSAIDs can inhibit PG synthesis almost completely, while

a residual edema still exists, and why PG synthesis can continue physiologically in tissues without the production of edema.

PGs and the pain of inflammation. PGE_2 contributes to pain by "sensitizing sensory nerve endings" towards Bk, but has little effect of its own (see review by Vane, 1976). The peripheral, analgesic activity of NSAIDs in CAR edema is in close agreement with their anti-inflammatory activity, and a reduction in CAR edema results in an "apparent" analgesic effect (263).

PG endoperoxides, TXA_2 and CAR edema. Pro-inflammatory activities of these short living molecules are difficult to assess. PGG_2 reduces local blood flow transiently, but then vasodilation dominates. Potentiation of Bk-induced plasma exudation was then observed, as would be expected from the formation of PGE_2 or PGI_2 (151). As discussed earlier it is unlikely that the endoperoxides normally accumulate when formed. TXB_2 has been found in CAR exudates (52) and the "importance" of TXB_2 as a leukotactic agent has been mentioned. Since platelets form TXA_2 and adhere mainly to venules in an inflamed tissue (287), one might speculate that TXA_2 may increase exudation by contracting endothelial cells in the post-capillary part of micro-circulation.

What is the origin of PGs in acute inflammation ? No direct answer is possible to this question, but platelets, vascular cells, tissue cells, and invading cells, such as neutrophils are possible candidates. It is suggested that phagocytosis of CAR by neutrophils was responsible for both release of lysosomal enzymes and PGs (262,274). Other reports indicate that in acute exudates from CAR impregnated sponge implants, the neutrophil invasion can be reduced (to 80%) without affecting PG formation (93,230), and that the presence of PGE preceded neutrophil invasion in urate crystal-induced inflammation (97).

Summary. Several cell types and mediators are probably involved in CAR edema. Most results indicate that in the absence of a certain type of mediator the edema will still develop. PGs are weak inducers of plasma leakage and have little chemotactic activity. PGs enhance the effects of mediators, such as H and Bk. This modulation, which seems to be of importance for edema development, is probably exerted through a local increase in blood flow. This idea explains why a physiological PG biosynthesis per se (for instance in kidney, seminal vesides, etc.) is not accompanied by exudation or attraction of neutrophils.

3. GENERAL METHODS

3.1. Animals.

Male Wistar rats, obtained from the animal breeding farm of TNO (Zeist, The Netherlands) were used, except for the experiment shown in fig. 6.2 (Chapter 6). The rats were fed with standard food (Hope Farms, Woerden, The Netherlands). EFA deficiency was induced by feeding pregnant rats a diet containing 4% hydrogenated cocosfat (prepared by Hope Farms, Woerden), starting 5 days before the expected day of delivery. After weaning, the newborn rats were kept on EFA deficient food. The EFAD rats were placed in "wire screen bottom cages". The development of EFA deficiency was monitored by weighing normal and EFAD rats once a week (see fig. 3.1). Apart from the diminished growth (± 35% reduction), some other symptoms of EFA deficiency were noticed: enhanced water intake, absence of yellowish-brown pigmentation of abdominal skin and some scaly lesions of the skin. In edema tests EFAD rats were compared with normal rats of approximately the same weight (220-270 g; see fig. 3.1). For platelet studies blood was obtained from normal and EFAD rats of identical age (18-23 weeks).

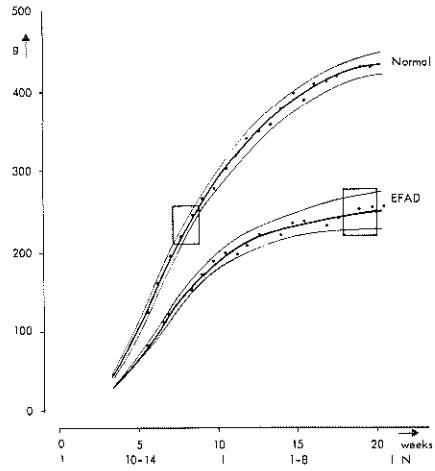


Fig. 3.1. Growth curves of normal and EFAD rats. The lines are drawn along the means of N average weights. These weights were determined by weighing N groups of five rats once weekly. The thin lines are drawn along the s.e.m.; squares indicate the weight ranges utilised in the edema experiments reported in the text.

3.2. Carrageenin-induced hindpaw edema (CAR edema).

Hindpaw edema was induced by injecting 0.1 ml carrageenin (CAR) or saline subplantar into the footpad (see fig. 5.1 for the site of injection). In one experiment (fig. 6.2) an additional 0.05 ml was injected into the footpad at 2h.

Saline. Sterile, pyrogen-free, 0.9% NaCl was obtained from the pharmacist (Dijkzigt Academic Hospital, Rotterdam, The Netherlands).

CAR (carrageenan, Viscarin, Marine Colloids, N.J., USA) was dissolved overnight at 40°C in distilled water, and diluted to 1% (w/v) while the solution was slowly stirred. In several experiments additional irritants were included in the CAR solution (see Chapter 6). For this purpose a solution containing 2% (w/v) CAR was prepared and diluted (1/1; v/v) with the other irritant. Thus, in all experiments 1 mg CAR was injected into the foot.

Edema measurements. Paw edema was measured with 3 techniques:

1. Diameter. Increase in paw thickness (Δ mm) was measured with the apparatus described by Bonta & Noordhoek (1973). Paw swelling was expressed as percent increase of the pre-irritant diameter: $(\Delta mm_{t=x}/mm_{t=0})100$

2. Volume. Increase in paw volume was measured using the mercury displacement method with an apparatus produced by U.Basile (Milan, Italy).

3. Weight. After killing with ether, the paws were severed at the tibiotarsal joint with a small guillotine and their weights measured. The differences (g) in wet weights between the control and inflamed paw served as a measure of the edema.

3.3. Measurements of prostaglandins, thromboxane A_2 and serotonin.

The compounds were bioassayed using isolated, superfused tissues (see 2.1.8). Contractions of the tissues, induced by addition of samples with unknown quantities of autacoids, were calibrated with standard doses of agonists. The selectivity of the assay was raised by using several tissues, arranged in a cascade (Vane 1964) and by addition of antagonists to the superfusing physiological salt solution. The antagonists prevented the tissues from responding to interfering substances. The sensitivity of the bioassay can be increased by means of a laminar flow technique (79). An improvement of the laminar flow technique permitted superfusion of more than one tissue (Bult et al., 1977).

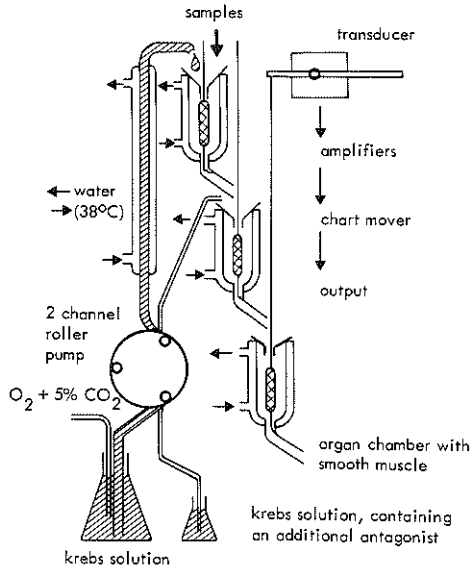


Fig. 3.2. Superfusion of isolated tissues, arranged in a cascade.

Cascade technique. Isolated tissues were arranged in a cascade as shown schematically in fig. 3.2. The organs were superfused (2.5 ml/min, 37°C) by means of a two channel roller pump (Verder, Vleuten) with gassed (95% O_2, 5% CO_2) Krebs, containing (mM): NaCl (118), KCL (4.17), $CaCl_2$ (2.5), $MgSO_4$ (1.18), KH_2PO_4 (1.18), $NaHCO_3$ (25) and glucose (5.6). The injection of substances onto the lower tissues was simplified by placing funnels on the organ chambers and this permitted the use of a relatively low flow rate, since it prevented changes in temperature and humidity in the organ chamber. Contractions were recorded with Harvard heart/smooth muscle transducers, connected either to a Rikadenki SA series multi-pen recorder, or to a Harvard chart mover.

Laminar flow technique. This elegant method was developed by Ferreira & De Souza Costa (1976). The tissue is superfused with a very thin layer of Krebs and is protected from desiccation by suspending it in mineral oil. An adaption (see fig. 3.3; for details: Bult, Parnham & Bonta, 1977) made it possible to use a laminar flow technique in a cascade. The technique gave satisfactory results with all the tissues that were used in the normal cascade, provided that:

1. All glasware was siliconized,
2. Thick threads were used to attach the tissues to the transducers,
3. The bath was filled with Krebs and the mineral oil was poured down the

inside of the bath after the installation of the tissues was finished, 4. The flow rate of the Krebs was directed (if necessary) to the correct side of tissue strips (original inside (in vivo) for vascular tissues and original outside (in vivo) of stomach strips).

The increase in sensitivity is demonstrated in fig. 3.4, where a normal cascade (already optimally sensitive, flow rate 2.5 ml/min) has been compared with the laminar flow technique (0.1 ml/min). Due to the fact that the bolus doses of PGE_2 are 25 fold less diluted in the laminar flow, the same factor predicts the apparent increase in sensitivity. Experimental data confirmed this. Doses of PGE_2 (ng), needed for half-maximal contractions were: cascade 59.6 ± 7.3 (12) and laminar flow technique: 2.4 ± 0.35 (10). The ratio between these values is: 24.8 ± 4.7 (see fig. 3.4).

Tissue selection and preparation. The following tissues were used:

Rat stomach strip (RSS). Rats were killed by a blow on the head and bleeding. The stomach was dissected out and two strips of about 2 mm were cut from the pale ruminal part of the stomach (Vane, 1957). The strips reached a stable base line within 120 min. This tissue is very sensitive to 5HT and PGs, especially PGE_1 and PGE_2. It is rather non-selective since it is also contracted by PGI_2, $F_{2\alpha}$, D_2, G_2, H_2 (see fig. 3.5, and ref. 98,114,170). Generally PGE_2 and 5HT were used as standards.

Rat colon (RC). Pieces (2 cm) of colon were dissected out, their contents were washed out and adipose tissue was removed. The upper end was closed with a thread, while the distal end was left open. Test substances were added after

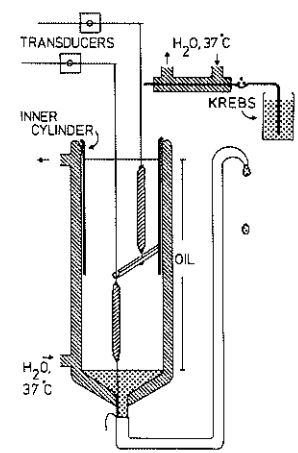


Fig. 3.3. Organ bath, containing an inner cylinder with a small, sloping drain, in which the upper tissue was secured. At the beginning of the experiment, the level of the parafin oil was adjusted with the syphon.

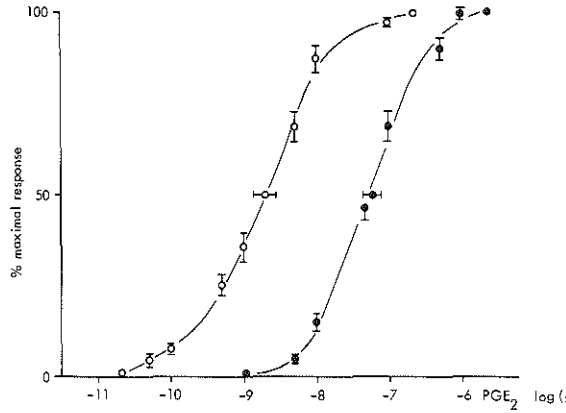


Fig.3.4. The apparent sensitivity of rat stomach strips in a normal cascade (closed circles, n=12) and in a cascade with a laminar flow (open symbols, n=10). The results, expressed as percentages of the maximal contraction, were obtained with the cumulative technique. The flow rates were 2.5 ml/min (normal cascade) and 0.1 ml/min (laminar flow cascade). Log D50 values: -7.25±0.12 (2.5 ml/min) and -8.62±0.09 (0.1 ml/min).

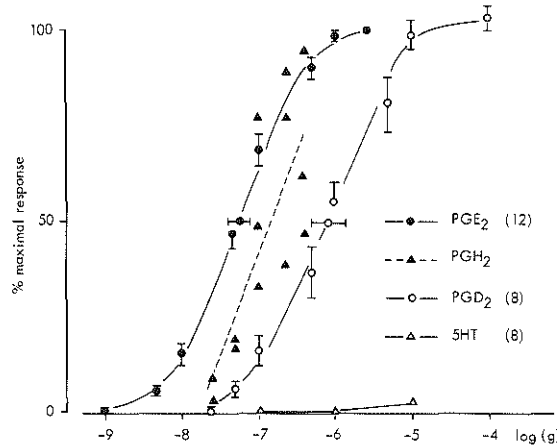


Fig. 3.5. Cumulative dose response curves to PGE_2, PGD_2 and 5HT in superfused (2.5 ml/min) rat stomach strips. Triangles represent single doses of PGH_2, from which the linear regression was calculated. All results are expressed as percentage of maximal PGE_2 contraction. Log D50 values: -7.25±0.12(PGE_2), -6.83(PGH_2), -6.12±0.20 (PGD_2) and > -5 (5HT). All antagonists were present in the Krebs.

a stabilizing period of 1h. Spontaneous activity was often present. The colon is about equally sensitive to PGE_2 and $PGF_{2\alpha}$ as shown in fig. 3.6. PGH_2 and its stable analogue 9,11 EM (upto 100 ng, 0.1 ml/min) did not contract this tissue. PGE_2 and $PGF_{2\alpha}$ were used as standards. The RC may be used in combination with a RSS to discriminate between PGE_2 and $PGF_{2\alpha}$ (as shown in fig. 3.6), since the RSS is less sensitive to $PGF_{2\alpha}$ than to PGE_2.

Rabbit aorta strip.(RAS). Male New Zealand white rabbits (2.5-3.5 kg) were blown on the base of the skull and exsanquinated. The chest was opened and the aorta removed. A spiral strip, about 2.5 mm wide was cut, and pieces of 3 cm length were used. Strips were often stored overnight at 4°C before use. The tissue is stimulated by 5HT, PGG_2, PGH_2 and especially TXA_2 (179). The classical PGs are also active but much higher doses are needed (see Bult & Bonta, 1976a). PGH_2 and 9,11 EM were used as standards.

Rabbit coeliac artery (RCA). After dissection the artery was freed from other adjacent tissues, spiralized, and a 2 cm strip (close to aortic trunk) was used. The tissue was stored (4°C) overnight before use. It is selective towards AA metabolites, since it is stimulated by TXA_2 (Bunting et al., 1976), but neither PGE_2 nor the endoperoxide PGH_2 had any effect (Bult & Bonta, 1976). In another report (47) a relaxation is described in response to PGE_2 or PGH_2. This discrepancy might be explained by a difference in preparation technique (storage at 4°C overnight) or by a different loading of the tissue. TXA_2, with its half life of 32 s, is of little use as standard and is not yet available in a highly purified form. Therefore, the 9,11 EM synthetic analogue of PGH_2 was used. In pharmacological respect this substance has the interesting property that it mimicks TXA_2 and not PGH_2 or PGG_2.

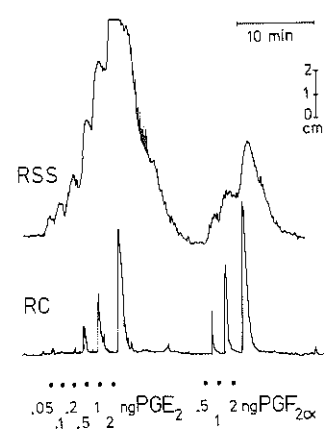


Fig. 3.6. Dose response curves to PGE_2 and $PGF_{2\alpha}$. The cascade consisted of a rat stomach strip (RSS) and a rat colon (RC), which were superfused with a laminar flow (0.38 ml/min). Test doses of PGE_2 and $PGF_{2\alpha}$-tromethamine salt ($PGF_{2\alpha}$) were given in 50 µl volumes.

Assessment of activities. Agonist solutions were freshly prepared before each experiment. After equilibration of the tissues, 0.05 or 0.1 ml (2.5 ml/min) or 0.01-0.05 ml (0.1 ml/min) was injected into the Krebs (Eppendorf pipette). Assessment of unknown activities was carried out by bracketing assay. An interesting discovery was that a "cumulative" procedure of obtaining dose-response curves produced data, such as maximal contraction and the logarithm of the dose of an agonist achieving a half-maximal contraction (log D_{50}), that were not significantly different from those obtained by a conventional stepwise procedure (see Bult & Bonta, 1976a, and fig. 3.7). In the cumulative

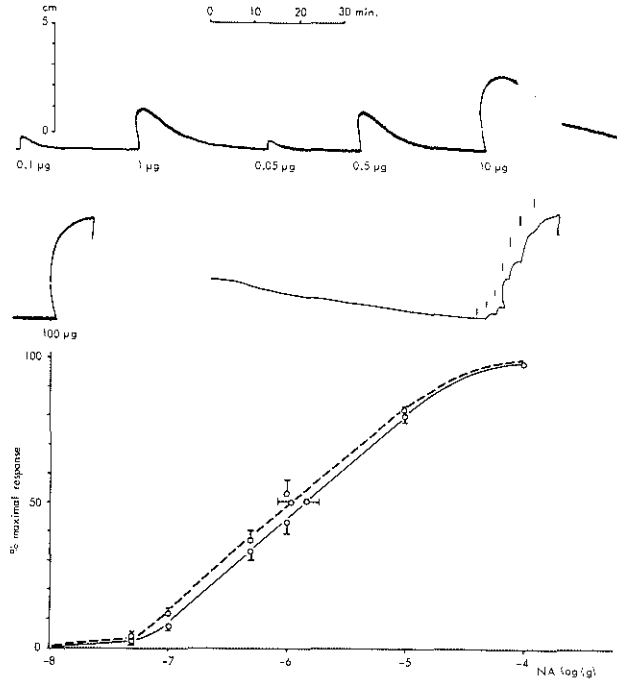


Fig. 3.7. Dose response curves obtained with the step-wise procedure (--o--) and with the cumulative technique (—o—). Results (n=8) were obtained on superfused (2.5 ml/min) rabbit aorta strips, stimulated by noradrenaline (NA), and expressed as percentages of the maximal contractions obtained using the step-wise technique. The upper traces show a single aorta strip, responding to step-wise doses of NA (up to 100 µg) and finally to cumulative doses of NA. In the latter case, 0.01,0.05, 0.1, 0.5, 1.0, 10 and 100 µg NA was given at the vertical bars. In these experiments, phentolamine (0.1 mg/l) was included in the Krebs instead of phenoxybenzamine. Log D_{50} values: -5.95 + 0.14 (step-wise) and -5.80 + 0.11 (cumulative).

technique, increasing doses were given 1 to 2 min after each other. This technique has a considerable time-saving effect, especially with sluggishly responding tissues such as rabbit arterial and rat stomach strips.

Antagonists. Substances, other than the AA-metabolites can stimulate or relax the tissues. A number of these materials can be couteracted by antagonists. A mixture of these antagonists (98) was included in the Krebs, in order to block acetylcholine, α- and β-adrenergic agonists, histamine, 5HT and PG-biosynthesis. Thus, for routine assay of AA-metabolites, the tissues were superfused with: atropine sulphate, 0.1 mg/l; phenoxybenzamine-HCl, 0.1 mg/l; sotalol-HCl, 0.1 mg/l; mepyramine-HCL, 0.1 mg/l; methysergide hydrogen maleinate, 0.1 mg/l; and indomethacin, 1.0 mg/l. Sometimes, one of the antagonists was omitted from the Krebs and added to selective tissues, via the second channel of the roller pump.

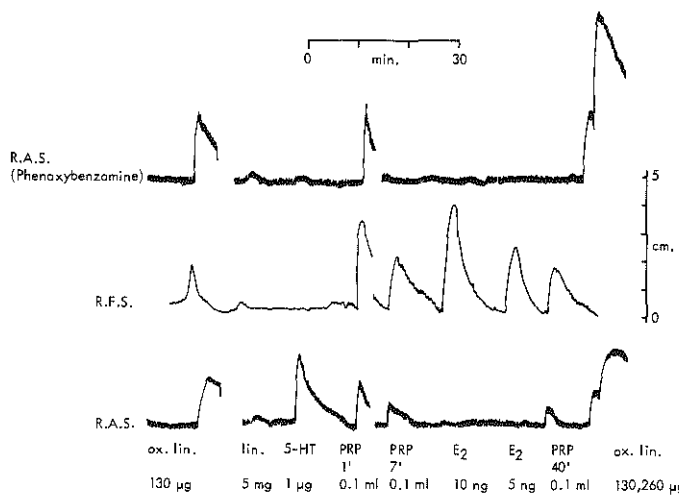


Fig. 3.8. Phenoxybenzamine blocks 5HT-induced contractions of the rabbit aorta strip (RAS). The cascade consisted of 1 RAS (incubated overnight with 3 µM phenoxybenzamine at 4°C), 1 rat fundus strip and 1 rabbit aorta strip (incubated overnight at 4°C, without phenoxybenzamine). The Krebs (2.5 ml/min) contained 0.05 µM methysergide and other antagonists (with phentolamine instead of phenoxybenzamine). The tissues were calibrated with PGE_2 (E_2), 5HT, linoleate (lin) and oxygenated linoleate (ox. lin). Rat PRP (0.1 ml) was also tested at time intervals of 1, 7 and 40 min after addition of collagen (40 µg/ml). Oxygenated linoleate was prepared according to Whitney Warton (1974), as described earlier (41). RFS: rat fundus strip= rat stomach.

In initial experiments, phentolamine-HCl (0.1 mg/l), a competitive α-blocker, was used instead of the irreversible blocker phenoxybenzamine. This permitted the use of high doses of noradrenaline for calibration of the aorta strips. However, it then appeared that under these conditions methysergide only partially blocked 5HT-induced RAS contractions (see Bult & Bonta, 1976a and fig. 3.8).

The nature of the 5HT blockade by methysergide was further investigated. The RAS exhibited tachyphylaxis towards 5HT. This resulted in a decreased maximal contraction without significantly influencing the log D_{50} of 5HT (see Bult & Bonta, 1976a). The estimated affinity (pA_2) of methysergide for the 5HT receptor was: $pA_2 = 7.8 \pm 0.2$ (first curves) or $pA_2 = 7.5 \pm 0.3$ (third or later curves). These values are low in comparison to the pA_2 (10.4 ± 0.4) given for the RSS (Frankhuizen & Bonta, 1974). Moreover, methysergide showed only competitive inhibition on the RAS whereas it has an additional, strong, non-competitive character in the RSS (Frankhuizen & Bonta, 1974). Pretreatment of the RAS with phenoxybenzamine (1 mg/l, 4°C, overnight) or replacement of phentolamine by phenoxybenzamine, resulted in a blockade of 5HT (upto 10 µg, 2.5 ml/min) in the presence of methysergide. A qualitative example of such blockade is shown in fig. 3.8. Thus, with phenoxybenzamine as well as methysergide in the antagonist mixture, a reasonable assessment of AA-metabolites seemed to be impossible. However, not all potential agonists are blocked (eg. Bk, Angiotensin II), and mixtures of agonists (eg. PGs + ADP or PGs + 5HT) may interfere with each other. Thus, extraction and separation of AA-metabolites is a prerequisite for quantitative determination. For TXA_2 this is impossible at the moment.

3.4. Extraction and separation of PGs.

Extraction. After addition of either ^{3}H-PGE_1 or ^{3}H-$PGF_{2\alpha}$ to the sample, an aliquot (usually 1 ml) was taken, and 0.9% NaCl (28 µM indomethacin) was added to make the final volume 5 ml). This was stored (0-120 min) on ice. Immediately after acidification to pH 3 (0.1 N HCl, volume predetermined, dependent on buffering capacity of sample) 5 ml diethyl ether was added and mixed on a vortex mixer. After centrifugation (10 min, 900 g, 4°C), the ether phase was aspirated and the procedure repeated with another 5ml ether. The combined ether fractions were dried under reduced pressure (37°C) and a small volume of ethyl acetate was added. If the extracts were not directly used

they were stored at -20°C (N_2 atmosphere). The efficiency of the extraction procedure, as determined by recovery of ^{3}H-PGE$_1$, was 85 ± 2% (30).

In some experiments, a column separation step was included. Aliquots (1 ml) of centrifugated plasma (900 G, 0°C, 30 min) were applied on Amberlite XAD-2 (BHD) columns (bed volume 1.0 x 3.0 cm; 138). The flow rate had been adjusted to 0.5 ml/min, and the columns were washed with 30 ml saline (containing 28 µM indomethacin). The PGs were eluted with 5ml methanol (recovery (%): ^{3}H-PGE$_1$: 87 ± 2 (5), ^{3}H-PGF$_{2\alpha}$: 88 ± 3 (5)). The methanol eluates were stored at -20°C under N_2. After evaporation of methanol (stream of N_2, 60 min, 37°C), the residual water was diluted to 5 ml, acidified to pH 3 with 1 M citric acid and extracted three times with 5 ml chloroform. This method had some advantages when it is partially automated:

1. A large number of samples was conveniently transferred in a more PG stabilizing medium (methanol- H_2). 2. Most proteins were washed away, which promoted phase separation during extraction. The introduction of an additional purification step was a disadvantage.

