THE EFFECTS OF NOVEL PROSTAGLANDIN ANALOGUES ON PLATELET FUNCTION

by

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The work described in this Thesis was performed by myself (unless otherwise stated) in the Department of Pharmacology, University of Edinburgh, between 1978 and 1981.

Certain sections of the work described in this Thesis have been published.

JONES, R.L., SUTHERLAND, ROMA A. and WILSON, N.H. (1981). Binding of tritium-labelled 9,11-epoxymethano PGH_2 to human platelets. Br. J. Pharmac. 73, 304-305P.

ARMSTRONG, ROMA A., JONES, R.L., PEESAPATI, V. and WILSON, N.H. (1981). Effect of the thromboxane antagonist EP045 on arachidonic acid responses in the rabbit, guinea pig and sheep. Br. J. Pharmac. 74, 790-791P.

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CONVENTIONS AND NOMENCLATURE

Whenever possible, trivial names of prostaglandins have been used in the text. The systematic names of all C_{20} prostaglandins referred to in this Thesis can be deduced from the examples given below; the nomenclature for other prostaglandins is explained in the general introduction (Chapter I).

Trivial name	Systematic name
PGE_1 (prostaglandin E_1)	11α,15(S)-Dihydroxy-9-Ketoprost-13 trans-enoic acid.
11-deoxy-PGE_1	15(S)-Hydroxy-9-Ketoprost-13 trans-enoic acid.
PGE_2	11α,15(S)-Dihydroxy-9-Ketoprosta 5 cis, 13 trans-dienoic acid.
PGE_3	11α,15(S)-Dihydroxy-9-Ketoprosta 5 cis, 13 trans, 17 cis-trienoic acid.
$PGF_{2\alpha}$	9α,11α,15(S)-Trihydroxyprosta 5 cis, 13 trans-dienoic acid.
PGD_2	9α,15(S)-dihydroxy-11-Ketoprosta 5 cis, 13 trans-enoic acid.
PGG_2	15(S)-Hydroperoxy-9α,11α-peroxido-prosta 5 cis, 13 trans enoic acid.
PGH_2	15(S)-Hydroxy-9α,11α-peroxidoprosta 5 cis, 13 trans-dienoic acid.
PGI_2	11α,15(S)-Dihydroxy-9-deoxy-6,9 - epoxy-5 Z, 13-trans prostadienoic acid.

In drawings of chemical structures, stereochemistry is not implied unless specifically indicated; a thickened or dotted line denotes a substituent located respectively above or below the plane of the paper. Throughout this Thesis, two different diagrammatic representations have been used for the structure of PGH_2/PGH_2 analogues and TXA_2. The more common representation of PGH_2 (Fig. A) is equivalent to

that shown in Fig. B. The benefit of the second representation (Fig. B) is that thromboxane mimics such as 11,9-epoxymethano PGH_2 appear to have a structure more similar to that of TXA_2 itself.

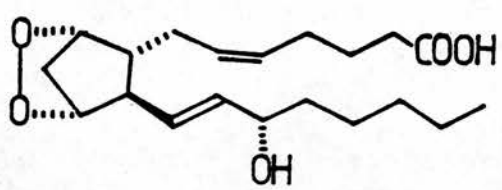


FIGURE A