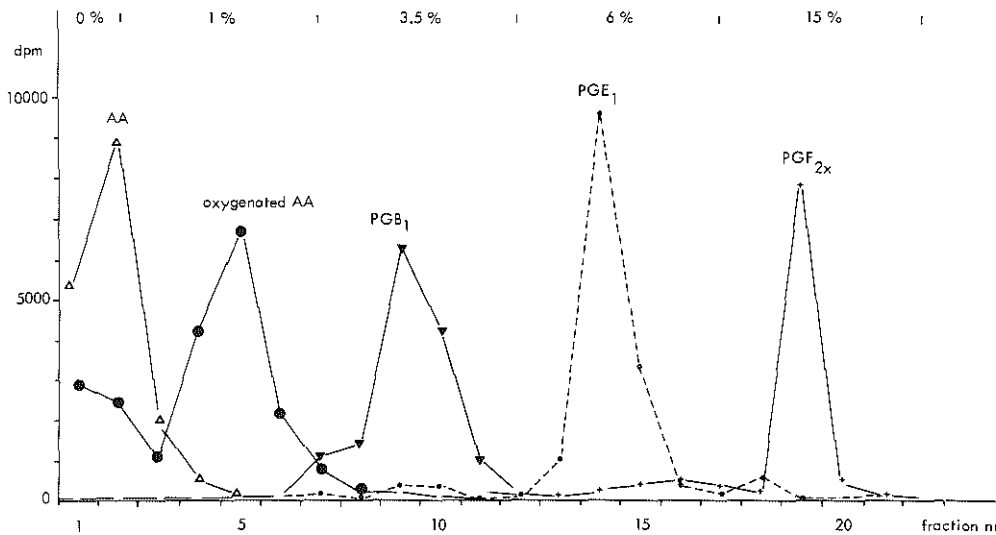


Fig. 3.9. Separation of PGB, PGE and PGF classes on silicagel columns. Tritiated AA (26,000 dpm), oxygenated AA (26,000 dpm), PGB_1 (20,000 dpm), PGE_1 (20,000 dpm) and $PGF_{2\alpha}$ (15,000 dpm) were loaded on silicagel columns, and eluted with chloroform, containing increasing volume percentages of methanol (% in fig.). Fractions (2 ml) were collected, transferred into scintillation vials, dried under N_2 at 37°C and counted. ^{3}H-PGB_1 was prepared by heating ^{3}H-PGE_1 (100°C, 10 min) in 0.1 N NaOH, and extracted at pH 3 in chloroform. Oxygenated ^{3}H-AA was prepared by the soya bean lipoxygenase method (see 278 and Bult & Bonta, 1976a).

Separation of PGs by silicagel column chromatography (see fig. 3.9.). The dried extracts were reconstituted in 0.5 ml chloroform (with 1 vol % methanol) and loaded onto silicagel columns, as where 2 washings with 0.2 ml chloroform. The method of Hillier and Dilley (1974) was followed, using the same type of columns (bed volume 0.5 x 8.0 cm), but the silicagel (Merk, kieselgel, 70-325 mesh) was activated (120°C) overnight. Elution of different PG classes was performed using chloroform, containing increasing percentages (by volume) of methanol: 5 ml 0% (fatty acids), 10 ml 1% (15 hydroxyarachidonic acid), 10 ml 3.5% (PGA + PGB), 10 ml 6% (PGE) and 6 ml 15% ($PGF_{2\alpha}$). The polarity needed for elution of PGA + PGB and PGE was somewhat higher than reported. No appreciable dehydration of PGE_1 was noted. The average flow rate ranged between 0.27 and 0.33 ml/min (gravity).

Separation by TLC. Dried extracts were dissolved in a small volume of ethyl acetate and spotted on Fertigplatten Kieselgel 60 F 254, 0.2 mm (Merk). The plates were developed with freshly prepared chloroform-methanol-acetic-acid-water (90:8:1:0.8, by volume) until the front was 17 cm from the origin (System C, Nugteren and Hazelhof, 1973).

3.5. Malon dialdehyde (MDA) assay.

MDA was measured spectrophotometrically after formation of the MDA-thiobarbituric acid (TBA) adduct. Trichloroacetic acid (1 ml, 15% in 1 N HCl) was added to 1 ml PRP (prepared as described in 4.3), and after mixing, the precipitated proteins were removed by centrifugation (15 min, 4°C, 900 G). The supernatant was filtered through a cotton wool plug and 1 ml TBA reagent was added to 1 ml filtrate. After heating (15 min, 100°C), the absorption was measured (532 nm, 20°C, 1 cm). TBA reagent was prepared daily by dissolving 0.5 g TBA in 5 ml 5N NaOH, to which 30 ml water were added. The solution was brought to pH 9-10 with 70% perchloric acid and diluted to 50 ml with water (1% TBA). Just before use, 2 volumes of the 1% TBA solution were mixed with 1 volume 7% perchloric acid, giving the final TBA reagent.

Standard. 1,1,3,3-tetraethoxypropane (TEP) was used as standard and hydrolyzed during heating (15 min, 100°C) of a mixture of 1 ml TEP ($0\text{-}10^{-5}$ M), 1 ml 15% TCA (in 1 N HCl) and 2 ml TBA reagent. The extinction coefficient (532) was: 1.46 ± 0.02 (7) $L.mole^{-1}.cm^{-1}$. If the MDA-TBA complex was formed in the presence of precipitated proteins (as described by Stuart et al., 1975), reduced extinction coefficients were measured with increasing doses (0-10

mg/ml) of albumin.

Recovery. A possible disadvantage of this MDA assay is the loss of MDA as a result of binding to proteins and loss of MDA after removal of denaturated proteins. When MDA (10^{-5} M) was incubated with PRP no significant loss was observed.

Determination of MDA in PRP. The method can provide satisfactory results in PRP. PRP and PPP (final volume 1 ml) were stirred with a 12 x 8 x 8 mm bar at 100 rpm. Under these conditions saline-treated PRP did not produce more TBA positive material than saline-treated PPP. Stronger stirring resulted in high extinction values of saline-treated PRP. Rat plasma contained a large amount of TBA positive material and incubations with PPP or PRP, stimulated with saline, were absolute requirements for a reliable measurement of MDA production by platelets. In table 3.1 the net MDA production by collagen aggregated rat platelets is shown. Extinction values of PPP have been subtracted before calculating the amount of MDA produced by platelets. The reaction seemed to be cyclo-oxygenase specific, since it was inhibited by indomethacin. Thus, it is unlikely that the AA lipoxygenase (2.1.7) contributed to MDA production.

Table 3.1. Measurement of TBA positive material in rat PRP (4.3).

Pretreatment	MDA (nmol/10^9 platelets)
Saline (50 µl)	0.47 ± 0.06 (10)
IM (10 µM)	0.02 ± 0.05 (5)
TYA (30 µM)	0.10 ± 0.03 (5)

PRP was preincubated with saline, indomethacin (IM) or eicosatetraynoic acid (TYA) and aggregated with collagen (40 µg/ml). After 5 min the reaction was stopped by addition of 1 ml 15% TCA as described above. TBA positive material was measured as described.

3.6. Additional techniques.

Fatty acid determination of total lipids from erythrocytes. After centrifugation (30 min, 950 G) of blood (4.3) plasma and "buffy" coat were removed. The erythrocytes were washed three times with 10 mM EDTA in saline. Lipids in 5 ml packed cells were isolated (Folch et al., 1957) by two extractions with 19 volumes of chloroform-methanol (2:1, v/v). Five mg dried extract was heated (2h, 70°C) in 9 ml methanol-HCl (2.6 gr HCl/100 ml methanol) under N_2.

Then 2 ml 8 N NaOH were added, and the mixture was again heated (1h, 70°C, N_2 atmosphere). The saponified mixture was acidified (5 N H_2SO_4, pH 4) and extracted three times with 25 ml pentane. The combined pentane fractions were dried (Na_2SO_2) and methylesters were prepared with a fresh ethereal diazomethane solution (N_2 atmosphere). The pentane fraction was filtered, and dried (37°C) under reduced pressure. The dried residu was dissolved in a drop of pentane and 1-2 µl was used for analysis. Fatty acids were separated on a 4 ft x 3mm column (3% EGSS-X on gaschrom Q, Chrompack, The Netherlands) in a Hewlett Packard 9750 gas chromatograph. Temperatures: injector, 240°C; column: 175°C; flame ionization detector, 250°C. The equivalent chain length values were compared with those of a mixture of fatty acids and with the data of Hoffstetter (1965). Weight percentages were determined by triangulation.

Liquid scintillation counting. In most experiments recovery of ^{3}H-PGs was measured in duplicate (or triplicate) samples. Aliquots were dissolved (if necessary after evaporation of the strongly quenching chloroform) in 10 ml scintillation fluid (1,4 dioxane, containing per liter: 60 g naphthalene, 4 g PPO, 0.2 g POPOP and 100 ml methanol). Radioactivity was measured in a Packard Tri Carb Model 3375 liquid scintillation spectrometer. External standardization was used for quench correction. The conversion of cpm into dpm was performed automatically. Average efficiencies: ^{3}H, 43%; ^{14}C, 75%. Dual labelling: ^{3}H, 39%; ^{14}C, 55%.

Chemicals. PGE_1, PGE_2 and $PGF_{2\alpha}$-tromethamine salt were gifts from Dr.J.E. Pike (Upjohn Company, Kalamazoo, Michigan); 9,11 (15 S-hydroxy-9,11-(epoxymethano)-prosta-5 Z,13 E dienoic acid) was a gift from Dr.G.L.Bundy (Upjohn Company, Kalamazoo, Michigan); PGH_2, PGD_2, 15-hydroperoxyarachidonic acid, dihomo-γ-linolenic acid, and 5,8,11 eicosatrienoic acid were gifts from Dr.D.H.Nugteren (UnileverResearch, Vlaardingen, The Netherlands). PGE_2, PGE_1, $PGF_{2\alpha}$ (10 mg/ml) and PGD_2 (50 µg/ml) were dissolved in ethanol, stored at -20°C, and these stock solutions were diluted just before use. PGH_2 was stored in diethyl ether (5 µg/ml) at -70°C. After evaporation of the ether, glucose-free Krebs was added and the biological activity of an aliquot was immediately tested. 15-Hydroxyperoxy AA (100 µg/ml) in ethanol was treated in the same way as PGH_2. Eicosatetraynoic acid was a gift from Dr.A.L.Willis (Roche Products, Welwyn Garden City, England), and dissolved in ethanol (10 mg/ml). Other fatty acids were stored in hexane (N_2 atmosphere, -20°C) and

just before use, solutions were made up in saline, containing 0.02% Na_2CO_3. Ethanol (final concentration 10%) was sometimes needed in order to obtain homogenous emulsions, In these cases saline-Na_2CO_3 with 10% ethanol served as a control. BPP_{9a} was a gift of Dr.L.J.Greene (Ribeirao Preto, Brasil). The following chemicals were purchased: arachidonic acid (99%), Sigma; 1-^{14}C-arachidonic acid (55 mCi/mmol), 5,6 ^{3}H-PGE_1 (59 Ci/mmol) and 9 ^{3}H-$PGF_{2\alpha}$ (15 Ci/mmol): The Radiochemical Centre, Amersham, England; linoleic acid and serotonin creatinine sulphate, Merck; bradykinin and methysergide hydrogen maleinate, Sandoz; ADP, Boehringer Mannheim; indomethacin, Merck, Sharp & Dohme; aspirin (calcium acetylsalicylate), Amsterdamsche Chinine Fabriek ACF; heparin and dexamethasone, Organon Oss, The Netherlands; 1,1,3,3-tetraethoxypropane, Merck-Schuchardt; thiobarbituric acid and atropine-sulphate, Merck; phenoxybenzamine-HCl, Ciba-Geigy; phentolamine-HCl, Smith, Kline & French Labs; sotalol-HCl, Mead-Johnson; mepyramine-HCl, SPECIA; adrenaline-bitartrate, Fluka A.G.

Presentation and statistical analysis of data. All data are expressed as means $\pm$ standard error of the mean, and when necessary, the number of observation is given between brackets {mean $\pm$ s.e.m. (n)}. If M values were below the limit of detection (a), this is indicated by : < a(M). When 2 groups of data were being compared, a two tailed Student's t-test was employed. If there seemed to be either a non-normal distribution, or marked differences in the variances of the two groups, the non-parametric Wilcoxon test was employed. For comparisons of more than 2 groups, a one way analysis of variance (ANOVA) was carried out. The F-ratio (c) and the degrees of freedom (a,b) are given: F_0 (a,b)=c. If an overall treatment effect existed, Duncan's new multiple range statistic was employed. For comparison of several treatments with one control the test of Dunnett was used (see fig. 6.3).

4. THROMBOXANE AND PROSTAGLANDIN BIOSYNTHESIS AND RAT PLATELET BEHAVIOUR

4.1. Abstract.

PGE and thromboxane A_2 (TXA_2) were formed during incubation of heparinized rat platelet rich plasma (PRP) with collagen or arachidonic acid (AA). The endogenous release of these products by EFAD platelets was drastically reduced. The oxygenated, endogenous AA products probably amplified aggregation induced by threshold doses of collagen. The addition of exogenous AA (or PGH_2) to PRP did not completely mimick the collagen-induced formation of products, since more stable PGs and little TXA_2 were formed in absence of aggregation. Circumstantial evidence indicated that TXA_2 was the aggregation-inducing substance in rat PRP. Aggregation induced by ADP or high doses of collagen probably proceeded independently of TXA_2 synthesis.

4.2. Introduction.

Platelets were chosen as an in vitro model of endogenous PG biosynthesis for several reasons: 1. It is relatively easy to obtain platelets. 2. Large amounts of oxygenated AA metabolites may be formed upon stimulation of platelets. 3. During their isolation, artificial PG release seems to be negligible, since homogenization -with concomitant PG synthesis- is not necessary, in contrast to spleen cells, kidney cells, etc.

Human platelets played a central role in the discoveries of TXA_2, of PGI_2 and of the first lipoxygenase in cells other than plant cells (see 2.1 and 2.4). PG-endoperoxides and TXA_2 are implicated in the mechanisms of aggregation of human platelets (see 2.4). However, little is known about AA metabolism in rat platelets. Thus, it was of interest to see if rat platelets release PGs and TXA_2 during aggregation, and to evaluate the significance of this release for their behaviour. Rat platelets differ from human platelets in some respects: they are not aggregated by adrenaline, show no biphasic aggregation in response to ADP (see 2.4 and 175) and their aggregation is not inhibited by PGD_2 (154,183).

The studies were mainly restricted to the following questions: 1. Are rat platelets a useful in vitro model for the assessment of PG and TXA_2 synthesis? 2. Is it possible to demonstrate a substantial reduction in endogenous PG release during EFA deficiency? 3. What is the importance of oxygenated products of AA in rat platelet behaviour?

Thus, rat platelet aggregation and release of AA-derived products, induced by various substances, were studied in PRP. Then, the influence of EFA deficiency on rat platelet behaviour was investigated. Finally, results obtained with cyclo-oxygenase inhibitors indomethacin and eicosatetraynoic acid (TYA; see 2.2) will be presented. TYA is also an inhibitor of platelet AA-lipoxygenase (117).

4.3. Methods.

Preparation of heparinized platelet rich plasma (PRP). Blood was obtained by cardiac puncture of ether anaesthetized normal and EFAD rats (see 3.1). The syringe (10 ml) and the needle (1.2 x 40 mm) were rinsed with 0.3 ml heparin solution (Thromboliquine, Organon, Oss, The Netherlands; 5000 i.u./ml). After centrifugation (15 min, 200 G) the PRP was carefully removed and diluted with platelet poor plasma (PPP), which was obtained by centrifugation (30 min, 900 G), to an OD (602 nm, 1 cm) of 0.9. PRP was prepared daily and stored (20°C) in capped, plastic vials. During the whole procedure glass contact was avoided. For some samples the platelet number was determined in a Coulter counter, giving the following results (cells x $10^{-3}/\mu l$): normal PRP, 669 ± 25 (10) and EFAD PRP, 729 ± 31 (5).

Aggregation. Aggregation was studied with the turbidimetric method (Born, 1962), using 8 volumes PRP, which were placed in siliconized, flat bottom cuvettes (6.5 x 45.0 mm) in an aggregometer (Payton Ass. Inc., Buffalo, N.Y.). PRP was preincubated for 3 min with 1 volume saline or Tyrode (mM: NaCl, 137; KCl, 2.7; $MgSO_4.7\ H_2O$, 1.1; NaH_2PO_4, 0.42; $NaHCO_3$, 11.9; pH 7.4), while stirring at 900 rpm and 37°C. At zero time the aggregation was started by the addition of 1 volume Tyrode (with inducers) or 1 volume collagen suspension. Collagen (Bovine achilles tendon, Sigma) was suspended in 18 mM acetic acid at a final concentration of 0.4 mg/ml. Drugs were preincubated during 3 min. The final volume of the incubations was either 0.25 or 0.5 ml. Aggregation was expressed as percent increase in light transmission; Δtransmission PPP-PRP=100%.

Bioassay. The release of mediators was assessed on a cascade of isolated tissues (see 3.3), either directly or after extraction and separation of PGs by column chromatography (see 3.4). The amounts of biological activities were expressed as ng/ml incubation mixture.

Radiochemical assay of oxygenated products of AA. In some experiments PRP was stimulated with collagen, 0.1 mM or 0.6 mM AA, in the presence of ^{14}C-AA (final volume 0.5 ml). Indomethacin (20 µl, final concentration 28 µM) was added at 8 min after starting the aggregation, and the suspensions (0.5 ml) were extracted and the PGs were separated by TLC (see 3.4).

4.4. Results.

4.4.1. Normal rat platelets and PG biosynthesis.

Aggregation in normal PRP, induced by various substances is shown in fig. 4.1. ADP (0.11, 0.21, 0.43, and 0.85 µg/ml) and collagen induced a single wave of aggregation. AA (182 µg/ml) on occasions induced only "shape change" of the platelets, indicated by increased turbidity and diminished oscillations, but the same dose, in another PRP, induced a monophasic aggregation. PGH_2 at 0.5

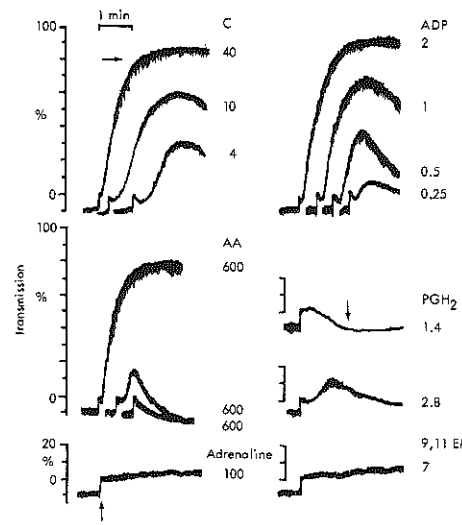


Fig. 4.1. Examples of aggregation of rat PRP. After 3 min preincubation (not shown) different substances were added. This is indicated by the increased transmission, due to dilution of the PRP (see for example adrenaline, ↑). A subsequent decreased transmission and dimished oscillations, indicating that the platelets are changing their shape, is often observed (see for instance lower doses of ADP and PGH_2, ↓). When the platelets aggregate the transmission increases, and large oscillations occur (→). The latter are due to aggregates that whirl around. C: collagen; 9,11 EM: 9,11 epoxymethano-analogue of PGH_2. All concentrations in µM, except for collagen (µg/ml).

µg/ml (1.4 µM) only resulted in shape change of platelets, whereas 1 µg/ml gave a small aggregation. Adrenaline (upto 100 µM) and 9,11 EM (upto 2.55 µg/ml) failed to aggregate rat platelets in PRP. The reasons for the variations in AA-induced aggregation in rat PRP are not exactly clear at this moment.

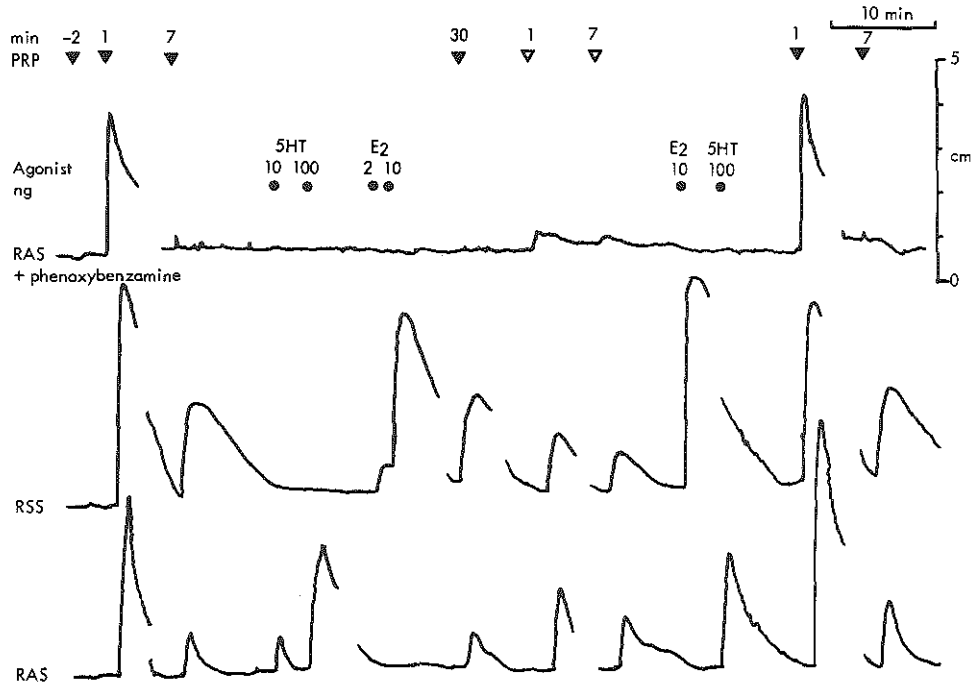


Fig. 4.2. Qualitative example of the release of different products during aggregation. PRP was preincubated with saline (▼) or 0.3 mM TYA (▽). At different times (min), before and after addition of collagen (40 µg/ml), aliquots (0.1 ml) were tested directly on a cascade of isolated tissues (2.5 ml/min), consisting of a rabbit aorta strip (RAS), pretreated with phenoxybenzamine, a rat stomach strip (RSS) and a control rabbit aorta strip. The Krebs contained methysergide and phentolamine (see 3.3). The tissues were calibrated (●) with serotonin (5HT) and PGE_2 (E_2).

Collagen-induced release of PG and TXA_2. Qualitative examples of biological activities, released by aggregating rat platelets, are shown in figures 3.8, 4.2 and 4.4. PRP was incubated with collagen and aliquots were tested on a cascade of isolated tissues. Fig. 4.2 shows that at 7 and 30 min after starting aggregation, both a stable PG-like activity (RSS) and 5HT-like activity (RAS) were present. Like PGE_2, the former activity is completely trapped during passage through a small Amberlite XAD-2 column, whereas the latter behaves like 5HT in being only partially trapped (Bult & Bonta, 1976a).

During the aggregation (1 min), an additional, labile rabbit aorta contracting substance (RCS) is formed. RCS contracts the RAS in the presence of

phenoxybenzamine and is neither PGE_2 nor 5HT. RCS disappears within 6 min, thus behaving like TXA_2 or PG endoperoxides. It is likely that both this labile activity and PGE-like activity are oxygenated AA products, since TYA blocks their formation. It is worth noting that TYA in this high concentration did not suppress the 5HT release (RAS,7 min). The RCS, which is not 5HT, MDA, ADP or hydro(per)oxy fatty acid (Bult & Bonta, 1976a) is probably TXA_2, because of the following characteristics:

1. RCS was lipid-soluble, since it was trapped by quick filtration through a small Amberlite XAD-2 column (Bult & Bonta, 1976a).
2. The half-life ($t_{\frac{1}{2}}$) of RCS, after removal of platelets in order to avoid de novo formation, was 32 ± 6 s (5), whereas the half-life of PGH_2 in PPP was significantly longer (125 ± 46 s (8), $p < 0.05$, Wilcoxon). In order to determine $t_{\frac{1}{2}}$ of TXA_2, the platelets were removed (within 30 s) twenty s after addition of collagen, by filtration through a milipore filter (0.45 µM pore size) under reduced pressure. Thereafter, aliquots were tested at different times (0, 20, 40, 80 s) on a cascade with 3 aorta strips and the $t_{\frac{1}{2}}$ values were estimated from linear plots of the logarithms of rabbit aorta contractions against time. The $t_{\frac{1}{2}}$ of RCS is identical to the $t_{\frac{1}{2}}$ of TXA_2, generated by washed human platelets (see 120, and 2.1). The estimated $t_{\frac{1}{2}}$ of PGH_2 in plasma was much shorter that the values obtained in buffer (120,189). This finding has been confirmed in human PRP (232).
3. RCS stimulated the rabbit coeliac strip (see fig. 4.4). This tissue is not contracted either by PGH_2 or by PGE_2 (47; Bult & Bonta, 1976b), whereas it is contracted by TXA_2 (47).
4. The appearance of RCS is inhibited by NaN_3, whereas PG-like (or PG endoperoxide-like) activities are unaffected (Bult & Bonta, 1976b). A similar result has been obtained with TXA_2 synthetase inhibitor (172) imidazole (Vincent & Zijlstra, personal communication).

These results confirm the suggestion that RCS is mainly composed of TXA_2 (120), although it has recently been reported that TXA_2 has a longer $t_{\frac{1}{2}}$ in human plasma (232). This discrepancy might be due to species differences or due to differences in the technique employed, since only the disappearance of biological activity was measured in the present study.

The stable PG-like material (PGL) behaved like PGE_2 during differential bioassay on the rat stomach strip and rat colon. The high concentrations, obtained by direct bioassay on the RSS (44 ± 3 ng PGE/ml); see table 4.1) seemed rather suspect since other reports indicated that citrated human PRP ge-

nerated about 10 times less PGE_2 in response to collagen (eg. 227,232). The latter finding was confirmed: in 3 samples of citrated human PRP, stimulated with collagen (67 µg/ml), 8.0, 4.5 and 4.8 ng PGE/ml were found, whereas PGE was undetectable (<2 ng PGE/ml) in 3 samples of aspirin pretreated (1 mg/ml) human PRP. In order to substantiate the results obtained by direct bioassay, the PGE of aggregated rat PRP was partially purified (see table 4.1). After extraction and column chromatography the major PG activity was indeed found in the PGE fraction. Moreover, like PGE_2, its biological activity disappeared after heating (5 min, 100°C) in 0.1 N NaOH, whereas the activity of $PGF_{2\alpha}$ did not.

Table 4.1. Generation of PGs by normal PRP: a comparison between direct bioassay and assay after extraction and column chromatography.

	Activity on RSS	PPP n=5	PRP + $^3HPGE_1$ n=5	PRP + $^3HPGF_{2\alpha}$ n=5
Direct	PGE_2 (ng/ml)	<5	44 ± 3	-
	$PGF_{2\alpha}$ (ng/ml)	<50	-	>150
PGE fraction	PGE_2 (ng)	-	23 ± 3	19 ± 2
(6% Methanol)	recovery (^3H,%)	-	49 ± 2	-
	PGE_2 (ng/ml)	-	46 ± 6	-
PGF fraction	$PGF_{2\alpha}$ (ng)	-	<5 *	<4; 7 **
(15% Methanol)	recovery (^3H,%)	-	-	52 ± 4
	$PGF_{2\alpha}$ (ng/ml)	-	-	<8; 3.5 **

Aggregation was induced by collagen (40 µg/ml, 5min). After addition of $^3HPGE_1$ (77.000 dpm) or $^3HPGF_{2\alpha}$ (25.000 dpm) and centrifugation (30 min, 900 G), an 0.1 ml aliquot of the supernatant was bioassayed directly (cascade with 2 rat stomach strips and a rat colon, 2.5 ml/min). A further 1.0 ml was extracted (using Amberlite XAD-2 columns) and added to silicagel columns (see 3.4). The fractions, eluted with 6% and 15% methanol in chloroform, were used for bioassay. These samples were dried, 0.2 ml Krebs was added, and after mixing, 2 doses (0.02 and 0.1 ml) were used for bioassay (cascade: see above), and 0.01 ml (triplicate) was used for liquid scintillation counting in order to determine the final recovery.
* = 5 pooled fractions; ** 4 pooled fractions.

The mean of the differences between direct assay and assay of the partially purified PGE compounds (- 3 ± 5, n=5; calculated from data in table 4.1) was not significantly different from zero ($p < 0.05$, paired Student's t-test). Thus, a PGE (probably E_2) is the major contributor to the PGL observed with direct bioassay of aggregated rat PRP.

The relationship between collagen concentrations and both PGE formation and aggregation are shown in fig. 4.3. A linear regression was calculated between the logarithm of the collagen dose and the amounts of PGE. Since the 95% confidence limits of its slope do not include zero, a linear regression is present between 4 and 40 µg/ml. No correlation was found between percentage of aggregation and PGE formation (Spearman test, $p > 0.05$).

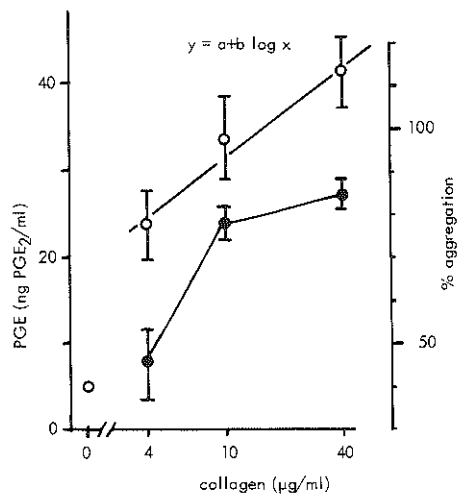


Fig. 4.3. Influence of collagen concentration on aggregation (●) and PGE formation (o) by normal PRP. After incubation (5 min) the platelets were removed by centrifugation (30 min, 900 G). The presence of PGs in the supernatant was assessed by adding 0.05 and 0.2 ml, cumulatively, to a cascade (2.5 ml/min) of 2 rat stomach strips and 1 rat colon. Cumulative doses of PGE_2 and $PGF_{2\alpha}$ served as standards. Since little $PGF_{2\alpha}$ seemed to be present, the results are expressed as ng PGE_2/ml, assessed on the 2 stomach strips. The mean value of the 4 estimations was calculated. PGE was undetectable in saline treated PRP (<5 ng/ml). Each point is the mean of 5 incubations.
a= 14.6; 95% confidence limits: $-0.7 < a < 29.9$. b= 17.2; 95% confidence limits: $7.8 < b < 26.6$.

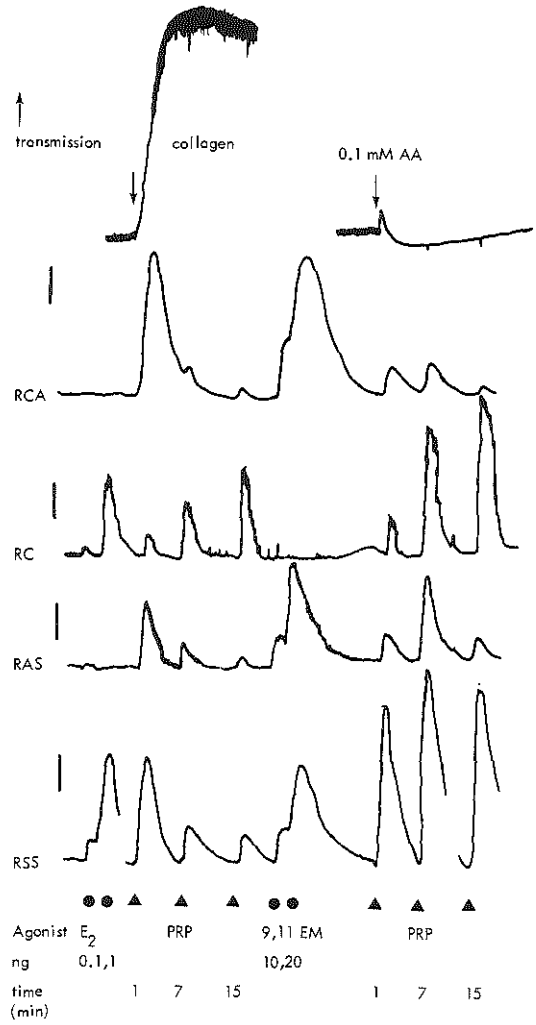


Fig. 4.4. Qualitative comparison of the aggregation, and the release of different biological activities by PRP, stimulated with collagen (40 µg/ml) or AA (0.1 mM). For collection of PRP and aggregation, see 4.3. Collagen and AA were added at zero time(↓). The light transmission was monitored as shown in the upper tracings (↑). Samples (50 µl) of the incubations were added directly to a cascade (0.1 ml/min) of superfused rat colon and rabbit coeliac artery strip, and to a second cascade (0.1 ml/min) of superfused rabbit aorta and rat stomach strips, at 1, 7, and 15 min after the start of the aggregation. The differences in sensitivity of the tissues (especially the RCA and RC) is shown by test doses of PGE_2 and the 9,11 epoxymethano analogue of PGH_2 (9,11 EM). The vertical bars represent 1 cm.

ADP-induced release of PGE. A supra-maximal dose of ADP (25 µM, cf. fig. 4.1) induced very little release of PGE in rat PRP. Five min after addition of either Tyrode or ADP, platelets were removed by centrifugation (30 min, 900 G), and 1 ml was extracted (using XAD-2 columns, see 3.4). Extracts (n=5) were then tested for biological activity (2 x RSS). Results (number of observations between brackets): Tyrode, <4 (5); ADP, 5.0 ± 0.2 (3), < 4 (2) ng PGE_2/ml.

AA-induced release of PGs. AA (0.1 mM) induced shape change without aggregation, as shown in fig. 4.4. However, PG endoperoxides had been formed, since RC, RAS, RSS and RCA were contracted. Several differences between collagen- and AA-induced formation of biological activities appeared. With AA, more stable PGE was formed (RC and RSS, 15 min), but TXA_2 concentrations seemed to be much lower (RCA, 1 min) and their formation seemed to be more prolonged and less explosive. Similar results, obtained in another experiment, are given in fig. 4.5. Again, in the absence of aggregation (0.1 mM AA) considerable PGE, but little TXA_2, was formed when compared with collagen-induced aggregation. Even if aggregation occurred (0.33 mM AA), the ratio between the activities

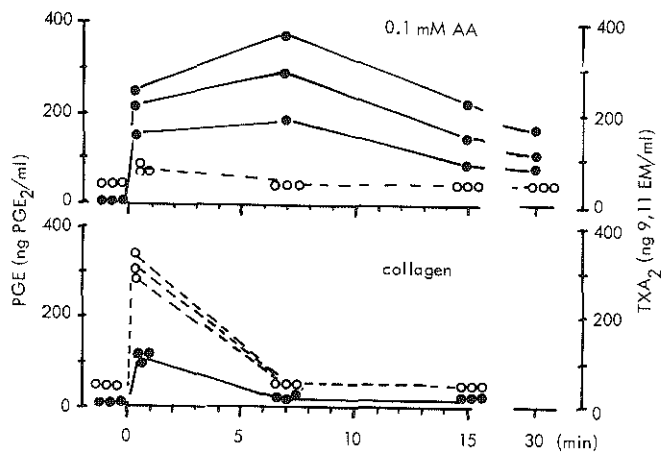


Fig. 4.5. Addition of collagen (40 µg/ml) or arachidonic acid (AA) to rat PRP led to different ratios between TXA_2 (--o--) and PGE (—●—). Their release was assessed on a cascade (0.1 ml/min) composed of rabbit coeliac artery strip (RCA, sensitive to TXA_2) and a rat stomach strip (RSS, sensitive to PG endoperoxides and PGs) at different times after addition of collagen or AA. Results are expressed as ng PGE_2/ml (RSS) and ng 9,11EM/ml (RCA). AA (0.1 mM) did not induce aggregation.

on rabbit coeliac artery and rat stomach strip remained reversed (results not shown).

In order to verify these observations, PRP was incubated with different amounts of AA and a fixed amount of ^{14}C-AA and the products were separated by TLC. TXB_2 has been reported to chromatograph between $PGF_{2\alpha}$ and PGE_2 in the system used (Dr. D.H.Nugteren, Unilever Res., Vlaardingen, personal communication; 158,178). With 0.1 mM AA, few ^{14}C oxygenated products were detectable (fig. 4.6). However, when lysed PRP was incubated with 0.1 mM AA, peaks appeared at the expected TXB_2 site and a small amount of radioactivity co-chromatographed with PGE_2. A similar pattern was obtained if a higher (0.6 mM) concentration of AA was used, which induced a small, reversible aggregation of the platelets. Both aggregation, and the appearance of radioactivity at TXB_2 and PGE sites, were blocked by indomathacin. Finally, less polar radioactive products were formed. The solvent system used is not very suitable for the separation of hydroxyfatty acids, such as HHT and HETE (see 158), but the formation of the less polar products was suppressed by indomathacin (28 μM). Even in the presence of 0.6 mM AA, PRP produced few oxygenated products, since less than 1% of all ^{14}C AA was converted.

In similar experiments, both the biological activity and the radioactivity

Table 4.2. Determination of lytic activity of AA.

	LDH (U/l)	%	n
PPP	44 ± 3	2.6 ± 0.4	3
PRP + saline	85 ± 10	4.9 ± 0.7	3
PRP + 1 mM AA	112 ± 12	6.5 ± 0.8	5
Lysed platelets	1720 ± 50	100 ± 3	3

Lactate dehydrogenase (LDH) was measured in PPP, lysed PRP and PRP that had been incubated for 5 min with saline or AA (1 mM). PPP was prepared from PRP (7 x 10^8 pl/ml; 4.3). Lysed platelets were obtained by resuspending the remaining platelet pellet in distilled water (0°C), in a volume equal to that of PPP. LDH was measured with a Monotest LDH opt, nr. 158186, Boehringer . The differences between incubations of PRP with saline or with 1 mM AA were not significant (Student's t-test, $p > 0.05$).

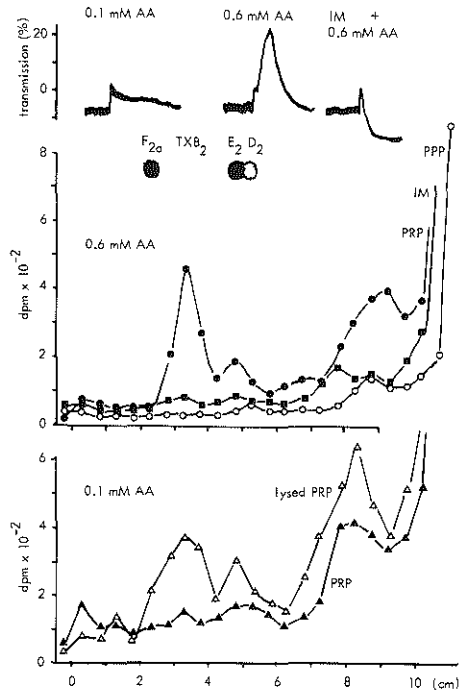


Fig. 4.6. Stimulation of rat PRP with different concentrations of AA. Lower section: separation of radioactive products produced by PRP and lysed PRP in the presence of 0.1 mM AA. Middle section: reference spots of PGs and separation of radioactive products produced by PPP and PRP, with or without indomethacin (IM), during incubation with 0.6 mM AA. Top section: corresponding aggregation patterns. PRP (0.4 ml, 2.4 x 10^8 cells) was preincubated (3 min) with saline (50 µl) or IM (28 µM). AA (1.0 or 6.0 mM, 50 µl, containing 7.4 x 10^5 dpm ^{14}C-AA) was added and after 8 min the reactions were stopped by addition of IM (see 4.3). A sample of PRP was lysed ultrasonically (10 s) in the presence of 0.1 mM AA. The products were extracted, separated by TLC and radioactivity was determined in 0.5 cm silicagel strips (3.4 & 3.6). Not shown: AA reference (13.5 cm) and front (17.3 cm). TXB_2 moves between $PGF_{2\alpha}$ ($F_{2\alpha}$) and PGE_2 (E_2) in this solvent system. D_2: standard of PGD_2.

were measured in the silicagel fractions. Moreover, tritiated $PGF_{2\alpha}$ and PGE_1 were added as internal standards. An example is shown in fig. 4.7. After incubation of PRP with 0.1 mM AA some biological activity was found at the PGE_2/PGD_2 site, but ^{14}C radioactivity was undetectable. A higher dose of AA (0.6 mM) did not cause aggregation of the PRP (not shown), but small ^{14}C peaks appeared in the PGE_2/PGD_2 region and the TXB_2 region. The peaks were of about

equal heights. As expected, a larger amount of PGE_2 was measured by bioassay (RSS). In some other experiments relatively more PGF-like activity was found to be present. In the fraction, which contained the peak of tritiated PGE_1 marker, the amount of PGE_2-like activity was 1.5 ng. According to the amount of ^{14}C dpm, approximately 9 ng was to be expected. The reason for this discrepancy between the calculated and the estimated amounts of PGE_2-like activity is unclear at this moment.

When collagen was used to aggregate PRP, which was preincubated with

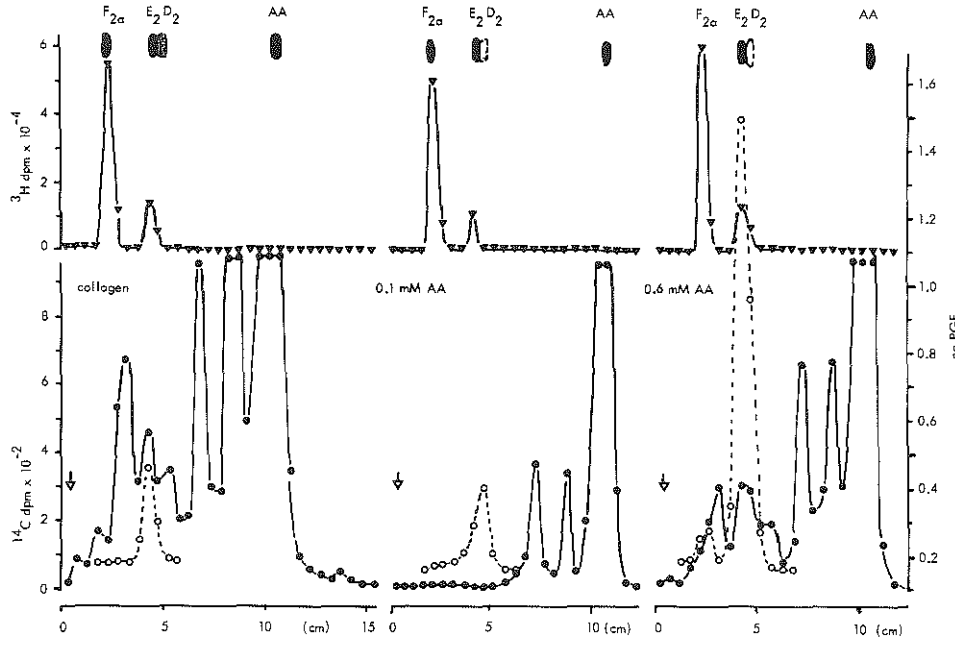


Fig. 4.7. Formation of AA-derived biologically active and radioactive products by rat PRP, stimulated with collagen (40 µg/ml) or AA (0.1 and 0.6 mM). In the examples shown, no aggregation occurred with 0.1 and 0.6 mM AA. PRP (0.4 ml, 0.29 x 10^9 cells) was preincubated with saline (0.05 ml) or ^{14}C-AA (1.2 x 10^6 dpm). After 3 min, AA (0.05 ml, 1 and 6 mM with 1.0 x 10^6 dpm ^{14}C-AA) was added to the saline-preincubated samples. Collagen (0.05 ml, 0.4 mg/ml) was added to ^{14}C-AA-preincubated samples. After 8 min, indomethacin (28 µM) was added, together with 3.6 x 10^4 dpm ^3H-PGE_1 and 14.4 x 10^4 dpm ^3H-$PGF_{2\alpha}$. After extraction and separation by TLC, both radioactivity (dpm ^{14}C= ●, dpm ^3H=) and biological activity (--o--) were determined in 0.5 cm fractions (see 3.4 & 3.6). Biological activity was determined on a RSS (0.1 ml/min) and expressed as ng PGE_2/fraction (see 3.4) The spots refer to references of PGE_2 (E_2), PGD_2 (D_2), $PGF_{2\alpha}$ ($F_{2\alpha}$) and AA on the same plate. TXB_2 moves between $F_{2\alpha}$ and E_2 in this solvent system.

^{14}C-AA, the ratio between the ^{14}C peaks at the TXB_2 and PGE_2 sites, was shifted towards the thromboxane pathway. This confirmed the results obtained by direct bioassay. Again biological activity was found at the PGE_2 site. When compared with the samples that were stimulated with exogenous AA, the amounts of less polar radioactive products were higher when PRP was stimulated with collagen.

It is unlikely that AA-induced lysis of the platelets, since significant leakage of the cytoplasmic marker, lactate dehydrogenase (LDH), was not observed with AA upto 1 mM AA (as shown in table 4.2). Addition of the lysed platelet preparation (see table 4.2) to PRP at a final concentration of 16%, failed to induce aggregation. Finally, the AA-induced aggregation in PRP was inhibited by indomethacin (28 μM), whereas ADP-induced aggregation was not suppressed at this concentration of indomethacin (see table 4.6). These results ruled out the possibility that AA-induced aggregation was due to ADP, that had been liberated from lysed platelets.

4.4.2. Influence of EFA deficiency on rat platelet behaviour.

A fatty acid analysis gives the best indication of EFA deficiency. For practical reasons the fatty acid spectrum of normal and EFAD rats was determined in erythrocytes, and not in platelets. The erythrocytes of 4 normal and 4 EFAD rats were used for a fatty acid analysis, as shown in table 4.3 .

Table 4.3. Fatty acid composition of total lipids from normal and EFAD rat erythrocytes.

Fatty acid	Normal % weight	EFAD % weight
16:0	29.8 ± 1.3	28.9 ± 0.9
18:0	20.0 ± 1.5	17.5 ± 2.1
18:1, n-9	10.2 ± 1.1	19.8 ± 0.7
18:2, n-6	7.6 ± 0.7	0.8 ± 0.04
20:3, n-6	0.2 ± 0.04	tr.
20:3, n-9	tr.	8.7 ± 0.9
20:4, n-6	18.6 ± 1.7	4.5 ± 0.6

The fatty acid analysis was performed as described in 3.6.
tr.: trace (< 0.1%). Each value is the mean of 4 rats.

The linoleic acid family (n-6) was decreased in favour of the oleic acid (n-9) family in EFAD cells, although a residual percentage of AA (24%) was still present.

Aggregation, induced by several stimuli, was measured in both normal and EFAD PRP (table 4.4). The PG endoperoxide PGH_2 gave small, but equal responses in both types of PRP. Preincubation of PRP with a high concentration of linoleic acid, in order to suppress binding of PGH_2 by albumin, did not result in enhanced aggregation. This enormous linoleic acid concentration did not influence the shape of platelets in either type of PRP. Much smaller doses of AA induced an indomethacin-resistant swelling (cf. fig. 4.1). No significant differences were observed between EFAD and normal PRP in any condition ($p > 0.05$, one way analysis of variance ; ANOVA).

ADP induced equal aggregation in normal and EFAD PRP, except for the lowest dose, when EFAD PRP aggregated less than normal PRP. EFA deficiency did

Table 4.4. Comparison between aggregatory capacity of normal and EFAD PRP.

Aggregation inducer		Normal PRP % aggregation	EFAD PRP % aggregation	
PGH_2	1 µg/ml	25.0 ± 5.5 (4)	17.9 ± 4.0 (3)	
LA(10 mM) + PGH_2	1 µg/ml	14.7 ± 4.3 (3)	15.5 ± 2.7 (3)	
ADP	2.50 µM	95.7 ± 2.6 (5)	90.8 ± 1.5 (5)	
	0.80 µM	76.9 ± 5.4 (5)	75.7 ± 3.9 (5)	
	0.25 µM	25.9 ± 3.0 (5)	14.5 ± 2.4 (5)	*
collagen	40 µg/ml	95.2 ± 1.6 (5)	95.3 ± 1.6 (5)	
	4 µg/ml	53.6 ±10.3 (5)	3.1 ± 1.5 (5)	**

PRP (200 µl) was preincubated (for 3 min at 37°C) with 25 µl Tyrode or with 25 µl Tyrode containing 100 mM linoleic acid (LA), and aggregation was started by addition of 25 µl inducer. Results are expressed as percentages of maximal aggregation, induced by 40 µM ADP, added in 10 µl at 5 min after starting aggregation. ANOVA of PGH_2 effects: $F_0(3,12) = 1.11$, $F_{0.95}(3,12) = 8.74$, $p > 0.05$. * = $p < 0.05$, ** = $p < 0.01$ (Wilcoxon test, two-sided, EFAD vs normal).

not alter the aggregating efficiency of high collagen doses (40 µg/ml), but almost completely (94%) prevented the aggregation induced by a dose giving approximately an half maximal response in normal PRP.

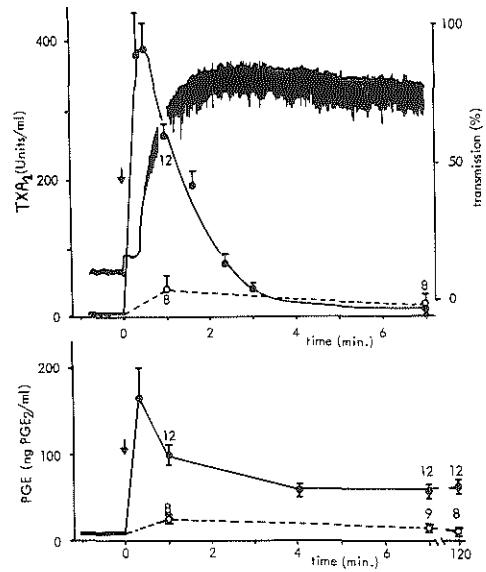


Fig. 4.8. Time courses of aggregation and generation of TXA_2, PG endoperoxides and PGE by normal (—●—) and EFAD (--o--) PRP, aggregating after addition of collagen (40 µg/ml, arrow). Aliquots (0.1 ml) were added directly to superfused (2.5 ml/min) strips of rabbit aorta and rat stomach. The upper part shows an aggregation pattern, together with release of TXA_2 (expressed as Units/ml; 1 Unit= 1 ng PGH_2). The lower part shows rat stomach contractions, calibrated with PGE_2. Unless otherwise indicated each point represents the mean of 4 observations.

EFAD PRP, stimulated with high doses of collagen, formed far less TXA_2 (84% reduction at 1 min) and less PGE (75% reduction at 7 min, 89% reduction at 120 min) than normal PRP. These data were obtained by direct bioassay of TXA_2 and PG release, assessed on RAS and RSS, as shown in fig. 4.8. Biological activities were not detectable during preincubation. At 20 s after the addition of collagen, when aggregation was starting, TXA_2 was almost maximally generated by normal PRP. TXA_2 levels were unaltered at 30 s , when light transmission increased steeply. At 1 min, when aggregation was completed for 75%, TXA_2 was already disappearing and when maximal aggregation was observed (3 min), hardly any TXA_2 was detectable. EFAD PRP generated only 16% of the TXA_2 released by normal PRP, but aggregation of EFAD PRP was unaltered with this high (40 µg/ml) dose of collagen (see also table 4.4). The contractions of the RSS showed a similar pattern, but a residual, stable PGE

was observed (7 min, 120 min). The generation of PGE by EFAD PRP was markedly suppressed. The initial peak of RSS-stimulating activity was possibly composed of a mixture of PG-endoperoxides.

Finally, the cyclo-oxygeanse activity of both types of PRP was tested in the presence of exogenous AA (fig. 4.9). Normal PRP generated increasing amounts of PGE (assessed on a RSS, and expressed as ng $PGE_2/10^9$ platelets) when increasing concentrations of AA were added. Since PGE was assessed at 7 min after addition of AA, it might be partly composed of PG endoperoxides, due to the fact that PG production seemed to be more prolonged when platelets were stimulated with exogenous AA (fig.4.4). A relatively high concentration of indomethacin inhibited the PGE production in the presence of 0.66 mM AA. EFAD PRP generated as much as PGE as normal PRP, when incubated with 0.33 mM AA. In similar experiments MDA production was measured in both types of PRP. Like the release of TXA_2, MDA formation needed higher concentrations of AA. The data represent the net MDA formation by platelets, since the extinction measurements were corrected for incubations with PPP.

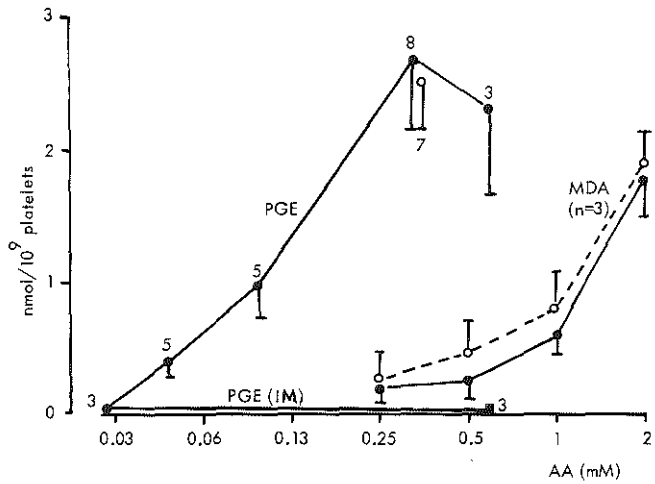


Fig. 4.9. Comparison of cyclo-oxygenase activity in normal (—●—) and EFAD PRP (--o--). After preincubation (3 min), PRP was exposed to different concentrations of AA. The amount of biological activity was assayed on rat stomach strips, 7 min after starting the aggregation. Although this activity probably consisted of a mixture of stable PGs and PG endoperoxides (see time curve in fig. 4.4) it is expressed as nmol $PGE_2/10^9$ platelets. PGE was also measured in the presence of 1.38 mM indomethacin (IM). In a separate experiment MDA was measured as described in the text (3.5).

No differences were observed between normal and EFAD PRP with respect to MDA production. Thus, the reduced endogenous generation of PG endoperoxides (and PGE) and TXA_2 by EFAD PRP is not due to a diminished cyclo-oxygenase activity.

Finally, high doses of collagen induced an equal release of 5HT-like material from both normal and EFAD PRP (table 4.5). Significant differences were not observed at 7 min (assessed on RAS), nor at 120 min (assessed on RSS). An unimpaired release of ^{14}C labelled 5HT by EFAD PRP, in response to high doses of collagen, has been described previously (260). Therefore, the above findings were not further substantiated by a fluorimetric assay.

Table 4.5. Direct bioassay of 5HT-like material, released from normal and EFAD PRP during collagen (40 µg/ml) induced aggregation.

Tissue	Time min	Normal PRP µg 5HT/ml (n)	EFAD PRP µg 5HT/ml(n)
RSS	120	0.32 ± 0.05 (6)	0.21 ± 0.07 (4)
RAS	7	0.46 ± 0.06 (5)	0.43 ± 0.07 (4)

Small aliquots of aggregated PRP (5-10 µl) were added to a superfused (2.5 ml/min, Krebs without methysergide) RSS. In this way, PGE was diluted too much to cause any contraction by itself. The activities on the RAS (2.5 ml/min, without phenoxybenzamine) were assessed after disappearance of TXA_2 in 0.1 ml aggregated PRP. With both methods, no statistical differences were found between EFAD and normal PRP ($p > 0.05$, Student's t-test).

4.4.3. Effects of drugs on platelet aggregation and PG biosynthesis.

IC50 values for TYA and indomethacin are given in table 4.6. Indomethacin was more potent than TYA as an inhibitor of endogenous TXA_2 and PGE production. Both drugs were equally active against TXA_2 and PGE release. Doses of indomethacin, which suppressed endogenous TXA_2 and PGE release by 50%, also produced half-maximal inhibition of aggregation induced by a threshold dose of collagen. This threshold dose of collagen (2 µg/ml) produced approximately 50% aggregation in normal PRP. Indomethacin (upto 200 µM) failed to suppress aggregation induced by a dose of ADP (0.1µM) that produced 50% aggregation. Finally, indomethacin did interfere with MDA production by AA stimulated PRP.

Table 4.6. Inhibitory effects of indomethacin and TYA in PRP.

Effect	Inducer	Indomethacin IC50 (μM) (n)	TYA IC50 (μM) (n)
TXA_2 (1 min)	Col., 40 μg/ml	5.5 ± 1.0 (8)	61.2 ± 8.6 (5)
PGE (7 min)	" , "	8.2 ± 1.4 (7)	53.2 ± 5.1 (5)
Aggregation	Col., 2 μg/ml	5.5 ± 1.5 (5)	-
Aggregation	ADP, 0.1 μM	> 200 (3)	-
MDA (5 min)	AA, 1 mM	5.0 ± 1.5 (5)	-

PRP was at random preincubated (3 min) with Tyrode or Tyrode containing 5 to 6 different concentrations of drugs, and then exposed to the inducers of aggregation. The IC_{50} was estimated from linear plots of response vs log (dose of inhibitor). The number of estimated IC_{50} values is given (n). The following effects were measured: the release of TXA_2 (at 1 min, mm contraction of superfused RAS, 2.5 ml/min, induced by 0.1 ml PRP), the release of PGE (at 7 min,ng PGE_2/0.1 ml, assessed on superfused RSS, 2.5 ml/min), release of MDA (see 3.5) and percent maximal aggregation (see 4.3). Release of TXA_2 and PGE was determined in the same incubations. IC_{50} values of both TYA and indomethacin against TXA_2 and PGE formation showed no statistical differences($p > 0.05$, Paired Student's t-test). Col.: collagen.

TYA and indomethacin had little effect on release of 5HT and aggreation induced by high doses of collagen. TYA (310 μM) then inhibited the release of TXA_2 by 89 %, but release of 5HT was unaltered (see Bult and Bonta,1977c and fig. 4.2). Indomethacin (152 μM) completely blocked TXA_2 and PGE production, while the release of 5HT was only inhibited by 50%.

Finally, table 4.7 demonstrates the inhibitory effects of imidazole on MDA production and platelet aggregation. The two inhibitory effects appeared to parallel one another.

Table 4.7. Imidazole inhibits aggregation and release of MDA by PRP.

	Aggregation %	MDA (μM)	%
Saline	100 (2)	3.84 ± 0.15 (7)	100 ± 4
Imidazole	17 ± 1 (2)	0.96 ± 0.11 (7)	25 ± 3

PRP was preincubated (3 min, 37°C, 900 rpm) with either saline or imidazole (100 μg/ml). Aggregation was induced by 1 mM AA and MDA production was measured as described in the text (3.6).

4.5. Discussion.

Collagen caused aggregation of rat platelets in PRP, with concomitant release of MDA, PG-like material (PGL) and RCS. MDA, PGL and RCS production were suppressed by cyclo-oxygenase inhibitors, like indomethacin and TYA. The biological properties of RCS are in agreement with those of TXA_2, as discussed in 4.4.1. Since $PGF_{2\alpha}$, PGD_2 and other AA metabolites are much less active than PGE_2 on the RSS (see fig. 3.5), it is likely that PGE compounds were the main contributors to the stable PGL, observed on the RSS during direct assay of aggregated PRP. Indeed, PGL cochromatographed with PGE_2 (and ^3H-PGE_1) on TLC plates and was eluted with ^3H-PGE_1 from silicagel columns. Unlike $PGF_{2\alpha}$, it lost its activity during treatment with NaOH. Because of these characteristics it was assumed to be PGE.

PGE formation by rat PRP is dependent on the dose of collagen. Collagen enhances AA release from human and rabbit platelets, and free AA is almost completely oxygenated (20,25,225). Therefore, the enhanced PGE formation with increasing doses of collagen is most likely due to an increased availability of AA. The finding that PGE is the major classical PG formed during aggregation with collagen, is consistent with observations in human PRP.

The amounts of PGE, released by collagen, are higher in heparinized rat PRP (approximately 50 ng PGE/ml) than in citrated human PRP (less than 10 ng PGE/ml). The results with human PRP were in agreement with other reports (eg. 231). The discrepancy in release of PGE by human and rat PRP may be explained in several ways, such as species differences, differences in platelet number, and in anticoagulant used and finally, the collagen concentration may directly influence the release of PGE (see above). It seems unlikely that the direct bioassay was inaccurate, since partial purification of PGE yielded similar data as direct assay (table 4.1). Moreover, following separation by TLC, relatively high amounts of endogenous PGE were again detected in collagen aggregated rat PRP.

It is possible that the anticoagulant may have influenced platelet activity and subsequent PG release. In other studies citrated PRP has often been used, which was not the case in the present work. In one report (Dray et al., 1976), plasma and serum PGE_2 levels (ng/ml; radioimmunoassay) in humans and rats have been compared: Human plasma: 0.005, rat plasma: 0.107; human serum: 0.60, rat serum: 3.35. Since serum PGs are probably formed by thrombin-stimulated platelets, these data indicate that rat platelets also formed more

PGE_2 in the absence of any anticoagulant. At least a part of the difference is probably explained by a higher number of platelets in rat PRP and rat blood (21). Moreover, rat platelets tend to be larger, judged from the optimal settings of the Coulter counter.

At the start of the aggregation induced by a high dose of collagen, there was a peak of TXA_2, which had already tapered off before maximal aggregation was reached (fig. 4.8). Only during aggregation with threshold doses of collagen did the cyclo-oxygenase products seem to be important for rat platelet behaviour. Under these conditions EFA deficiency markedly reduced aggregation (table 4.4) and indomethacin suppressed aggregation by 50% in doses that also caused a half-maximal inhibition of PGE and TXA_2 production (table 4.6). These IC50 values were obtained after 3 min preincubation. A shorter preincubation (1 min), resulted in higher IC50 values (data not shown), which might be explained by the irreversible nature of NSAID action (see 2.2.2). The free levels of the inhibitors were probably much lower (159).

Thus, threshold doses of collagen almost entirely failed to aggregate EFAD PRP, which released only limited amounts of PGE and TXA_2 (fig. 4.8). Unfortunately, the AA levels in platelets were not determined in this study, but erythrocytes of EFAD rats showed a marked reduction of AA. Since both cell-types are derived of bone-marrow, a similar pattern is expectable in EFAD platelets. It is likely that the reduction in EFAD platelets is even greater than in erythrocytes, since their turnover is higher. Haddeman and Hornstra (1974) measured AA in platelet lipids and observed about 93% reduction in EFAD platelets and a diminished collagen-induced aggregation. Cyclo-oxygenase activity and sensitivity to PGH_2 were unaltered in EFAD platelets (fig 4.9)

Higher collagen doses induced aggregation and release reaction 1 in rat PRP, independently of the AA-derived products (table 4.5). The doses of indomethacin needed for inhibition of aggregation with higher collagen doses, are about 100 times higher (260) than those required for inhibition of PG synthesis, and other mechanisms must be involved. This is reinforced by the observation that the IC50 of indomethacin against aggregation by EFAD platelets is over 0.02 M, whereas an increased sensitivity of EFAD PRP is to be expected when cyclo-oxygenase products are necessary for aggregation with high doses of collagen (260). The mechanisms of aggregation by "high" doses of collagen are unclear as yet, but it is unlikely that a residual release of PG-endoperoxides, during EFA deficiency or in the presence of high doses of NSAIDs is responsible for a normal release reaction and aggregation by high

doses of collagen. Moreover, when rat PRP was incubated with exogenous AA, in low concentrations, a reproducible formation of PGs was observed without aggregation. This implies that rat PRP can form PG endoperoxides from exogenous AA without subsequent aggregation. Similar results have been obtained in dog PRP (55). Besides, only limited aggregation was observed with high doses of PGH_2 (1 µg/ml). The doses of endoperoxide needed for maximal aggregation of human PRP are below 0.5 µg/ml (116,120). Finally, 9,11 EM, an endoperoxide analogue, failed to induce aggregation, whereas 200 ng/ml results in complete aggregation of human PRP (Malmsten, 1976). All these experiments indicate that rat platelets are less sensitive to exogenous PG endoperoxides than human platelets.

During stimulation of rat PRP with AA, in doses which failed to aggregate platelets, there was very little TXA_2 synthesis. In comparison with the endogenous formation, the ratio between PGE and TXA_2 was shifted towards the former. Higher doses of AA induced an indomethacin (28 µM) - sensitive aggregation. These doses were higher than those required in rabbit (max. aggregation at 60 µM) or human PRP (max. aggregation with 500 µM; 259), but the AA-induced aggregation showed daily variations in rat PRP. This is possibly explained by variations in binding of AA plasma proteins. Aggregation was accompanied by the appearance of TXA_2, although its ratio towards PGE was still different from that obtained with endogenous release. Preloading of the PRP with linoleic acid resulted in enhanced formation of TXA_2 (results of 3 experiments, not shown here). The release of TXA_2 also tented to be longer than the explosive, endogenous generation. Thus, the total synthesis of TXA_2 could be greater than that expected from the direct bioassay, which might also be inaccurate due to interference with substances other than PGs, TXA_2, ADP and 5HT. Moreover, since TXA_2 can not be quantified by bioassay the same experiments were repeated with ^{14}C AA. TXB_2 and PGs were separated by TLC, and their formation was followed by measuring radioactivity. The RF values of $PGF_{2\alpha}$ (0.12), PGE_2 (0.27), PGD_2 (0.29) and AA (0.73) were somewhat lower than those given by Nugteren & Hazelhof (1973). After aggregation, radioactive products were detected which behaved like TXB_2, PGE_2 and/or PGD_2. Their appearance, as well as that of some less polar products, was inhibited by indomethacin. Thus, these peaks were due to cyclo-oxygenase activity, and not to co-chromatography of AA with phospholipids or other materials.

The results thus obtained supported the previous, bioassay findings that rat platelets formed larger amounts of PGE_2, when compared with washed human

platelets (117,119) and that the formation of TXB_2 only became important if aggregation took place. Since PRP, instead of a washed cell suspension was used, the results do not permit a direct comparison with most other papers. In PRP, the platelets were probably less damaged, but it had the disadvantage that only very little AA was oxygenated. Moreover, in contrast to other papers, the biosynthesis of oxygenated products was also monitored in the presence of doses of AA that did not elicit aggregation.

The "abundant" formation of PGE_2. Although no ^{14}C PGE_2/PGD_2 spots were detectable after incubation of PRP with 0.1 mM AA, the more sensitive laminar flow bioassay indicated the presence of PGE_2. During lysis the conversion of free AA was fascilitated and both ^{14}C-PGE_2 and ^{14}C-TXB_2 became detectable. After aggregation, either with collagen or with 0.6 mM AA, a higher TXB_2 peak appeared, but in contrast to results with human platelet suspensions, it was not 100 times as high as the PGE_2 peak (117). A similar finding has recently been described. Microsomal preparations from rat platelets produced considerably less TXB_2 than similar preparations from either human (Dr. Helen White, Wellcome Res., N.C., U.S.A. personal communication) or guinea pig (S.Abrahams et al., 1977) platelets. Thus,the TXA_2 synthetase pathway seems less predominant in rat platelets and as a result, more classical PGs appear.

PGE_2 formation in the absence of aggregation. If no aggregation occurred, even less TXB_2 was formed (eg. see fig. 4.7). This confirmed the bioassay results showing that the ration TXA_2/PGE was reversed when 0.1 mM AA, instead of collagen, was used to stimulate PRP. Thus, exogenous AA can lead to even more predominant PGE production. This observation may be explained in several ways. It is possible that rat platelet TXA_2 synthetase has a low affinity for PG endoperoxides, or that the subcellular sites of some of the cyclo-oxygenase enzymes are not completely identical to that of the PGH_2-TXA_2 isomerase. However, experiments with washed rat platelets are necessary to substantiate the finding that the TXA_2/PGE ratio was reversed when exogenous AA was used to stimulate the platelets.

Whichever explanation is correct, the experiments indicated that stimulation of intact, non-lysed platelets with exogenous AA, failed to mimick all events that take place during collagen-induced aggregation in PRP. This was more or less to be expected. Even if all endogenous AA is derived from the plasma-membrane (which is not yet known), it does come from those phospholipids (eg. Blackwell et al., 1977) that are predominantly present at the inside of the platelet plasma-membrane (54,244). Thus, exogenous AA first has

to penetrate to the inside of the platelet in order to become equivalent to the endogenous cyclo-oxygenase substrate. In PRP a considerable amount of exogenous AA, or exogenous PGH_2 (178) will never reach platelet cyclo-oxygenase or TXA_2 synthetase, due to the fact that it is trapped by albumin. Aggregation, induced by exogenous AA, coincided with TXA_2 formation. Thus, it is likely that TXA_2 is the aggregation-inducing substance, either directly or after induction of the release of ADP. The importance of TXA_2 is also indicated by the finding that imidazole, an inhibitor of PGH_2-TXA_2 isomerase (168), inhibits aggregation and also MDA release (and TXA_2 release, bioassayed on rabbit coeliac artery; J.E.Vincent and F.J.Zijlstra, personal communication). The experiment with imidazole supported the suggestion that in platelets MDA is derived to a large extent from TXA_2 (111).

ADP induced aggregation in rat PRP independently of cyclo-oxygenase products. During aggregation induced by a high dose of ADP, very little PGE was released. Moreover, for the inhibition of aggregation by threshold doses of ADP, enormous concentrations of indomethacin (above 0.1 mM) were needed. Abrahams et al., (1977) and Haddeman & Hornstra (1974) came to similar conclusions in citrated rat PRP. Apparently rat platelets, which display only a single wave of aggregation, lack the aspirin sensitive secondary aggregation of human platelets. However, EFA deficiency led to reduced aggregation induced by threshold doses of ADP. The difference was less prominent than in collagen-induced aggregation, and might be due to membrane alterations, but no evidence was obtained for this suggestion.

5. THE PRESENCE OF PROSTAGLANDINS IN CARRAGEENIN-INDUCED HIND PAW EDEMA.

5.1. Abstract.

Release of prostaglandin-like material (PGL) during the development of carrageenin-induced pedal edema (CAR edema) was demonstrated with a perfusion technique. The release of PGL, which was already detectable after 1 hour, was not due to an experimental artefact. These findings have been confirmed in more quantitative experiments. Both indomethacin (2.5 mg/kg) and EFA deficiency reduced the release of PGL in CAR edema. A parallel decrease in swelling was also observed. This supports the suggestion that an endogenous cyclo-oxygenase product, formed during CAR edema, has a pro-inflammatory activity in the early phase of this model of acute inflammation.

5.2. Introduction.

Carrageenin-induced pedal edema (CAR edema) is one of the most frequently used acute anti-inflammatory models. Reliable measurements of the mediators thought to be involved are scarce (see 2.5). Thus, the widely accepted hypothesis (Di Rosa et al., 1971) that PGs are involved in this edema from 3 h onwards, has never been supported by data on local levels of PGs, since in Willis' (1969) report, neither saline control values nor data on the time of collection were given. In order to substantiate PG mediation, attempts were undertaken to detect PGs in CAR edema under carefully controlled conditions.

In spite of the fact that CAR-induced pleurisy is a more suitable model for quantitative measurements of mediators in edema fluid, the pedal edema was chosen for the following reasons:

1. It is far more widely used than CAR pleurisy for the assessment of the anti-inflammatory activities of drugs.
2. It is still used as a model for the evaluation of the roles of mediators in an acute inflammatory reaction (eg. Moncada et al.,1973). The results thus obtained, are often extrapolated to inflammation in general, although the validity of such extrapolations is questionable.
3. It differs from the pleurisy model in some pharmacological respects, especially during its initial phase (Vinegar et al., 1976).

The collection of exudate is a technical problem, which prevented most authors from investigating mediator levels during CAR edema. In these studies

an old perfusion technique, first described by Rocha e Silva & Antonio (1960) was used. The method was modified for later experiments, which permitted a quantification of the efficiency of the perfusion. Since anaesthesia might interfere with the development of CAR edema, the rats were anaesthetized just before the start of the perfusion. With this method, the effects of EFA deficiency and indomethacin treatment were investigated.

5.3. Methods.

5.3.1. Perfusion method 1 (see fig. 5.1.).

Male Wistar rats (180-250 g) were randomized and anaesthetized with urethane (25%)-chloralose (2%) and fixed on a warming apparatus (37°C). Polythene cannulae (diameter= 3 mm) were inserted, subcutaneously, through a small incision in the lateral skin of the tarsus and pushed into the subplantar region (see fig. 5.1). Perfusion of the paw with 6% dextran, in sterile,

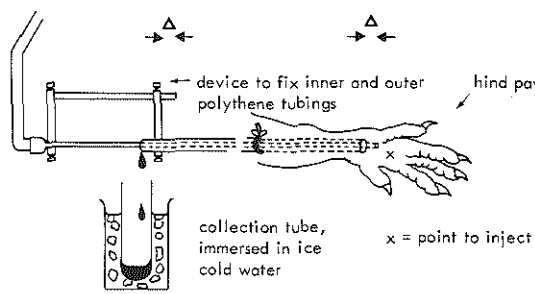


Fig. 5.1. Coaxial perfusion method (after Rocha e Silva, 1960). The anaesthetized rats were fixed on a warming apparatus (37°C). After insertion of the cannulae, sterile saline, at 37°C was supplied to the inner cannula using an infuse system. The extension of the inner cannula (Δ mm) was adjusted with the device shown left.

pyrogen-free saline, was performed via an inner cannula (diameter 1 mm) that protruded ± 3 mm beyond the outer cannula into the subcutaneous space. The infused fluid was collected through the outer cannula. The first perfusate (30 min, 4 ml/h) was discarded. Then, the perfusion was continued (30 min, 2 ml/h) in order to obtain basal levels of mediators. Thereafter, either CAR (1 mg) or 0.1 ml saline was injected into the foot (see 3.2). At different times after injection of saline or CAR the perfusion was again started for 30 min (2ml/h). Perfusates were collected in siliconized tubes on ice.

Biological activities in the perfusates were assessed directly on a cascade (2.5 ml/min) of 2 rat stomach strips. Methysergide was omitted from the Krebs and introduced into the Krebs superfusing the lower tissue (see 3.3). The rats were anaesthetized during the development of the edema, in contrast to method 2.

5.3.2. Perfusion method 2.

For later experiments an improved technique was used. Randomized normal and EFAD (see 3.1) male Wistar rats (180-220 g) were injected intravenously with 0.1 µCi ^{125}I-human serum albumin (The Radio Chemical Centre, Amersham). Their hindpaws were then injected with one of the two treatments: saline (0.1 ml) in one paw only, or CAR (1 mg) in one paw and saline in the contralateral paw. Perfusion was started 4h after these injections, at a flow rate of 5ml/h.

Installation of the cannulae. Only one paw, either CAR- or saline-treated, was perfused and three different protocols were used:

A. The rats were anaesthetized with pentobarbitone (200 mg/kg), 3.75h after injection of the irritants. The stainless steel cannulae (see fig. 5.2) were then inserted and the perfusion was started (at 4h).
B. The rats were anaesthetized (see A) and indomethacin (5 mg/kg, i.v.) was then administered, 5 min before installation of the cannulae and the perfusion was started (at 4h).
C. The rats were anaesthetized (see A), and killed (ether) 5 min before the installation of the cannulae and perfusion was started (at 4h).

Two different stainless steel, outer cannulae were employed, with different diameters (1.1, and 2.0 mm). The inner cannulae consisted of a polythene tube, which was secured with flexible tygon^{R} tubing around the outer tube (see fig. 5.2). The tubes were discarded after use and the stainless steel

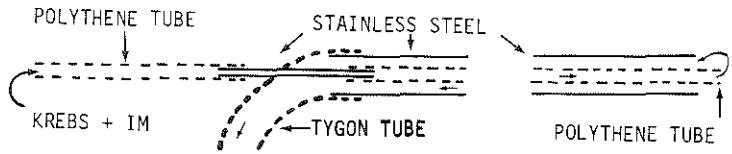


Fig. 5.2. Schematic drawing of the cannulae used in perfusion method 2. IM: indomethacin.

cylinders were cleaned by ultrasonication in a detergent solution, washed several times with tap and distilled water, acetone and ethanol, dried and finally siliconized.

Krebs, containing 28 μM indomethacin (IM), was used as the perfusion fluid, and perfusates were collected on ice, prior to extraction (see below). The following parameters were measured:

In vivo:

1. Paw diameter at 0h and 4h (see 3.2).
2. ^{125}I-radioactivity in inflamed paw and the contralateral control paw (at 4h). In order to measure radioactivity, the paws were placed in a fixed position under a gamma scintillation counter (Berthold, type 52 20/20 H, Wildbad, GFR) connected to a rate meter-integrator (Berthold, type LB 241 K, Wildbad, GFR). The counting efficiency was 37%. Δ dpm (difference between control and treated paw) gives the amount of ^{125}I albumin exudation. Percent exudation was calculated as (Δ dpm/dpm control paw) x 100.

In perfusates:

3. Volume (ml).
4. Protein content in 0.1 ml, according to Lowry et al. (1971). Bovine serum albumin was used as standard.
5. Radioactivity (dpm) in 0.1 ml was determined with an automatic gamma counting system (Nuclear Chicago,1185 Series). The average counting efficiency was 67%. The recovery of ^{125}I albumin from the exudate was calculated as{dpm/Δ dpm (in vivo)} x 100 x volume x 10 .
6. The perfusates were extracted (3.4) and PG-like activity (PGL) was bioassayed on a cascade of RSS and RC (0.1 ml/min), and expressed as ng PGE_2. The values were uncorrected for recovery (recovery of ^{3}H-PGE_1: 91 ± 2%, 16).

After removal of cannulae and killing the rats:

7. Paw weights (see 3.2).
8. Radioactivity in severed paws (see perfusates, 5).

5.4. Results.

The results obtained with anaesthetized rats (method 5.3.1) are summarized in table 5.1. PGL was generally not detectable in untreated paws. The prolonged presence of the cannulae, failed to release PGL, as indicated by saline

treatment. PGL was released after injection of CAR, and was already detectable at 1 and 2 hours. At 4 and 6 hours after application of CAR the amount of PGL that was removed by the perfusion was significantly higher than before the administration of CAR.

Table 5.1. Collection of prostaglandin-like activity (PGL) from rat hindpaws before (-30 min) and after treatment with CAR or saline.

Perfusion time	Saline treated PGL	CAR treated PGL	
h	ng PGE_2/perfusate	ng PGE_2/perfusate	
-0.5-0	< 1.0 (8)	< 1.0 (10), 1.1 (1)	
1-1.5	< 1.0 (2)	< 1.0, 1.3 (2)	
2-2.5	< 1.0 (2)	2.0 (1)	
4-4.5	< 1.0 (2)	2.9 ± 0.3 (4)	*
6-6.5	< 1.0 (2)	3.3 ± 1.2 (4)	*

Since the efficiency of the perfusion method was not estimated, the values are expressed as ng PGE_2/perfusate. Numbers of observations are given in brackets. *= $p < 0.05$, when compared with zero-time controls (Wilcoxon test).

Comparison of levels of PGL obtained using different (A, B and C) perfusion methods.

Fig. 5.3 shows the correlation between protein content of the perfusates and the recovery of ^{125}I labelled albumin from the CAR exudate at 4h.

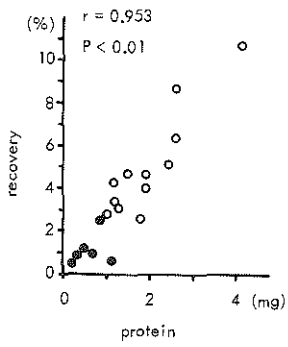


Fig. 5.3. Correlation between protein content of perfusates and percent recovery of ^{125}I albumin from the exudate. The data were obtained with killed animals (method C). The paws were perfused with Krebs, containing 28 µM indomethacin. Recovery (%) and protein content (mg) were measured as described in 5.3.2.
●= diameter outer cannulae= 1.1 mm
o= diameter outer cannulae= 2.0 mm

A positive correlation existed between these activities, but the recovery was low, even with the larger cannulae, which have been used in the following experiments.

The effects of the different perfusion methods on PG release are shown in table 5.2. It should be noted that PGE levels in rat plasma were below 50 pg/ml (n=3), as determined on the RSS (0.1 ml/min), after extraction of 3 ml plasma and TLC, and correction for recovery of ^{3}H-PGE$_1$. Thus, the 1.29 ng PGE$_2$, which was detected in perfusates from saline-treated paws, using method A, was probably an artefact, due to damage of small vessels or other tissue constituents. The presence of considerable amounts of protein in those perfusates also indicated tissue damage. Killing the rats (Method C) reduced the amount of protein in perfusates of saline treated paws and tended to lower PGL levels. This may imply that damage of vessels is responsible for artificial protein leakage and for the greater part of the PGL in saline-treated paws, perfused using Method A. Treatment of the rats with indomethacin (Method B), prior to insertion of the cannulae, also reduced the artificial release of PGL to levels below the limit of detection.

Table 5.2. Comparison of different perfusion methods.

Perfusate	Saline-treated paws.		
	Method A	Method B	Method C
protein (mg)	0.81 ± 0.32	0.31 ± 0.21	0.06 ± 0.01*
recovery of ^{125}I (%)	---	40 ± 20	52 ± 28
PGL (ng PGE$_2$)	1.29 ± 0.8	< 0.04*	0.19 ± 0.12
PGL (ng PGE$_2$/exudate)	---	< 0.24	0.30 ± 0.09
	Carrageenin-treated paws.		
protein (mg)	---	1.72 ± 0.43⊕	2.17 ± 0.83⊕
recovery of ^{125}I (%)	---	2.8 ± 0.7	6.1 ± 2.0
PGL (ng PGE$_2$)	---	0.18 ± 0.07⊕	0.39 ± 0.15
PGL (ng PGE$_2$/exudate)	---	6.4 ± 1.2⊕	7.2 ± 1.4⊕

Paws were perfused at 4 h after injection of saline or carrageenin. Method A, anaesthetized rats; Method B, anaesthetized rats,treated with indomethacin (5 mg/kg,i.v.) 5 min before insertion of cannulae; Method C, killed rats (see 5.3.2). Protein, recovery of ^{125}I albumin and PGL were measured as described in 5.3.2. The *in situ* amount of PGL in the exudate was calculated using the percent recovery of ^{125}I, and expressed as ng PGE$_2$/exudate. Values represent mean ± s.e.m of 4 experiments. * = $p < 0.05$, Method B or C vs Method A; ⊕ $p < 0.05$, carrageenine vs saline (Wilcoxon test,two tailed)

The recovery of ^{125}I albumin from saline-treated paws (determined with Methods B and C) showed considerable variation. This is explained by the small amount of exudation in these paws, which was hardly detectable in vivo. In the eperiment shown in table 5.2, the increase in exudation due to saline, expressed as a percentage of the radioactivity in the untreated paw (5.3.2), was 7.4 ± 8.7 % (n=8) (cf. CAR-treated paws in table 5.3). Thus, in some cases no exudation was detectable, although a small amount of radioactivity appeared in the perfusates. In these situations the recovery was, arbitrarily, assumed to be 100%. On the other hand, some exudation was sometimes present, but hardly any radioactivity was collected by the perfusion, possibly as a consequence of incorrect placing of the cannula.

When corrected for recovery, the highest value calculated was less than 240 pg PGL and on occasions even less than 40 pg PGL was present in the exudate of saline-treated paws (Method B).

Since Method A was less suitable for detection of PG release in CAR edema, Methods B and C were used exclusively. Apparently, only a small, variable percentage of all extravascular albumin was removable from the CAR exudate. In perfusates from CAR-treated paw, higher amounts of PGL were detectable, the difference being significant with Method B. The variation in the PGL values was reduced after correction for the recovery of the perfusion. Thus, measurement of the recovery of the perfusion method is essential for an estimate of PGL levels in the exudate in situ. At 4h, the estimated in situ levels of PGL in CAR exudates were significantly increased (table 5.2), when compared with saline-treated paws (Methods B and C).

Effects of indomethacin and EFA deficiency on levels of PGL in CAR edema.

EFA deficiency and treatment with indomethacin resulted in lower levels of PGL in CAR exudates. The results of these experiments are given in table 5.3. PGL was undetectable in perfusates of inflamed paws of EFAD rats. In perfusates of inflamed paws of indomethacin-treated normal rats, PGL was undetectable in 3 out of 4 samples. After correction for recovery of ^{125}I albumin, the estimated in situ levels of PGL in CAR edema, were significantly reduced in EFAD rats and in indomethacin-treated rats. Inflamed paws of EFAD rats displayed reduced swelling (%), but protein leakage, measured in vivo with ^{125}I albumin and assessed from the protein contents of the perfusates, was not reduced in EFAD rats. Indomethacin suppressed swelling as well as

protein exudation.

Table 5.3. Influence of indomethacin (IM) and EFA deficiency on PGL levels and exudation in CAR edema at 4h.

	Normal (8)	IM (4)	EFAD (4)
in vivo			
paw diameter (Δ mm)	5.0 ± 0.28	3.1 ± 0.28**	4.4 ± 0.34
paw swelling (%)	132 ± 6	74 ± 7**	101 ± 8*
exudation ^{125}I (%)	707 ± 93	332 ± 91*	568 ± 177
perfusate			
volume (ml)	2.58 ± 0.13	2.93 ± 0.34	2.38 ± 0.05
protein (mg)	2.06 ± 0.33	0.79 ± 0.14**	2.87 ± 0.49
recovery of ^{125}I (%)	4.96 ± 0.69	6.69 ± 2.82	7.49 ± 1.34
PGL (ng PGE_2)	0.25 ± 0.05	0.16; < 0.04 (3)*	< 0.04*
exudate (in situ)			
PGL (ng PGE_2)	5.33 ± 5.0	2.58; < 1.32 (3)*	< 0.8**
protein (mg)	43.0 ± 5.0	17.4 ± 4.5**	39.0 ± 2.6
swelling (g)	0.99 ± 0.09	0.61 ± 0.11*	0.57 ± 0.05*

Indomethacin (2.5 mg/kg, s.c.) was administered at -30 min and 3h. Perfusion with Krebs, containing 28 μM indomethacin was started 4 hours after subplantar administration of CAR (Method C). For details: see 5.3.2. * = $p < 0.05$, ** = $p < 0.01$, Wilcoxon test. Numbers of observations are given between brackets.

5.5. Discussion.

A perfusion method (Rocha e Silva & Antonio, 1960) was used to obtain exudate of CAR edema for assessment of PG-like activities. PGL was only detectable in one out of 19 perfusates before injection of the irritants. After application of CAR, increased amounts of PGL were measurable in the perfusates. Saline treatment did not raise the levels of PGL. Since PGL was undetectable in saline-treated paws, it is unlikely that tissue irritation by the prolonged presence of cannulae is a source of PGL.

After application of CAR, the levels of PGL increased with time, from 1h onwards. This indicated that the, so-called, "PG phase" of CAR edema probably started before 3h, in contrast to an earlier suggestion (70). The number of observations in the first 2 hours were, however, too few to justify statiscally

significant conclusions.

The method of Rocha e Silva & Antonio (1960) had several disadvantages such as the prolonged anaesthesia of the rats. Moreover, it did not provide any insight into the in situ PG levels. During edema development, part of the exudate is probably pushed into the perfusion system, since an additional (3 rd) period of perfusion (data not shown) always removed less PGL, which was often undetectable.

In later experiments, the following improvements were implemented:

1. The rats were not anaesthetized during the development of the edema.
2. The efficiency of the perfusion was quantified.
3. Indomethacin was included in the perfusion fluid in order to stop de novo formation of PGL during its collection.
4. The perfusates were extracted before assay.
5. The sensitivity of the bioassay was drastically increased.

A disadvantage which was also noticed, was the release of PGL, as a consequence of tissue damage during insertion of the cannulae, despite the presence of indomethacin in the perfusion medium.However, this method still seems more subtle than squeezing the exudate out of amputated paws, the method employed by Willis (1969). Thus, the only previous data on PGL levels in CAR edema, which have been obtained with a rather harmful mangling machine, lack reliability, since data on non-inflamed paws were omitted.

Simple protein measurements revealed that artificial leakage was greatly reduced when blood circulation through the paw had been terminated by killing the rat (Method B). A complete reduction of the artificial PG release was obtained by administration (i.v.) of indomethacin just before the insertion of the cannulae. Perfusates from inflamed paws contained more PGL then perfusates of saline-treated paws. This difference was significant when the artificial PG release during the operation was blocked by indomethacin (Method B). Indomethacin was also included in the Krebs used to wash out the subplantar area of the paws. If it was omitted, higher levels of PGL were observed in the perfusates (data not shown). This difference is explainable by biosynthesis of PGs in the coaxial cannula and in the collecting tube. Thus, in vitro PG biosynthesis by the cells that were washed away or stick to the superfusing system was then probably assessed. Since the main purpose of this study was to measure in situ PG levels, and more appropriate techniques are available for the assessment of in vitro PG biosynthesis by different celltypes (see 2.4.,

and Zurier, 1976), indomethacin was always included in the perfusing Krebs.

The efficiency of the perfusion was estimated from the amount of ^{125}I albumin, an exudate constituent which permitted *in vivo* detection, that was trapped. It was assumed that all ^{125}I albumin, that had accumulated in the inflamed paw, was present in an extravascular compartment. This assumption is not necessarily correct, since part of the increase might be due to an enhanced blood volume of the paw. However, other studies (Vincent & Zijlstra, submitted for publication) indicated that blood volume, measured with ^{51}Cr labelled erythrocytes, increased by approximately 10 percent, whereas the accumulation of ^{125}I albumin averaged 700%. Thus, the greater part of ^{125}I albumin was indeed extravascular. A significant, positive correlation was present between the estimated recovery and the protein content of perfusates. Thus, the method permitted estimation of total protein leakage. Since classical PGs exhibit weak binding to albumin (eg. 222), it was assumed that the recovery, determined with albumin, was also applicable to PGL. Circumstantial evidence for this assumption was provided by the reduction of the variation in PGL levels in perfusates, after correction for recovery.

The efficiency of the perfusion was low, which is not surprising, since CAR edema develops in the whole paw, whereas only the subplantar, subcutaneous region was perfused. Moreover, it has been reported on basis of histology that a large part of the edema is "tissue" bound, which means that the edema develops within interstitial and muscle tissue (eg. Bechara et al., 1976). Thus, only extravascular fluid, in the proximity of the inlet of the coaxial cannula was likely to be collected. Indeed, no correlation seemed to be present between perfusion time and the efficiency of trapping ^{125}I albumin (see also results with anaesthetized animals).

In perfusates from inflamed paws, an acid, ether-extractable substance was detectable, that stimulated a RSS in the presence of a complete mixture of antagonists. The quantity of this material was expressed in terms of ng PGE_2/exudate. The amounts were too small to permit a better characterization, eg. by NaOH treatment. It is not unlikely that, during CAR edema endoperoxide metabolites, other than classical PGs, are formed in large amounts. Experiments with ^{14}C-AA, and subsequent extraction and separation on TLC have, however, not yet been performed. Similar *in situ* amounts of PGL were found in several experiments, using Methods B and C (compare table 5.2 & 5.3). Since the weight of the exudate at 4h was approximately 1 g (table 5.3), it would appear that at least 5 ng PGL/ml is then present in the CAR exudate. Since

PGs other than E_2 (eg. D_2 or I_2) are less potent stimulators of the RSS (see table 2.1 and fig. 3.5), these quantities of PGL are minimum estimates. PGE_2, in doses of 1 ng, increases bloodflow significantly (Johnston, Hay & Movat, 1976) without affecting exudation. Thus, the PGL present in CAR edudate may contribute to this pro-inflammatory activity of PGs.

Although 5 ng PGL/ml seems quite a low level, it is at least 100 times higher than the plasma concentration of PGE_2, which was below 50 pg/ml. In order to achieve such a concentration gradient between plasma and exudate, PG biosynthesis must be extremely active, if a free exchange of PGs exists between plasma and exudate. Alternatively, the half life of PGs in exudates will be much longer if such a free exchange does not exist as suggested in ref. 24, since 15-hydroxy-PG dehydrogenase activity was absent in the exudate of CAR-induced granuloma (Ohuchi et al., 1976). In fact, such prolonged persistence of PGs in an exudate seems a possible explanation for the high levels of PGs that are claimed to be present in exudates of CAR-induced pleurisy (50,105, 262) and CAR-induced granuloma (191,273,274). Unfortunately, data on the turnover of PGs and other putative mediators in exudates are lacking, despite the availability of labelled compounds and suitable models like CAR-induced pleurisy.

In accordance with in vitro observations (see 4.4), indomethacin (2.5 mg/kg) reduced PGL levels in CAR edema. The present results directly support the suggestion (eg. 83,255,274) that in CAR-induced hind-paw edema, a model that is so often used for the assessment of anti-inflammatory activity of NSAIDs, indomethacin exerts its activity through inhibition of PG release. In three perfusates, no PGL was detectable and the calculated in situ quantities in the exudates were significantly reduced. This inhibition coincided with reduced ^{125}I albumin leakage, a reduced protein content of the exudate and reduced swelling.

The results with EFAD rats directly support the idea that these animals can serve as a model in which PG biosynthesis is of minor importance. In the absence of PGs the development of CAR edema was impaired in EFAD rats. Interestingly, protein exudation, measured with two methods, was not significantly reduced during EFA deficiency. This difference between edema development and protein exudation is not explainable by a reduction in PG biosynthesis, since it was not observed in indomethacin-treated rats. It may be due to changes in the functional properties of venules, or other blood vessels, during EFA deficiency. It is worth noting that EFA deficiency affects several

membrane properties (see 2.3.3). Because of the unchanged protein exudation, a smaller difference in osmotic pressures between intra- and extravascular compartments is likely to occur. This will facilitate water accumulation, and thus swelling of the tissue compartment in EFAD rats in spite of the reduced biosynthesis of PGs. Thus, any drug that interferes with the "enhanced" protein leakage during EFAD conditions, will further reduce edema formation. Moreover, the appearance of substrates that bind to albumin will be facilitated by the unchanged leakage of proteins in the inflamed area.

In conclusion, experiments with both EFAD and indomethacin-treated rats indicated that PG biosynthesis contributed to the development of CAR edema.

6. THE CONTRIBUTION OF PROSTAGLANDINS TO THE DEVELOPMENT OF CARRAGEENIN-INDUCED HIND-PAW EDEMA.

6.1. Abstract.

The reduction (from 2h onwards) of carrageenin-induced hindpaw edema (CAR edema) in EFA deficient rats was probably caused by a shortage of PG precursors. The local supplementation of arachidonic acid (AA) restored CAR edema to normal levels in EFA deficient rats. This suggested that the cyclo-oxygenase activity of EFA deficient rats was unaltered (cf. Chapter 4). Moreover, EFA deficient rats showed an unaltered response towards edemagens (bradykinin and serotonin). PGE_1 was a weak edemagen in the rat paw, $PGF_{2\alpha}$ was inactive. Circumstantial evidence indicated that the bradykinin mediated component of CAR edema was unaltered during EFA deficiency.

Simultaneous injection of PGE_1, AA or dihomo-γ-linolenic acid, together with CAR, enhanced CAR edema in normal rats. The stable 9,11 epoxymethano analogue of PGH_2, eicosatetraynoic acid and 5,8,11 eicosatrienoic acid were inactive in this respect. The possibility that release of a vasodilator PG endoperoxide-derived product is a rate limiting factor in CAR edema development, is discussed.

6.2. Introduction.

Non-steroidal anti-inflammatory drugs, which are cyclo-oxygenase inhibitors, suppress carrageenin-induced pedal edema (CAR edema). Therefore, it is generally accepted that PGs are involved in this process (see 2.5.2). In the previous chapter it has been demonstrated that prostaglandin-like material (PGL) accumulated in the exudate during CAR edema development.

The role of PGs in CAR edema was further characterized using essential fatty acid deficient (EFAD) rats, which have a shortage of arachidonic acid (AA), the main PG precursor, as shown by the data on erythrocytes (table 4.3). Moreover, studies on platelets of EFAD rats revealed that endogenous PG endoperoxide production, measured as release of PGE and TXA_2, was drastically diminished, but cyclo-oxygenase activity was unimpaired (see chapter 4).

Reduced CAR edema has been described in EFA deficient rats (Bonta et al., 1974), and has been confirmed and reinforced by measurements of PGL in CAR edema (see chapter 5). Since EFA deficiency does not only affect PG biosynthe-

sis (see 2.2.3), the decreased swelling might be due to alterations in other inflammatory parameters. Some of the alternatives have been ruled out in the experiments described in this chapter.

AA was administered locally to EFA deficient rats, in order to investigate whether PG precursor shortage or a change in cyclo-oxygenase activity was responsible for the suppression of CAR edema. Local administration of AA potentiated CAR edema in normal rats (eg. 149), but it was not known whether dihomo-γ-linolenic acid (PGE_1 precursor) or eicosatrienoic acid (n-9), which accumulates during EFA deficiency (see 2.3), were able to enhance CAR edema.

The inflammatory activities of a stable PG endoperoxide analogue (9,11 EM), PGE_1, $PGF_{2\alpha}$, serotonin (5HT) and bradybinin (Bk) were investigated, both in normal and in EFAD rats. In order to evaluate the Bk component of CAR edema in EFAD rats, the effects of a bradykinin potentiating peptide (BPP_{9a}) were tested in both types of rats.

6.3. Methods.

Animals. Both normal and EFAD male Wistar rats (220-280 g) were used (see 3.1). In the experiment shown in fig. 6.2, another inbred strain (R x U, C.P.B., Medical Faculty, Erasmus University, Rotterdam) was used.

Hind paw edema. Edema was evoked by subplantar injection of 0.1 ml sterile pyrogen-free 0.9% NaCl, 0.1 CAR (1%), 0.1 ml irritant or a combination (in 0.1 ml), and increase in paw-diameter or paw-volume was measured (see 3.2).

PGE_1, $PGF_{2\alpha}$, 9,11 EM, 5HT and Bk were dissolved in sterile pyrogen-free 0.9% NaCl. For the administration of the combination of fatty acids or PGE_1 and CAR an aliquot of the fatty acid stock solution (see 3.6) was dried under reduced pressure, if necessary, and dissolved in 1 volume ethanol (10 mg/ml). 9 Volumes of 0.9% NaCl, containing 0.2% Na_2CO_3, were then added, giving a solution of 1 mg fatty acid/ml. This solution was diluted 1:1 (v/v) with 2% (w/v) CAR. Thus, the paws were injected with 1 mg CAR and 0.05 mg fatty acid in 5% ethanol and 0.1% Na_2CO_3. Controls received the same mixture without fatty acid.

In one experiment (fig. 6.2), arachidonic acid (0.05 ml, 1 mg/ml, prepared as described above) was administered at 2h after the injection of CAR. Controls received 0.05 ml 0.9% NaCl containing 0.1% Na_2CO_3 and 5% ethanol.

Bradykinin potentiating peptide (BPP_{9a}) was dissolved (1 mg/ml) in sterile, pyrogen-free 0.9% NaCl and was diluted (1:1, v/v) with 2% CAR.

6.4. Results.

6.4.1. CAR edema in normal and EFAD rats.

The EFAD and control rats used in these studies were of different ages (see 3.1). This does not seem to have been of importance, since the age of the rats had little influence on the development of edema in normal rats as shown in fig. 6.1.

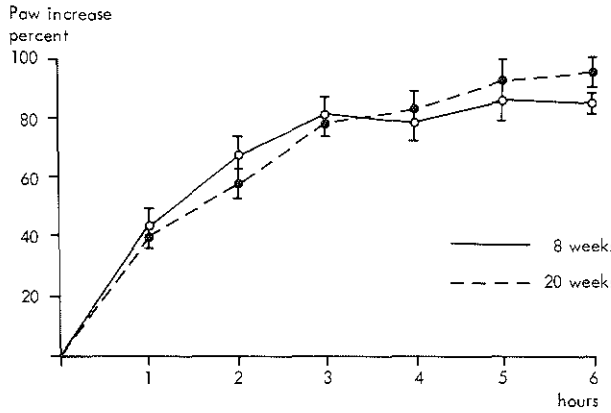


Fig. 6.1. CAR edema in normal rats of different age. Both hind paws of 5 rats of 8 weeks and 20 weeks were injected with 1 mg CAR and swelling was expressed as percent increase in diameter.

The reduced CAR edema of EFAD rats (see 34) was confirmed in several experiments, in both R x U and Wistar rat strains. An example is given in fig. 6.2. The swelling in EFAD rats differed significantly from that in normal rats after 2h (Student's t-test: $p < 0.05$). The difference between both types of rats disappeared after injection of 50 µg AA into each paw at 2h (Student's t-test: $p > 0.05$, 3h, 4h). Vehicle-injected paws of EFAD and normal rats differed significantly from each other at each later measuring point ($p < 0.01$).

The responsiveness towards different putative mediators was assessed in both normal and EFAD rats (fig. 6.3). PGE_1 alone, in doses of 5 ng and 10 ng, caused a small swelling (30 min) that was statistically different from the saline controls. Only 20 ng PGE_1 induced swelling which was 5% above saline levels (30 min) in normal rats. EFAD rats seemed more sensitive to PGE_1, since 5 ng PGE_1 already produced a swelling, which was 5% higher than that due to saline. No dose-relationship was found for PGE_1 in EFAD rats. Therefore, the higher sensitivity of EFAD rats towards PGE_1 when compared with normal rats,

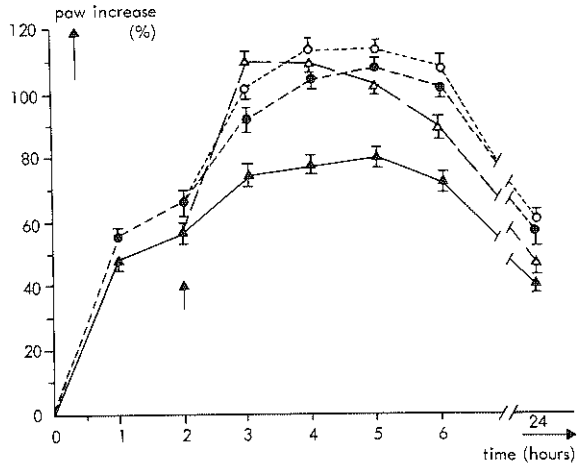


Fig. 6.2. Effects of AA on CAR edema in 20 normal and 20 EFAD rats. In this experiment R x U rats were used. CAR was injected at 0h into both paws. Groups of 10 rats received 50 µg AA or Na_2CO_3 (0.1%, 0.05 ml) in both paws after 2h (↑). Swelling was determined by diameter measurements (see 3.2). Normal rats: ●, Na_2CO_3, ○, AA (50 µg); EFAD rats: ▲, Na_2CO_3, △, AA (50 µg).

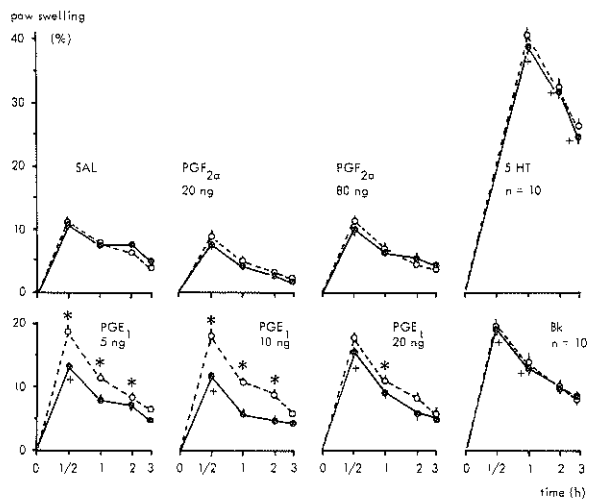


Fig. 6.3. Paw swelling induced by saline, PGE_1, $PGF_{2\alpha}$, 5HT (5 µg) and Bk (5 µg) in normal (—●—) and EFAD (--○--) rats. The irritants were injected in both paws. Swelling is expressed as percent increase in paw diameter. The numbers of observations were 20, unless indicated otherwise. +: $p < 0.05$, normal rats, treatment vs saline (Dunnett's test); *: $p < 0.05$, EFAD vs normal rats (Student's t-test).

disappeared at 20 ng PGE_1 per paw. Thus, PGE_1 alone is a very poor edemagen in this dose range. $PGF_{2\alpha}$ was not an edemagen in the rat paw. Bk and 5HT, in the high doses applied, are indeed edemagens, but no significant differences were observed between normal and EFAD rats.

Bradykinin potentiating peptide (BPP_{9a}) enhanced CAR edema in normal rats (fig. 6.4). This effect was apparent between 1 and 5 hours. BPP_{9a} also increased CAR edema in EFAD rats. The increase was approximately equal in both types of rats.

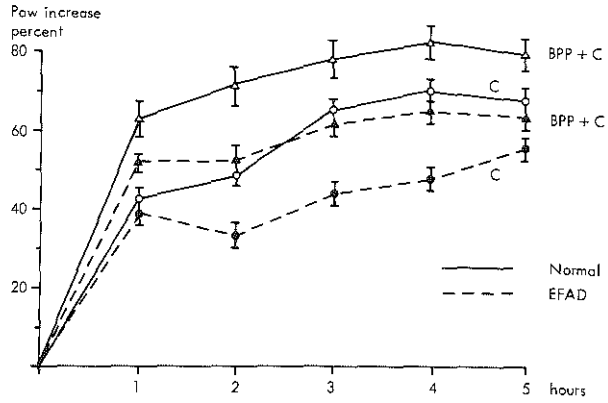


Fig. 6.4. Potentiation of CAR edema (C) by simultaneous subplantar injection (BPP + C) of a synthetic bradykinin potentiating peptide (BPP_{9a}, 50 µg) in both paws of 5 normal and 5 EFAD rats. Swelling is expressed as percent increase in paw diameter.

6.4.2. Effects of locally administered fatty acids and PGs on CAR edema in normal rats.

AA (50 µg) potentiated CAR edema in normal rats, when given simultaneously with CAR (see fig. 6.5). After 2h the difference between AA- and vehicle-supplemented CAR edema had disappeared ($p > 0.05$, Wilcoxon test). It is worth noting that the same dose of AA had much less effect when given at 2h after injection of CAR (fig. 6.2.). A qualitatively similar, but less pronounced enhancement of CAR edema was obtained by simultaneous administration of CAR and dihomo-γ-linolenic acid (50 µg), precursor of PGG_1. 5,8,11 Eicosatrienoic acid, which is not a PG-precursor, and eicosatetraynoic acid, which is an oxygenase inhibitor, were without significant effects ($p > 0.05$, Wilcoxon test).

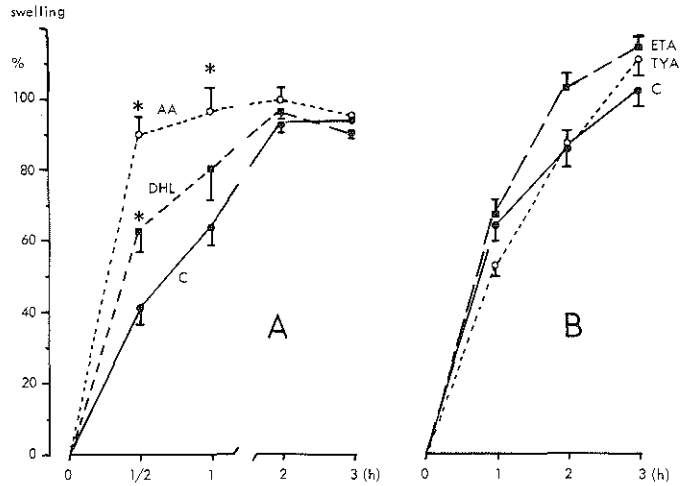


Fig. 6.5. Potentiation of CAR edema by the simultaneous administration of CAR (1 mg) and fatty acids (50 µg/paw; see 6.3). Both hindpaws of 4 (fig. A) and 5 (fig. B) rats were injected and the diameters of the paws were measured (see 3.2). Symbols: AA, arachidonic acid; DHL, dihomo-γ-linolenic acid; TYA, eicosatetraynoic acid; ETA, eicosatrienoic acid (20:3,n-9); C, carrageenin + vehicle without fatty acid. *: $p < 0.05$; fatty acid treated vs vehicle treated, Wilcoxon test.

Table 6.1. Indomethacin (IM) and aspirin (ASA) suppress AA-induced potentiation of CAR edema.

	mg/kg	CAR mm (increase)	CAR + AA mm (increase)	difference mm	%
SAL		1.48 ± 0.15	3.42 ± 0.13	1.94 ± 0.19	100 ± 9
IM	5	1.56 ± 0.14	2.61 ± 0.13	1.05 ± 0.19	51 ± 9
ASA	125	1.33 ± 0.10	2.28 ± 0.11	0.95 ± 0.15	46 ± 7

Six groups of 4 rats received indomethacin, aspirin or saline (SAL, 0.2 ml/kg) subcutaneously, 30 min before injection of CAR (1 mg) or CAR + AA (0.05 mg) in both paws. The increase in paw diameter (mm) was measured after 30 min.

Pretreatment of the rats with either indomethacin (5 mg/kg) or aspirin (125 mg/kg) inhibited the potentiation due to AA (table 6.1). The drugs were administered 30 min before the injection of the irritants, and paw swelling was measured after 30 min. Both NSAIDs failed to inhibit CAR edema at 30 min. Thus, the potentiation of CAR edema by AA was, at least partially, due to

conversion of AA into PGs.

Fig. 6.6. shows that CAR edema developed almost maximally within 1 hour when PGE_1 (50 ng) was given simultaneously with CAR. After 3h the difference between PGE_1 and vehicle treatment had disappeared ($p > 0.05$, Wilcoxon test). The higher doses of PGE_1 (0.1 mg) produced a similar potentiating effect. However, the reduction of the later phase (24h) of CAR edema became significant with this higher dose of PGE_1. This dose had systemic effects and caused diarrhoea.

A high dose (0.05 mg) of the stable 9,11 epoxymethano analogue of PGH_2 (9,11 EM) failed to induce swelling when given alone, and displayed neither inhibiting nor enhancing effects when given simultaneously with CAR (see fig. 6.7).

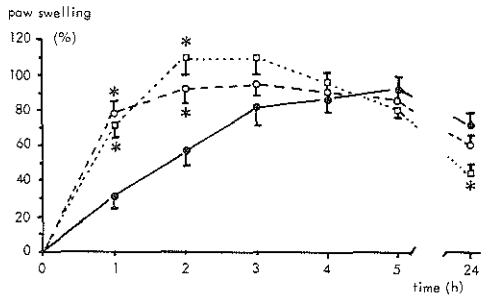


Fig. 6.6. Effects of PGE_1 on CAR edema. PGE_1 (50 ng or 0.1 mg) was injected simultaneously with 1 mg CAR (0.1 ml). —●—: CAR + vehicle without PGE_1; --○--: CAR + 50 ng PGE_1; ··□··: CAR + 0.1 mg PGE_1. Increase in volume was measured (n=4) and expressed as percent of the volume at zero time. *: $p < 0.05$ PGE_1, treated vs vehicle treated, Wilcoxon test.

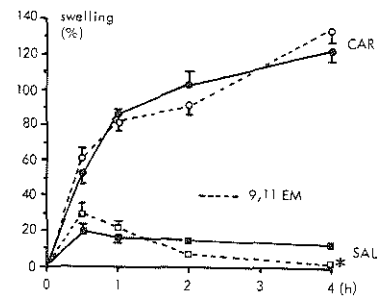


Fig. 6.7. Local effects of the PGH_2-analogue 9,11 EM (50 µg) in combination with saline (0.1 ml) or CAR (1 mg). SAL= saline with or without 9,11 EM; CAR= carrageenin with or without 9,11 EM. Swelling was expressed as percent increase in paw diameter. *: $p < 0.05$. 9,11 EM treated vs saline treated; Wilcoxon test.

6.5. Discussion.

It is generally accepted that PGs are involved in the development of CAR edema. This assumption is based on indirect evidence, such as sensitivity of the model to cyclo-oxygenase inhibitors (see 2.5, Vinegar et al., 1976, Vane 1976). Direct reliable measurements of PGs in the CAR pedal edema were lacking, but have been reported in chapter 5. Further indications for the pro-inflammatory involvement of PGs in CAR edema have been obtained, using a different approach, in previous experiments carried out by Bonta et al. (1974). It was shown that PG precursor deficient rats displayed a reduced CAR edema, which was almost insensitive towards inhibition by indomethacin. The reduction was rather selective, since chicken egg white-induced edema, in which amines are probably the most important edemagens, was unaltered during EFA deficiency. In the present study, these findings have been confirmed.

In all experiments, normal animals were matched for weight with EFA deficient animals. Thus, EFAD rats were older (see fig. 3.1). Since normal rats of both ages developed an equal inflammatory response, the reduced edema in EFAD rats was probably not due to age-differences. The CAR edema curves showed no biphasic components, but if edema, produced by saline (fig. 6.3) in the contralateral paw, had been subtracted a biphasic curve would have appeared. In most experiments described here, potentiation or reduction of CAR edema in the later phases, where saline-induced edema becomes insignificant, has been studied and therefore, saline controls were often not necessary. In accordance with other reports (eg. Lewis, A.L. et al., 1975), the maximal development of CAR edema showed daily variations (compare fig. 6.4 and 6.6).

If the reduced CAR edema in EFAD rats was due to PG precursor shortage, then local supplementation of AA should, at least partially, restore the swelling to normal levels. As shown in fig. 6.2, this assumption was shown to be true. The AA was administered 2h after the start of the edema, since from that moment the EFAD rats showed a clear reduction in swelling. It was assumed that in EFA deficient rats the effects of exogenous AA should be relatively short lasting and indeed, the AA enhancement tapered off from 3h after its addition. In normal rats, AA hardly enhanced the edema at all, when given at 2h.

The reduced edema in EFAD rats was not due to differences in sensitivity towards PGs of the E type (PGE_1), 5HT or Bk. PGE_1 was a weak edemagen in the

rat paw, which confirmed several more recent data (149,169,272). Interestingly, EFAD rats showed an enhanced sensitivity towards PGE_1. Bk and 5HT produced significant swelling, without any distinction being observed between normal and AA deficient rats. Thus, EFA deficiency did not interfere with the normal responses to 5HT and Bk and despite the increased sensitivity towards PGE_1, EFAD deficiency resulted in a decrease in the "so-called" PG-phase of CAR edema (Di Rosa et al., 1971).

The bradykinin potentiating peptide (BPP_{9a}), when given simultaneously with CAR, enhanced the edema in normal rats by up to 5 hours. This is in agreement with a report in which BPP_{9a} increased CAR edema when given 0 to 6h after CAR (Ferreira et al., 1974). These and other studies (Crunkhorn & Meacock, 1971) indicate that Bk is formed during the whole of the first 5h of CAR edema. The suggestion by Di Rosa et al. (1971) that Bk is released transiently after an initial release of 5HT and H has, therefore, to be rejected.

BPP_{9a} enhanced the swelling of EFAD rats to the same extent as that of normal animals. It appears that the kinin-mediated components of CAR edema remained unaltered during EFA deficiency. It is a well documented fact that Bk enhances PG release in several situations (see Vane, 1976; cf. table 2.2). Apparently its action as edemagen is not strictly dependent on PG release, since EFAD rats responded in a normal way to both Bk alone and to the potentiation of CAR edema by BPP_{9a}. A similar result with BPP_{9a} was obtained when PG biosynthesis was blocked by a high dose of indomethacin (81).

Local administration of AA at 2h after injection of CAR had little effect in normal rats (fig. 6.2). This picture changed when AA was injected concomitantly with CAR into paws of normal rats. In this way, the swelling developed maximally within 30 min. Availability of PG precursors may apparently be a limiting factor in the initial stages of CAR edema, and later administration of exogenous AA does not further increase the swelling. Non-specific potentiation by AA, not involving cyclo-oxygenase conversion of the fatty acid, seemed unlikely, since eicosatrienoic acid (n-9) failed to enhance the swelling. This fatty acid replaces AA during EFA deficiency and is not a cyclo-oxygenase substrate (see 2.1.2). Moreover, the AA-induced potentiation was susceptible to inhibition by cyclo-oxygenase inhibitors, like indomethacin and aspirin. Both of these findings are in agreement with reports in which AA-induced potentiation of CAR edema, was suppressed by NSAIDs, but not by corticosteroids and with the finding that linolenic acid (18:3,n-3) lacked

this potentiating capability (149,270,271)

The limited availability of PGs, in the early edema phase, is also demonstrated by the potentiation of the early phase of CAR edema by PGE_1. This effect of PGs of the E type has also been found by several other authors (Moncada et al., 1973, Lewis et al., 1975, Smith et al., 1974). Interestingly, PGE_1 in higher doses suppressed the swelling after 24h (see chapter 7).

Dihomo-γ-linolenic acid (20:3,n-6) was less potent than AA. This finding may be explained in several ways. It is possible that dihomo-γ-linolenic acid is less efficiently oxygenated than AA. A more tempting explanation is that both EFA's are equally efficiently converted into endoperoxides, but that the PG endoperoxides derived from AA yield more potent pro-inflammatory products. PGE_1 (derived from 20:3 (n-6)) and PGE_2 (derived from AA) are generally equipotent as -indirect- inducers of plasma leakage in the rat skin (eg. 60). Thus, endoperoxide-derived substances, other than classical PGs, might be of more importance for edema development. Since PGG_1 and PGH_1 are not suitable substrates for either TXA_2 synthetase (178,187) and PGI_2 synthetase, these pathways may deliver such pro-inflammatory substances. PGI_2, a potent arterial smooth muscle relaxant, seems the best candidate, since a drastic increase in local blood flow is the most important pro-inflammatory feature of PGs (see 2.5).

The stable 9,11 epoxymethano analogue of PGH_2 (9,11EM), which shares pharmacological properties with PGH_2 (Malmsten, 1976) and TXA_2 (see 3.2), but lacks their instability, seemed suitable for the evaluation of a possible involvement of TXA_2 (or PGH_2) in inflammation. In accordance with its lack of vasodilatory properties, a huge dose had no pro-inflammatory effect by itself nor in combination with CAR. More studies are needed to establish the pro-inflammatory role of PGI_2 or TXA_2, since both 6-keto- $PGF_{1\alpha}$ and TXB_2 have been detected in CAR-induced granuloma-pouch exudates (52,53).

7. ACUTE ANTI-INFLAMMATORY EFFECTS OF SOME DRUGS IN PROSTAGLANDIN PRECURSOR DEFICIENT RATS.

7.1. Abstract.

The anti-inflammatory activities of 2 types of anti-inflammatory drugs have been studied in EFAD rats, in which PG release is of minor importance. It appeared that the main anti-inflammatory activity of indomethacin was explainable by its inhibition of PG biosynthesis. This drug was hardly active against carrageenin-induced hindpaw edema (CAR edema) in EFAD rats. Aspirin and dexamethasone exhibited normal inhibition of CAR edema in this model. Thus, interference with PG biosynthesis did not fully explain their anti-inflammatory activities. Indirect evidence for the hypothesis that aspirin inhibited phosphodiesterase in vivo was not obtained. Finally, when PGE_1 was given systemically, its anti-inflammatory effects (see Chapter 6) prevailed. This effect was probably exerted via adenylate cyclase stimulation, since theophylline potentiated the anti-inflammatory effect of PGE_1. The finding that PGE_1 can suppress CAR edema indicates that PGs may modulate this inflammatory reaction.

7.2. Introduction.

Both NSAIDs and corticosteroids reduce carrageenin-induced hind paw edema (CAR edema), and the previous chapters have described the evidence which has been obtained for a pro-inflammatory role of PGs in the initial phase of this model. Part of this evidence was provided by studies on EFAD rats which displayed a reduced CAR edema. The reduction was explained by PG precursor deficiency and not by differences in cyclo-oxygenase activity, or sensitivity towards PGs and other mediators.

It is generally accepted that the anti-inflammatory activity of aspirin-like drugs is mainly due to suppression of PG biosynthesis (Vane, 1971, 1976). This attractive idea has recently received some criticism (eg. Smith, 1975). Corticosteroids also interfere with the cellular release of PGs (see Gryglewski, 1976; table 2.2.) and this might partially explain their anti-inflammatory activity. Therefore, it seemed worth testing the anti-inflammatory activity of 2 NSAIDs (aspirin and indomethacin) and a corticosteroid (dexamethasone) on CAR edema in EFAD rats, under conditions where the role of the arachidonate system has largely been ruled out.

Even relatively high doses of indomethacin were inactive in this poorly-developed model, while both aspirin and dexamethasone still displayed anti-inflammatory activity. A few attempts have been made to find evidence for the hypothesis that the alternative mode of action of aspirin was due to phosphodiesterase inhibition. Finally, the inhibition of paw swelling by PGE_1 was further investigated.

7.3. Methods.

CAR edema was induced in normal and EFAD rats (see 3.1 & 3.2). Drugs were given subcutaneously (2 ml/kg). Indomethacin, aspirin and dexamethasone were administered at $-\frac{1}{2}$ and 3h. The other drugs (PGE_1, theophylline) were injected (s.c.) 30 min before the start of the inflammatory response. Control animals received saline.

7.4. Results.

7.4.1. The anti-inflammatory activity of indomethacin and aspirin in normal and EFAD rats.

The inhibition of CAR edema by indomethacin is shown in fig. 7.1. Indomethacin was only partially effective in suppressing the reduced edema in EFAD rats. The reduction was significant at 2h whereas the inhibition of CAR edema in normal rats was significant from 2h onwards. The results were similar to those obtained in earlier experiments (see Bonta, Chrispijn et al., 1974 and Bonta, Bult et al., 1976), although the experiments on normal and EFAD rats were not done on the same day. In pilot experiments, which are not shown here, anti-inflammatory doses of aspirin and dexamethasone were selected that were approximately equipotent to the doses of indomethacin, used in normal rats. Subsequently, indomethacin and aspirin were tested at the same time in normal and EFAD rats. Complete swelling curves are not shown here, but have been published (Bonta, Bult et al., 1977). The measurements, at 5h are summarized in fig 7.2. Analysis of variance (ANOVA), indicated that significant differences existed between the different groups, which were further elaborated with Duncan's multiple range test. Indomethacin significantly ($p < 0.05$) reduced CAR edema in normal rats, although the inhibition was less than that usually obtained. Indomethacin did not significantly reduce the suppressed ($p < 0.05$) CAR edema in EFAD rats (cf. fig. 7.1). This was in contrast to as-

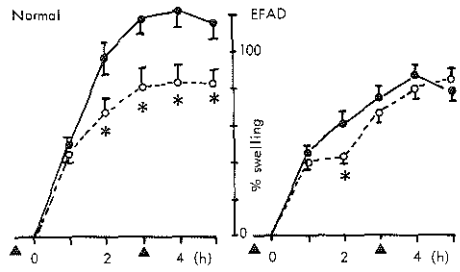


Fig. 7.1. Effect of indomethacin (--o--) on CAR edema in normal and EFAD rats. Indomethacin (2.5 mg/kg, s.c.) was administered to 5 normal and 4 EFAD rats at the points indicated by▲; controls (5 normal and 4 EFAD rats) received saline (2 ml/kg, s.c.). Results are expressed as percent increase in diameter of the paw. *: $p < 0.05$, Wilcoxon test. EFAD and normal rats were not compared statistically since the experiments were not performed on one day.

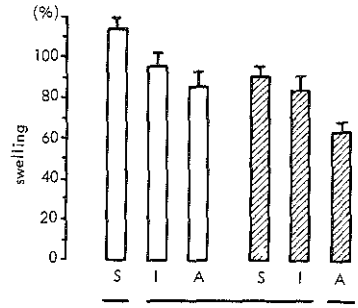


Fig. 7.2. Effects of indomethacin (I) and aspirin (A) on CAR edema in normal (open columns) and EFAD (hatched columns) rats. Results show percent increase in diameter of the left paw at 5h (4 rats/group). Indomethacin (2.5 mg/kg, s.c.), aspirin (125 mg/kg, s.c.) and saline (S, 2 ml/kg) were administered at $-\frac{1}{2}$h and 3h. ANOVA: $F_0(5,18) = 7.03$ $F_{0.95}(5,18) = 4.59$; $p < 0.05$. The means of the treatments that are not underscored with a common line, differ from one other significantly ($p < 0.05$, Duncan's test).

pirin , which caused a significant ($p < 0.05$) reduction of the edema in both normal and EFAD animals. Thus, the anti-inflammatory activity of indomethacin was largely dependent on inhibition of PG biosynthesis, whereas aspirin acted, at least partially, via other mechanisms.

7.4.2. The anti-inflammatory activity of dexamethasone in normal and EFAD rats.

The effects of dexamethasone on CAR edema in normal and EFAD rats have been presented earlier (Bonta, Bult et al., 1977a). The data at 5h after injection of CAR are summarized in fig. 7.3. ANOVA revealed statistical differences between 4 groups and Duncan's test indicated that dexamethasone significantly ($p < 0.05$) inhibited CAR edema in both EFAD and normal rats. Moreover, EFA deficiency again reduced CAR edema significantly ($p < 0.05$) when compared with normal rats. This indicated that this corticosteroid was still active, in rats which had a shortage of PG precursors. It must be assumed that dexamethasone interfered with other processes apart from liberation of AA.

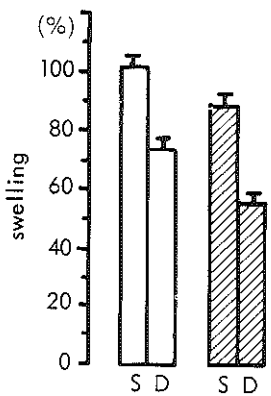


Fig. 7.3. Inhibition of CAR edema by dexamethasone (D) in normal (open columns) and EFAD (hatched columns) rats. The results show the percent increase in paw diameter. Dexamethasone (125 mg/kg) or saline (S, 2 ml/kg) was administered subcutaneously to groups of 5 rats at $-\frac{1}{2}$ and 3h. Swelling was measured 5h after subplantar administration of CAR. ANOVA: F_0 (3,16)= 42.3 $F_{0.99}$ (3.16)= 5.29; $p < 0.01$. Duncan's test:
normal rats; saline vs dexamethasone $p < 0.05$
EFAD rats; saline vs dexamethasone $p < 0.05$
normal rats vs EFAD rats (saline treated) $p < 0.05$

7.4.3. Anti-inflammatory effect of PGE_1 in normal rats.

The anti-inflammatory activity of PGE_1, which in vitro elevates cAMP levels of several cell-types (eg 11,215,270) and theophylline, an inhibitor of phosphodiesterase, was investigated. PGE_1 (1 mg/kg) and theophylline (75 mg/kg) had little anti-inflammatory activity when given separately, but their combined systemic administration completely abolished CAR edema (fig. 7.4). It is worth noting that PGE_1 displayed systemic effects, since it induced vasodilation (redness of hairless skin) and diarrhoea, and the combination of PGE_1 and theophylline was rather toxic, since 1 rat died.

It seemed likely that PGE_1 exerted its effect via stimulation of adenylate cyclase. The concomitant systemic application of carbachol (0.2 mg/kg),

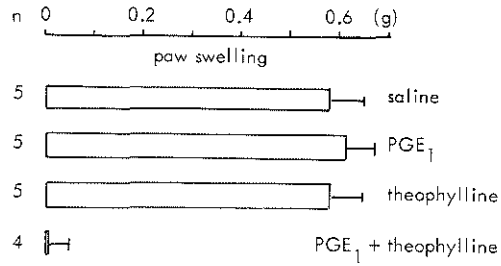


Fig. 7.4. Effects of systemic administration of PGE_1 and theophylline on CAR edema. PGE_1 (1 mg/kg), theophylline (75 mg/kg) and saline (2 ml/kg) were administered (s.c.) to groups of 5 rats, 30 min before application of CAR (1 mg) into the left paw. The contralateral paw was treated with saline; after 4h the rats were killed, and the differences in weight (g) between the left and right paws were determined (3.2).

a guanylate cyclase stimulator (eg. 270), together with theophylline (75 mg/kg) was without anti-inflammatory effect.

When given locally, acute, pro-inflammatory effects of PGE_1 (0.1 mg/paw) (see fig. 6.6) and theophylline (0.1 mg/paw; data not shown) prevailed. However, the late phase of CAR edema (24h) was always suppressed by PGE_1 (eg. fig. 6.6). Thus, combined systemic administration of PGE_1 and theophylline abolished CAR edema, whereas acute pro-inflammatory effects dominated when both drugs were given locally.

In several experiments PGE_1 and aspirin or indomethacin were administered systemically, and the anti-inflammatory activities of the seperate drugs and their combinations were investigated. An example of the anti-inflammatory effects of PGE_1 and aspirin is shown in fig. 7.5. Systemically asministered PGE_1 displayed a small ($p > 0.05$) edema suppressing effect at 4h after the injection of CAR. Inhibition of the edema by PGE_1 was more substantial and significant at 24h after application of CAR. Aspirin suppressed CAR edema, but PGE_1 failed to enhance the anti-inflammatory activity of aspirin (4h, 24h), in contrast to the potentiation observed with combination of PGE_1 and theophylline.

The results obtained with the combination of indomethacin and PGE_1 were very similar to those obtained with the simultaneous administration of aspirin and are, therefore, not shown. Again PGE_1 did not enhance the anti-inflammatory activity of indomethacin.

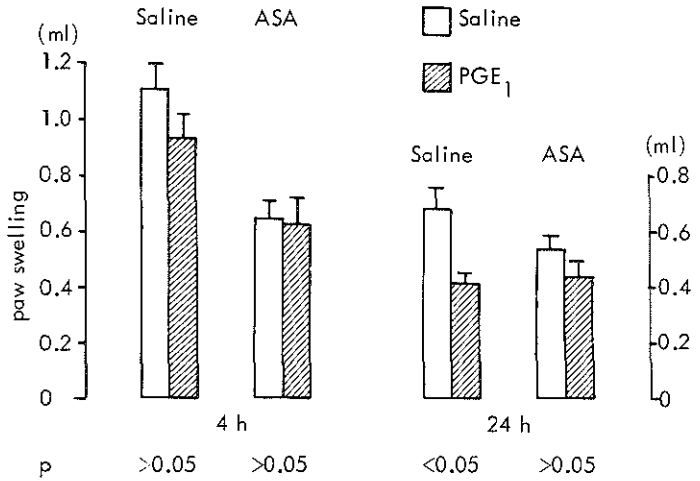


Fig. 7.5. Anti-inflammatory effects (4h and 24h) of systemically administered PGE_1 (hatched columns) and aspirin (ASA). CAR edema (1 mg/ left paw) was evoked in groups of 5 rats, pretreated (-30 min) with saline (2 ml/kg), aspirin (125 mg/kg, s.c.), PGE_1 (1 mg/kg, s.c.) or a combination of PGE_1 and aspirin. The administration of aspirin was repeated at 3h, while the other groups received saline. Swelling (ml) was measured volumetrically (see 3.2) at 4h and 24h after injection of CAR.
ANOVA, 4 hours: F_0 (3,16)= 6.70; $F_{0.99}$ (3,16)= 5.26; $p < 0.01$
Duncan's test: saline vs ASA, $p < 0.05$; saline vs PGE_1, $p > 0.05$
in aspirin treated rats, saline vs PGE_1, $p > 0.05$
ANOVA, 24 hours: F_0 (3,16)= 3.90; $F_{0.95}$ (3,16)= 3.25; $p < 0.05$
Duncan's test: saline treated rats, PGE_1 vs saline, $p < 0.05$. No other statistically significant differences.

7.5. Discussion.

In the work described in the previous chapters, several indications were obtained, that supported a possible pro-inflammatory role for cyclo-oxygenase metabolites in early CAR edema. The anti-inflammatory activity of 2 typical NSAIDs has now been evaluated in CAR edema under conditions where endogenous PG release is, for the greater part, absent (see chapters 4 and 5).

The anti-inflammatory activity of lower doses of indomethacin is probably largely dependent on cyclo-oxygenase inhibition. From 3h onwards, the dose of indomethacin used (2.5 mg/kg), which is around the ED50 value (Vinegar et al., 1976), had very little suppressive effect on CAR edema in EFA deficient rats. This dose does not inhibit neutrophil mobilization (Vinegar et al., 1976).

Even additional administration of indomethacin did not further suppress the late phase of CAR edema under EFAD conditions. However, indomethacin always had a small, but significant anti-edema activity at 2h during EFA deficiency (see also Bonta et al., 1974). This may be explained in several ways (vide infra and Bonta, Bult et al., 1977) but a plausible explanation seems that, even during severe EFA deficiency, some residual AA is always present in biological membranes (see table 4.3). This limited AA pool might be consumed during the initiation of CAR edema. Therefore, NSAIDs are likely to suppress the initial PG phase of CAR edema in EFAD rats. PG mediation of CAR edema was demonstrable from 2h onwards (see chapter 6), but it is likely that, with more sophisticated techniques, the PG phase will be shown to start even earlier (cf. Vane, 1976).

A dose of aspirin was selected in pilot experimets, which was generally about equipotent to the dose of indomethacin used in normal rats, and which again turned out to be around the ED50 value given by Vinegar et al., 1976. In contrast to indomethacin, this dose of aspirin displayed an anti-inflammatory activity during the whole course of CAR edema in PG precursor deficient rats. Thus, aspirin, which undoubtedly inhibits PG biosynthesis, can exert its anti-inflammatory activity, via other mechanisms, provided that the metabolism and/or tissue distribution of aspirin was not affected by EFA deficiency. A simple control experiment for testing this statement would be to administer both aspirin and indomethacin concomitantly to normal rats. An anti-inflammatory action of aspirin in normal rats, that were made PG deficient by means of indomethacin, would have supported the above statement.

There are several possible explanations for the additional anti-inflammatory activity of aspirin (see 2.2.2). It is likely that part of this activity is due to conversion to salicylate (see 148). This anti-inflammatory compound is devoid of significant anti-cyclo-oxygenase activity in vitro (see 2.2.2). Due to the scarcity of EFAD rats, the anti-inflammatory activity of salicylate has not yet been tested in EFAD rats. It would be expected to be as active as in normal rats. It seems unlikely that inhibition of neutrophil mobilization constitutes an alternative action. Both aspirin and indomethacin display such activity at higher doses (eg. 262), but only aspirin was active in PG precursor deficient rats. Moreover, both drugs were used in doses which do not affect neutrophil migration in CAR-induced pleurisy (see 262). Unfortunately, the invasion of neutrophils in vivo was not measured in EFAD rats, but EFA deficiency did not affect peripheral white cell patterns (Vincent et al.,1975).

Aspirin and indomethacin have been reported to inhibit phosphodiesterase in vitro (see 2.2.2). Elevation of cAMP levels of neutrophils in vitro leads to reduced release of lysosomal enzymes (eg. 270). Thus, inhibition of phosphodiesterase by aspirin might suppress inflammatory reactions, but no indications for such a role in vivo were obtained in the present study. The combination of PGE_1 and phosphodiesterase inhibitor theophylline totally blocked CAR edema. PGs of the E type are potent stimulators of adenylate cyclase in several cell types, including platelets (2.4), mastcells (11) and neutrophils (270). Thus, it is likely that the combined effect of PGE_1 and theophylline was due to enhancement of cAMP levels in several tissues. However, the inhibition was probably affected by "non-specific" systemic changes, since the combination was rather toxic (1 rat died) and might have involved a very marked hypotension

PGE_1 failed to enhance the anti-inflammatory activity of aspirin and indomethacin. Both NSAIDs, in this dose range, acted differently from theophylline. Additive anti-inflammatory effects of PGE_1 in combination with both NSAIDs have been described (69). The doses of the NSAIDs were, however, much higher than in this study. Thus, it is possible that theophylline and high doses of the two NSAIDs are indiscriminable from each other with respect to enhancement of their activity by PGs of the E type.

Dexamethasone was as active in normal rats as in EFAD rats. Thus, its anti-inflammatory activity seems to be independent of inhibition of local AA release. Inhibition of neutrophil mobilization, observed in CAR pleurisy and in several other models, therefore, seems a more likely mechanism of its anti-inflammatory activity (eg. Vinegar et al., 1976).

Finally, the question arises as to whether PGE_1 is pro- or anti-inflammatory. In accordance with most studies, local administration of PGE_1 enhanced acute CAR edema. However, 24h later the edema was reduced. The pro-inflammatory activity of PGE_1 is probably due to increased vasodilation and increased local blood flow, facilitating both exudation and the supply of circulating pro-inflammatory cells. As a result of this potentiated inflammatory response, the irritant may be removed more rapidly and the resulting edema after 24h will be reduced. Locally administered theophylline had a similar effect. The anti-inflammatory activity of systemically administered PGE_1 must be based on different mechanisms. Again, vasodilation was induced, since redness of the hairless skin parts was observed, but the initial stages of CAR edema were not potentiated. In fact, the anti-inflammatory effect of PGE_1 tended to start

earlier after systemic administration. A non-significant reduction of CAR edema was often noticed after 4h (cf. 69). This initial reduction might possibly be due to a general vasodilation, resulting in hypotension. However, after 24h, CAR edema was markedly depressed, while the rats seemed to be normal (no redness, no diarrhoea).

Thus, systemically administered PGE_1 exerted its anti-inflammatory effects at 24h via unknown mechanisms. Possibilities include an inhibitory action on release of amines by platelets (see 2.4) or mastcells (11), or an inhibition of release of lysosomal enzymes from neutrophils (270). These actions, which have all been demonstrated to occur in vitro, are exerted by those PGs which elevate cAMP levels (eg. PGE_1, PGI_2). The in vivo significance of these feed-back systems is, however, largely unknown. Since high (0.1 mg/ml) levels of PGE_1 are needed for inhibition of the relase of lysosomal enzymes from neutrophils in vitro (Weissmann et al., 1976), this mechanism seems of little importance for the inhibition of CAR edema in rats, treated with "only" 1 mg/kg PGE_1.

Whether endogenous PGs display an anti-inflammatory effect is not yet known. It is obvious that endogenous PGE_1 will be of little importance, in this respect due to the scarcity of dihomo-γ-linolenic acid in most tissues. Therefore, PGI_2 which is much more potent than PGE_1 as stimulator of platelet adenylate cyclase (99,242), seems to be a better candidate. The question as to whether PGs are pro- or anti-inflammatory might be dependent on the local or systemic site of action. It might also be possible that PGs, such as PGI_2, initially promote exudation via a marked increase in local bloodflow (see 2.5), whereas in a later stage of CAR edema the same type of PG may locally attenuate the inflammatory response via inhibition of the activity of invaded cells.

8. GENERAL DISCUSSION

8.1. Bioassay.

For the determination of nanogram quantities of prostaglandins (PGs), the cascade superfusion technique, described by Vane (1964), was used. The method permitted simultaneous parallel bioassay with several tissues (see 2.1.7). Suspension of the tissues in mineral oil (Ferreira and De Souza Costa, 1976), enabled detection of PGs in the picogram range. Thereby, bioassay reaches the sensitivity of radioimmunoassay. The laminar flow technique was adapted to superfusion of several different tissues (Bult et al.,1977, chapter 3). This improvement allowed a differential, direct bioassay or enhanced the specifity of the assay after extraction and silicagel chromatography of biological samples. A higher concentration of the agonist near its receptor site constitutes the fundamental difference between the mineral oil method and the classical cascade technique. It is important to realize that, using mineral oil, the sensitivity towards interfering substances is raised to the same extent as for the autacoids being tested. An illustration of this phenomenon is that upto 0.2 ml of rat platelet poor plasma (PPP) never contracted rabbit coeliac artery or rabbit aorta strips, superfused at 2.5 ml/min, whereas 0.05 ml PPP always induced a small contraction of the same tissues, superfused at 0.1 ml/min in mineral oil.

The superfusing Krebs contained a mixture of antagonists to rule out as many interfering substances as possible. In initial experiments, phentolamine was used to antagonize α-adrenergic agonists, and methysergide was used to block the effects of serotonin (5HT). Although the activity of 5HT on the rat stomach strips (RSS) was for the greater part blocked, 5HT then still contracted the rabbit aorta strip (RAS). This explained the release of both a labile and a stable rabbit aorta contracting substance (RCS) by aggregating platelet rich plasma (PRP). Only the labile component was liable to inhibition by indomethacin (Bult, Bonta et al., 1976). 5HT-induced contractions as well as stable RCS responses disappeared if the rabbit aorta was treated with the rather non-specific, irreversible α-blocker, phenoxybenzamine, together with methysergide. This indicated that methysergide alone is too week as a 5HT-antagonist to be used for reliable bioassays of TXA_2, which is probably the major constituent of RCS (120).

For the determination of the potencies of different agonists and antagonists the cumulative method for obtaining dose-response curves was applied

to the superfused organ cascade (Bult & Bonta, 1976a). This had a considerable time-saving effect and produced data, such as maximal contraction and log D50, that were not significantly different from those obtained by the stepwise procedure. These results indicated that the cumulative technique, which is often used for measuring dose-response curves with bathing tissues, is applicable to superfused tissues as well (Bult & Bonta, 1976a).

8.2. Platelet studies (Chapter 4).

Collagen-stimulated rat PRP released malondialdehyde (MDA) and materials which behaved like 5HT, PGE_2 and RCS (Piper & Vane, 1969) as determined by direct differential bioassay with a cascade of isolated tissues (Bult & Bonta, 1976b, 1977). The release of MDA, PG-like material (PGL) and RCS was greatly reduced during essential fatty acid (EFA) deficiency and was liable to inhibition by indomethacin, a cyclo-oxygenase inhibitor (Vane, 1971). These findings indicated that MDA, PGL and RCS were derived from PG endoperoxides.

PGL possessed pharmacological properties identiacal to those of PGE_2 and after extraction into an organic solvent, it co-chromatographed with PGE_1 in 2 systems. Therefore, it seems most probable that PGL was identical to a PGE (PGE_2). Since its pharmacological properties and its half life matched the characteristics of thromboxane A_2 (TXA_2; 47,120,178), RCS consisted probably mainly of TXA_2 (cf. 120). Release of both PGE and TXA_2 by rat platelets, aggregated with collagen in heparinized plasma, was qualitatively similar to that obtained with washed human platelets, stimulated with thrombin (119).

The endogenous formation of PGE was dependent on the dose of collagen. Endogenous AA, which is liberated from certain phospholipids during stimulation of washed human platelets, is, for the greater part, oxygenated by both cyclo-oxygenase and lipoxygenase (20,25). Thus, it seems likely that the enhanced formation of PGE by rat PRP was due to an enhanced availability of non-esterified AA with increasing doses of collagen. No data have been obtained which substantiated this assumption. However, it is likely that an endogenous PLA_2 is responsible for controlling the availability of AA, although an increased supply of AA may be achieved in several ways (see 2.1.2).

Aggregation by threshold doses of collagen was suppressed by indomethacin, in concentrations that were similar to those needed for TXA_2, PGE and MDA inhibition. This suggested that, under these conditions, the formation of cyclo-oxygenase products was essential for rat platelet aggregation. In EFAD

platelets, the formation of TXA_2 and PGE was drastically diminished during stimulation with supra-maximal doses of collagen. Decreasing the collagen dose would be expected to even further decrease the supply of cyclo-oxygenase products. As a result, it was not surprising that threshold doses of collagen, which seem to aggregate normal rat platelets largely by releasing oxygenated AA products, were less effective in EFAD PRP. Indeed, under conditions where AA supply was rate-limiting for aggregation in normal PRP, the EFAD platelets almost failed to aggregate. The impaired aggregation was not due to an altered sensitivity towards PG endoperoxides, and in the presence of exogenous AA the cyclo-oxygenase activity in EFAD platelets was unchanged. These findings are in agreement with the pro-aggregatory effects of either endoperoxides or TXA_2 in human platelets (116,120,231).

The lipoxygenase pathway may supply oxygenated fatty acids both in the presence of indomethacin and during EFA deficiency (119,186, see also 2.1.7). Under both conditions an impaired aggregation with threshold doses of collagen was observed, which indicated that the AA lipoxygenase pathway did not contribute to rat platelet aggregation in vitro.

Peak levels of TXA_2 coincided with the start of aggregation in rat PRP (Bult & Bonta, 1976b). This finding has been confirmed in washed human platelets, aggregated by addition of AA (101). In the latter report the levels of TXA_2 were measured by radioimmunoassay after conversion of TXA_2 into mono-O-methyl-TXB_2. This suggested that TXA_2 rather than the endoperoxides, was the pro-aggregatory metabolite of AA. If, as has been suggested (Haddeman et al., 1977), platelet MDA was mainly derived from TXA_2, and not from endoperoxides, the importance of TXA_2 in rat platelet aggregation was supported by the finding that both aggregation and MDA production were suppressed by imidazole, an inhibitor of PGH_2-TXA_2 isomerase (168).

Aggregation induced by higher doses of collagen, occurred independently of cyclo-oxygenase products. The possibility that residual TXA_2 formation was responsible for normal aggregation in the presence of indomethacin or during EFA deficiency, seemed unlikely. Doses of indomethacin and TYA which completely blocked TXA_2, MDA and PGE production in normal PRP, failed to interfere with aggregation and 5HT release. Even the combination of EFA deficiency and enormous doses of indomethacin did not suppress aggregation (Vincent et al., 1975). Thus, the mechanisms involved in aggregation with maximal doses of collagen are unclear. The attachment of a large number of collagen fibers may alter platelet membrane properties allowing Ca^{2+} influx, which will lead

to release of ADP and aggregation (see scheme in 2.4). It must be realized that rat platelets will aggregate as soon as ADP has been released, by whatever stimulus (see below).

PG biosynthesis was not involved in ADP-induced aggregation of heparinized rat PRP. Even supra-maximal doses of ADP did not release significant amounts of PGE. Moreover, the aggregation of rat PRP by threshold doses of ADP was not inhibited by indomethacin at concentrations which completely suppressed TXA_2, MDA and PGE release. The fact that threshold doses of ADP only partially aggregated EFAD PRP, seems to be in contradiction to this assumption, but may be explained by membrane alterations during EFA deficiency (see 2.3.3).

It is impossible to obtain information on the absolute amounts of TXA_2 using direct bioassay. Therefore, rat PRP was stimulated in the presence of ^{14}C-AA, and the formation of ^{14}C-TXB_2 and ^{14}C-PGE_2 was assessed. It is obvious that, in this way, not all the TXA_2 metabolites were measured, since conversion of TXA_2 to HHT and MDA (84,111,239), was not measured. In all experiments, whether in the presence of collagen or not, the ratio of TXB_2/(PGE_2+PGD_2) was much smaller than in studies with human platelets (eg. 119). Similar results, also indicating that the conversion of PG endoperoxides to TXB_2 is less predominant in rat platelet preparations, have recently been reported by other authors (Abrahams et al., 1977).

In the presence of exogenous AA, rat PRP formed PG endoperoxides with little release of TXA_2 (bioassay) or TXB_2 (TLC). At higher concentrations, AA induced both aggregation and a more significant release of TXA_2 (or TXB_2). This again suggested that the thromboxane pathway is more important for aggregation than endoperoxide formation per se. Possible explanations for the release of PGE without TXA_2 (or TXB_2), which seemed at first sight to contradict the results obtained with human platelets (120,178), have been proposed in section 4.5. Whatever explanation may be correct, the fact remained that experiments, where PG and TXA_2 biosynthesis was measured, following additions of exogenous AA to PRP, provided results that were not directly comparable to those obtained when endogenous AA was converted into PGE and TXA_2 by PRP. A similar discrepancy has been described in human PRP, where exogenous PGH_2 was not converted into TXA_2 (178).

The physiological significance of the observation that rat platelets can form PGE, and therefore PG endoperoxides, from exogenous AA, without aggregating, still has to be determined. However, it is likely that it is of little physiological importance, since even in inflammatory exudates, it is difficult

to imagine how free AA will ever reach concentrations of 0.1 mM. The situation changes, however, when high doses of free AA are administered locally to an organism (see 8.3).

In conclusion, it can be stated that rat platelet aggregation, induced in vitro by threshold doses of collagen, is amplified by cyclo-oxygenase products, particularly TXA_2. Both ADP and high doses of collagen may induce aggregation without the involvement of AA-derived products. The relevance of these in vitro studies to in vivo platelet behaviour is unclear. However, it is tempting to explain the tendency towards decreased arterial thrombosis formation in EFAD rats (review: Hornstra, 1973) on the basis of the decreased responsiveness of EFA platelets towards collagen.

Finally, rat platelet aggregation in vitro is certainly a good model for the evaluation of factors involved in PG biosynthesis. NSAIDs are active in this model, mainly because of inhibition of cyclo-oxygenase. Caution should, however, be exercised in extrapolating the results obtained in this in vitro model to inflammation. For instance, TYA, which blocked platelet cyclo-oxygenase, was without anti-inflammatory activity in CAR edema, when administered locally (upto 250 µg/paw).

8.3. Prostaglandins and carrageenin-induced hindpaw edema (CAR edema).

In the experiments described in chapter 5, the presence of prostaglandin-like material (PGL) in acute CAR edema (Winters et al., 1962) has been demonstrated using a coaxial perfusion method (214). Under carefully controlled conditions, very little, if any PGL was detectable in saline-treated paws. Thus, tissue damage was ruled out as a major source of PGL. An earlier report (Willis, 1969) claimed the presence of PGL in CAR pedal edema. However, this paper is not very reliable, since data on levels of PGL in saline-treated paws and on the time of collection of the exudate were omitted. Such control data are indispensable for an evaluation of the reliability of the techniques employed. In the present study, PGL was already detectable one or two hours after administration of CAR to the paw. This indicated that the involvement of PGs started in the very early phase of CAR edema. Vane (1976) came to a similar conclusion, using rats immunized against PGE_1. Both findings are not in agreement with the suggestion that the PG phase starts at 3h (70). PGL was statistically different from control values at 4 and 6h.

By measuring in vivo the exudation of ^{125}I albumin, which was administered (i.v.) before application of CAR, it was possible to test the efficiency of

the perfusion technique. The recovery of ^{125}I albumin from the exudate in the inflamed paw turned out to be very low. An estimation of in situ PGL levels 4h after the injection of CAR, indicated the presence of approximately 5 ng PGE_2 equivalents/ml exudate. The real value may have been higher, since it is not unlikely that PGs other than PGE_2, such as PGD_2, PGI_2 and its metabolite 6-keto $PGF_{1\alpha}$, -which are considerably less, or not active at all on the RSS- were formed to a large extent.

It is generally assumed that PG biosynthesis provides substances which act as pro-inflammatory mediators (Zurier, 1974, Vane, 1976). Evidence for pro-inflammatory effects of AA metabolites in CAR edema, was obtained in two different ways. Firstly, EFA deficiency resulted in a reduced AA content of rat erythrocytes and in reduced (80-90%) biosynthesis of TXA_2 and PGE by rat platelets. The yield of PGL from CAR edema (4h) in EFAD animals, turned out to be below the limit of detection, a finding which correlated with a reduced swelling. Secondly, it is wellknown that aspirin-like drugs reduce CAR pedal edema, a model which contributed largely to the discovery of indomethacin. Inhibition of PG biosynthesis has been claimed to be the major anti-inflammatory activity of indomethacin (Vane, 1971). Indeed, a reduction of CAR edema was associated with reduced PGL levels in situ in rats pretreated with indomethacin (2.5 mg/kg).

Thus, a pro-inflammatory, cyclo-oxygenase product was formed during acute CAR edema. In fact, it seemed that its formation was rate-limiting for the exudation, since simultaneous administration of either PGE_1 or AA, together with CAR, resulted in an almost maximal swelling within one hour (Chapter 6). The endogenous origin of PGL during acute inflammatory reactions is still a matter of debate, especially with respect to the role of neutrophils (92,93, 262). However, when exogenous AA is administered, the potentiation might be mediated by platelets. This is based on the observation that rat platelets can form PG endoperoxides, without aggregating and without forming the vasoconstrictor TXA_2 (Chapter 4). The PG endoperoxides preferently decompose to vasodilatory PGs, such as PGE_2, and are suitable substrates for PGI_2 synthetase which will again result in the formation of a pro-inflammatory vasodilator. Of course, it is also possible that a variety of other cell types transform exogenous AA into its pro-inflammatory products.

The involvement of cyclo-oxygenase in the potentiation of CAR edema by AA seems indisputable, since the potentiation was liable to inhibition by aspirin and indomethacin (see also 149,229). Moreover, 5,8,11 eicosatrienoic acid,

which accumulated during EFA deficiency, failed to enhance CAR edema. The observation that dihomo-γ-linolenic acid was less potent than AA in potentiating CAR edema suggested that possibly TXA_2 or PGI_2, rather than the classical PGs or PG endoperoxides, were of importance as pro-inflammatory substances.

The reduced edema in EFAD rats was used as a model in which PG release was of minor importance (see above) and the anti-inflammatory activities of NSAIDs (indomethacin and aspirin) and a corticosteroid (dexamethasone) were tested on this model. Exogenous AA restored the suppressed CAR pedal edema in EFAD rats. Due to the difficulty in obtaining exudate, no fatty acid analysis of exudate was carried out; the results obtained *in vitro* with platelets, however, indicated that cyclo-oxygenase substrate and not cyclo-oxygenase activity was diminished during EFA deficiency. Moreover, EFAD rats were equally sensitive to subplantar administration of Bk, 5HT and PGE. The bradykinin component of inflammation, which, in contrast to the suggestion of Di Rosa et al. (1971), lasted at least 5 hours (59,81), was unaffected in EFAD animals. This was judged from an experiment with the bradykinin potentiating peptide BPP_{9a}. Thus, PG precursor shortage, rather than alterations in PG-synthetizing capacity or changes in sensitivity towards other autacoids, was responsible for the impaired edema development during EFA deficiency.

Indomethacin was largely inactive in this PG precursor deficient model, especially during the later phase of the edema (4h), when PGs were not detectable (see Chapter 5). Thus, inhibition of PG biosynthesis (Vane 1971) seemed to be sufficient to explain the acute anti-inflammatory activity of this drug. The activity of indomethacin on the early phase (2h) of CAR edema in EFAD rats is most easily explained by a limited residual release of PGs during this phase, although no measurements of PGL were made in early CAR edema of EFAD rats to verify this assumption. At 4h, when indomethacin was inactive as an anti-inflammatory drug in EFAD rats, aspirin still exerted a marked, reproducible, anti-inflammatory activity. Therefore, the anti-inflammatory activity of aspirin is not completely explainable on the basis of its inhibition of PG synthetase. This finding may be explained in several ways (see Chapter 7), but the *in vivo* conversion of aspirin into salicylate (eg. 148) seems the most attractive, but fails to explain the mode of action of salicylate. It is well-known that salicylate is as potent as aspirin on CAR edema, but salicylate does not inhibit PG biosynthesis *in vitro* (eg. 82,230,254).

The anti-inflammatory activity of indomethacin and aspirin -in contrast to that of theophylline- was not potentiated by PGE_1. Thus, it was unlikely that both NSAIDs, in the doses used, exerted an *in vivo* action similar to

that of theophylline, namely phosphodiesterase inhibition.

Recently, it has been demonstrated that corticosteroids interfere with the liberation of AA, and thereby with the release of PGs (see review by Gryglewski, 1976, 2.2.2). Since dexamethasone was effective in the EFAD inflammatory model, its anti-inflammatory activity was not completely explainable by inhibition of PG release.

Finally, PGs may be regarded as modulators of the CAR response. All classical PGs fail to induce "true" inflammation on their own, but they potentiate responses to other autacoids (eg. Williams & Morley, 1973). Thus, CAR edema developed when PG biosynthesis was greatly reduced (NSAIDs, EFA deficiency), but the inflammatory process was less severe. The acute, pro-inflammatory effects of PGs are, at this moment, mainly explainable on the basis of their marked ability to increase local blood flow. On the other hand, certain PGs (eg. PGE_1) have anti-inflammatory effects on CAR edema (see Chapter 7) as well as on a chronic experimental inflammatory reaction (Bonta, Parnham et al., 1977). In both types of inflammatory models, the anti-inflammatory activity of PGE was potentiated by the phosphodiesterase inhibitor theophylline. However, the exact anti-inflammatory mechanism(s) and site of action of PGE_1 are unknown at this moment.

The latter observations question the anti-inflammatory mode of action of drugs that interfere with PG biosynthesis. Undoubtedly, NSAIDs reduce swelling and fever and are peripheral analgesics, but on the other hand, they may remove those endogenous PGs which possibly shorten the inflammatory process. Thus, a rational therapy of inflammation might be impossible with PG synthetase inhibitors. In general, anti-inflammatory -not anti-edemagen- drugs are used emprically, without basic knowledge about their mode of action. This is largely due to the fact that "much of what we know about the inflammatory process was known in 1900" (Ebert & Grant, 1974). Even for a simple model, such as CAR edema, it is almost impossible to construct a pathway leading to inflammation (Vinegar et al., 1976) and even less is known about endogenous factors which terminate this process.

CAR edema will probably not contribute to the development of new, empiric, anti-inflammatory agents of clinical importance. However, basic studies on this model, with recently developed research techniques, will probably be essential for understanding of exudation, changes in blood flow, properties for vascular endothelium, the origin, site of action and turnover of PGs and other autacoids, the attraction of inflammatory cells, and the mutual inter-

actions between these events. Therefore, CAR edema and similar "simple" models, are able to provide possible mechanisms for more complex, chronic situations, where, upto now, it has been impossible even to start developing an hypothetical step-by-step pathway leading to inflammation.

9. SUMMARY

This thesis describes studies carried out to investigate the importance of prostaglandin (PG) biosynthesis in the development of carrageenin-induced hind-paw edema (CAR edema) in the rat. In addition, PG and thromboxane A_2 (TXA_2) biosynthesis by rat platelets, and its relevance for aggregation, was studied in vitro. Some of the experiments were performed with essential fatty acid deficient (EFAD) rats, which contained very little arachidonic acid (AA), the main PG percusor.

The formation of PGs and/or TXA_2 was generally measured by bioassay on isolated tissues, using the cascade superfusion technique described by Vane (1964). It was found that a "cumulative" procedure for obtaining dose-response curves was conveniently applicable to superfused tissues. Under specified conditions it was possible to assay TXA_2 on a rabbit aorta strip, despite the concomitant presence of serotonin. Finally, the sensitivity of the bioassay was greatly improved by superfusing the tissues in the cascade with a laminar flow (chapter 3).

The in vitro experiments are summarized in chapter 4. Upon stimulation with collagen or AA, rat platelets in heparinized, platelet rich plasma, synthetized malondialdehyde (MDA), PGE and TXA_2. Following both types of stimuli, this biosynthesis was dose-dependent and liable to inhibition by indomethacin, aspirin and eicosatetraynoic acid. During EFA deficiency the release of PGE and TXA_2 was greatly reduced, but the activity of the cyclo-oxygenase, which catalyses the transformation of AA into PG-endoperoxides, seemed to be unaltered.

The oxygenated endogenous AA products, especially TXA_2, amplified the aggregation induced by threshold doses of collagen. The addition of exogenous AA to rat platelet rich plasma did not completely mimick the reactions induced by collagen, since, in the absence of aggregation, more stable PGs and less TXA_2 were formed.

Aggregation induced by ADP or high doses of collagen was independent of TXA_2 synthesis in rat platelets. Moreover, the platelets of this species are relatively insensitive to the exogenous PG endoperoxide PGH_2, and the stable 9,11 epoxymethano analogue of PGH_2.

The experiments described in chapter 5 were performed in order to detect PGs during CAR edema under carefully controlled conditions. For the collection of exudate from CAR edema, a coaxial perfusion method was used.

In perfusates from inflamed paws prostaglandin-like material (PGL) was detectable at 1, 2, 4 and 6 hours. PGL was neither measurable before aplication of CAR, nor after administration of saline to the paws.

These results were confirmed at 4 hours after administration of CAR, using a more quantitative approach. Both EFA deficiency and pretreatment with indomethacin (2.5 mg/kg) led to decreased biosynthesis of PGL in the inflamed area, which coincided with diminished swelling. Thus, during the development of CAR edema a pro-inflammatory PGL was formed.

The involvement of PGs in the development of CAR edema was further established in the experiments described in chapter 6. It appeared that the reduction of the CAR edema (from 2 hours onwards) in EFAD rats was most probably caused by a shortage of AA. The impaired inflammatory response in these animals was not due to diminished cyclo-oxygenase activity (cf. chapter 4). Moreover, the sensitivity towards edemagens (bradykinin and serotonin), as well as the bradykinin component of CAR edema, were unaltered in EFAD rats. These experiments further indicated that release of AA, and its subsequent conversion into a PG with "vasodilatory properties", is possibly a rate-limiting factor in edema development.

Finally, the anti-inflammatory activities of aspirin and indomethacin, which inhibit the cyclo-oxygenase, and dexamethasone, a corticosteroid that may interfere with the cellular liberation of PGs, were studied on CAR edema in EFAD rats in which endogenous release of PGs is of minor importance (see chapters 5 & 6). Indomethacin (2.5 mg/kg), almost failed to suppress the reduced CAR edema in EFAD rats. However, aspirin (125 mg/kg) and dexamethasone (0.125 mg/kg), in doses that were approximately equipotent to those of indomethacin in normal rats, still displayed anti-inflammatory activities in EFAD rats. Thus, the main anti-inflammatory activity of indomethacin was probably based on its inhibition of PG biosynthesis, but aspirin and dexamethasone possessed addional anti-inflammatory properties.

Circumstantial evidence for the hypothesis that inhibition of phosphodiesterase was responsible for the additional anti-inflammatory activity of aspirin, was not obtained in vivo. However, in these experiments, it was found that PGE_1 (1 mg/kg), when administered systemically, suppressed the later phase (24h) of CAR edema. This might indicate that PGs modulate this model of acute inflammation and that biosynthesis of PGs enhances the acute phase of the edema, but may also contribute to the termination of the inflammatory process.

10. SAMENVATTING

Het verkrijgen van meer inzicht in de rol van prostaglandines (PGs) in ontstekingsreakties was het doel van de experimenten die in dit proefschrift beschreven zijn. Daartoe werd het carrageenine (CAR) oedeem, een akute ontstekingsreaktie in de achterpoot van de rat als model gekozen. Omdat het moeilijk is prostaglandines op betrouwbare wijze in ontstoken weefsel te meten, werd ter ondersteuning van de resultaten tevens de biosynthese van prostaglandines in vitro bestudeerd. Dit gebeurde met geïsoleerde bloedplaatjes van de rat.

In hoofdstuk 2 is literatuur over verschillende aspecten van het onderzoek samengevat. Het bleek daaruit dat de vorming van prostaglandine-endoperoxiden uit arachidonzuur gekatalyseerd wordt door het enzyme cyclo-oxygenase. Uit de prostaglandine-endoperoxiden kunnen andere biologisch aktieve prostaglandines gevormd worden, zoals PGE_2 en prostacycline, of het instabiele thromboxaan A_2 (2.1). Niet steroïde ontstekingsremmende farmaka, zoals aspirine (acetylsalicylzuur) en indomethacine, onderdrukken PG-biosynthese omdat zij de cyclo-oxygenase remmen. Steroïde ontstekingsremmers, zoals dexamethason, kunnen prostaglandine vorming tegengaan door het beschikbaar komen van arachidonzuur te belemmeren (2.2). Bij het onderzoek is gebruik gemaakt van essentieel vetzuur (EFA) deficiënte ratten. Deze werden groot gebracht met een dieet zonder linolzuur, en kunnen daardoor geen arachidonzuur produceren. Een EFA deficiënt organisme zal dus minder substraat bezitten voor endogene vorming van PGs en thromboxaan A_2 (2.3). Vorming van thromboxaan A_2 treedt o.a. op bij de aggregatie van menselijke bloedplaatjes, en is één van de faktoren die van belang is voor de haemostatische funktie van bloedplaatjes. De toegenomen kans op bloedingen na gebruik van aspirine berust op de irreversibele remming van thromboxaan A_2 produktie (2.4). Tenslotte gaf de literatuur aanwijzingen dat prostaglandines een rol spelen bij het carrageenine oedeem.

In het experimentele deel van dit proefschrift werd nagegaan of PGs aantoonbaar waren in de ontstoken poten, en of PGs uitsluitend een ontstekingsbevorderende rol hebben. De proeven werden ten dele uitgevoerd met EFA deficiënte ratten. Met deze proefdieren werd onderzocht of de ontstekingsremmende werking van aspirine, indomethacine en dexamethason berustte op een onderdrukking van het vrijkomen van prostaglandines.

PGs en thromboxaan A_2, die veelal slechts in minieme hoeveelheden gevormd worden, werden bepaald met een biologische ijking. Geïsoleerde orgaanpreparaten

met glad spierweefsel, zoals strips van rattemaag en konijneaorta, werden onder elkaar bevestigd. Een fysiologische zoutoplossing werd via het bovenste weefsel successievelijk over de lagere organen geleid (cascade techniek). Door verschillende organen te kiezen, kunnen meerdere biologisch aktieve stoffen tegelijkertijd in één monster bepaald worden aan de hand van de contrakties van de geïsoleerde weefsels. De contrakties werden geijkt met standaarden van die stoffen, die de organen stimuleren (agonisten), terwijl storende stoffen geblokeerd werden met "antagonisten". Ten opzichte van andere meetmethoden heeft de biologische ijking één voordeel: door het dynamische, snelle karakter is het mogelijk zeer labiele verbindingen, zoals thromboxaan A_2 aan te tonen. In hoofdtuk 3 zijn modifikaties van de methode beschreven. Deze versnelden de bepaling en maakten het mogelijk om prostaglandines en thromboxaan A_2 in aawezigheid van serotonine te meten. De gevoeligheid van de ijking werd tenslotte vergroot met behulp van een "Laminar flow technique", waardoor het mogelijk werd PGE_2 vanaf 20-50 picogram (10^{-12}g) aan te tonen.

De experimenten met bloedplaatjes van de rat zijn samengevat in hoofdstuk 4. Er werd nagegaan of bloedplaatjes van EFA deficiënte ratten minder endogene prostaglandines en thromboxaan A_2 vormden. Tegelijkertijd werd de nog grotendeels onbekende rol van prostaglandine metabolisme voor het funktioneren van rattebloedplaatjes bestudeerd. Dit werd gedaan in plaatjes rijk plasma dat met heparine onstolbaar gemaakt was.

Na toedienen van collageen aggregeerden de bloedplaaajes en daarbij kwam malondialdehyde, thromboxaan A_2 en PGE vrij. Verschillende cyclo-oxygenase remmers onderdrukten de vorming van deze produkten. Thromboxaan A_2 was reeds 20 seconden na toedienen van collageen in maximale hoeveelheden ontstaan en was nog voor de voltooiing van de aggregatie in veel mindere mate aantoonbaar. De bloedplaatjes van EFA deficiënte ratten synthetiseerden slechts 10 à 20 procent van de normale endogene hoeveelheid PGE en thromboxaan A_2.

Bij drempeldoses collageen was prostaglandine-endoperoxide vorming noodzakelijk voor het funktioneren van rattebloedplaatjes. Dit bleek uit het feit dat dan nog maar 10% van de EFA deficiënte bloedplaatjes aggregeerde. Bovendien was indomethacine dan even effektief als cyclo-oxygenase remmer en als remmer van de aggregatie van normale bloedplaatjes. Uit experimenten met exogeen arachidonzuur bleek dat de cyclo-oxygenase aktiviteit van EFA deficiënte bloedplaatjes niet verminderd was. Bovendien werden aanwijzingen gevonden dat vooral de vorming van thromboxaan A_2 van belang is voor de plaatjesfunktie. De aggregatie, èn het vrijmaken van serotonine waren afhankelijk van de

thromboxaan A_2 vorming als de bloedplaatjes met hoge doses collageen gestimuleerd werden. Tot zover leken de thrombocyten van de rat op die van de mens. Er waren ook verschillen, omdat plaatjes van de rat meer PGE en relatief weinig thromboxaan A_2 vormden. Bovendien aggregeerden bloedplaatjes van de rat nauwelijks na toedienen van het prostaglandine endoperoxide PGH_2 en is hun aggregatie onder invloed van ADP niet afhankelijk van prostaglandine endoperoxide biosynthese.

Omdat vermoed werd dat prostaglandines ontstaan bij het carrageenine oedeem in de achterpoot van de rat, werd getracht om prostaglandines aan te tonen in het ontstoken weefsel. Ontstekingsexsudaat werd daartoe verzameld met een coaxiale perfusietechniek, zoals beschreven is in hoofdstuk 5. Inderdaad was 4 en 6 uur, en waarschijnlijk al eerder, na het begin van de ontsteking een prostaglandine-achtige materie aantoonbaar in het exsudaat. In tegenstelling tot de enige eerdere publikatie waarin dit onderzocht was, werd aangetoond dat de prostaglandine vorming geen artefakt was.

Met een belangrijk verbeterde proefopzet werden de waarnemingen, 4 uur na toedienen van carrageenine, bevestigd. Door gebruik te maken van radio-aktief gemerkt albumine was het mogelijk de efficiëntie van de perfusietechniek te kwantificeren. Indien artefakten tot een minimum beperkt werden, dan was in de ontstekingsvloeistof 5 à 7 nanogram PGE_2-equivalenten aantoonbaar. Omdat ongeveer 1 ml exsudaat gevormd was, was de lokale concentratie "PGE_2" minstens 100 maal groter dan die in bloedplasma. Als de ratten voorbehandeld werden met indomethacine (2.5 mg/kg) dan werd zowel PG-vorming als pootzwelling en eiwitlekkage onderdrukt. In EFA deficiënte ratten waren PG-vorming en pootzwelling afgenomen, maar de lekkage van plasma eiwitten was niet verminderd. Deze proeven wijzen erop dat tijdens de eerste uren van de oedeemvorming biosynthese van ontstekingsbevorderende prostaglandines plaatsvindt.

In hoofdstuk 6 is de bijdrage van PG-biosynthese tot het ontstekingsproces nader onderzocht. In EFA deficiënte ratten was de pootzwelling vanaf 2 uur na toedienen van carrageenine verminderd ten opzichte van normale ratten. Dit werd veroorzaakt door een verminderde beschikbaarheid van arachidonzuur, en niet door een afgenomen cyclo-oxygenase aktiviteit (zie ook in vitro proeven). Andere komponenten van het ontstekingsproces zoals de gevoeligheid voor plasmalekkage veroorzakende stoffen als bradykinine en histamine, waren onveranderd in EFA deficiënte ratten. Dit alles duidt aan dat biosynthese van prostaglandines bijdraagt tot het ontstekingsproces, en dat EFA deficiënte ratten, die minder endogene prostaglandines kunnen vormen, dientengevolge een vermin-

derde pootzwelling vertonen.

De beschikbaarheid van endogeen arachidonzuur is mogelijk een snelheidsbepalende faktor in de oedeemvorming. Een indirekte aanwijzing duidde erop dat niet de prostaglandine-endoperoxiden of stabiele PGs, maar veeleer thromboxaan A_2 of prostacycline de pootzwelling bevorderen. Anderzijds bleek uit de experimenten in hoofdstuk 6 dat een prostaglandine (PGE_1 in een latere fase (24h) de pootzwelling kan reduceren.

In hoofdstuk 7 werd onderzocht of onderdrukking van de biosynthese van prostaglandines (zie 2.2) het enige ontstekingsremmende effekt is van indomethacine, aspirine en dexamethason. Dit werd onderzocht in EFA deficiënte ratten in een fase van het ontstekingsproces (na 4 uur) waarin prostaglandines niet aantoonbaar waren in het ontstekingsexsudaat. Indomethacine (2.5 mg/kg) was niet in staat het verminderde carrageenine oedeem in EFA deficiënte ratten verder te remmen. De oedeemremmende werking van indomethacine berust dus grotendeels op onderdrukking van PG biosynthese. Omdat zowel aspirine als dexamethason de pootzwelling in EFA deficiënte ratten wel onderdrukten, moeten beide farmaka, naast een eventuele belemmering van het vrijkomen van prostaglandines, nog een andere ontstekingsremmende eigenschap bezitten. Het lijkt goed mogelijk dat splitsing van aspirine in salicylaat de alternatieve ontstekingsremmende werking van aspirine verklaart. Het werkingsmechanisme van de ontstekingsremmer salicylaat is nog onbekend, maar de stof remt de biosynthese van prostaglandines niet in vitro (zie 2.2). Tenslotte bleek opnieuw dat PGE_1, in farmakologische doses, de latere fase (24 uur) van de pootzwelling onderdrukte. Dit berustte waarschijnlijk op stimulatie van cyclisch AMP vorming, maar nader onderzoek is nodig om dit te bevestigen.

Dit alles wijst er op dat PGs het carrageenine oedeem moduleren. Zelfs als er veel minder prostaglandines gevormd worden, bijvoorbeeld na toediening van indomethacine of in EFA deficiënte ratten, dan gaat de ontstekingsreaktie door. Echter, biosynthese van prostaglandines versnelt en/of versterkt de ontwikkeling van het carrageenine oedeem. Of endogene prostaglandines, net als PGE_1, het proces eveneens bekorten is nog niet bekend, maar lijkt niet uitgesloten.

Nader onderzoek naar de ontstekingsremmende werking van PGs lijkt gewenst. Het zou kunnen leiden tot een herwaardering van de therapeutische waarde van ontstekingsremmende farmaka die voornamelijk werkzaam zijn via remming van PG-biosyntese. Hoewel farmaka, als indomethacine symptomen van een ontsteking onderdrukken, leidt hun toediening mogelijk tevens tot een onderdrukking van één der faktoren die een ontstekingsproces afremmen.

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Abbrevations.

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BBA : Biochim. Biophys. Acta

BBRC : Biochem. Biophys. Res. Commun.

PNAS : Proc. Natl. Acad. Sci. USA

PSEBM : Proc. Soc. Exp. Biol. Med.

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CURRICULUM VITAE

Na het behalen van het einddiploma Gymnasium β aan het Grotius College te Heerlen in 1967, ben ik begonnen met de studie Biologie aan de Rijksuniversiteit Utrecht. Het kandidaatsexamen B4 werd afgelegd op 8 juni 1970 en het doktoraalexamen, met als hoofdrichtingen biofysische chemie en biologische toxicologie en als bijvak biochemie werd behaald op 24 september 1973.

Vanaf 1 september 1973 tot 1 september 1977 was ik als wetenschappelijk medewerker in dienst van FUNGO en werkzaam bij de afdeling Farmacologie van de Erasmus Universiteit Rotterdam. Aldaar werd onder leiding van Prof. Dr I.L. Bonta het hier beschreven onderzoek verricht.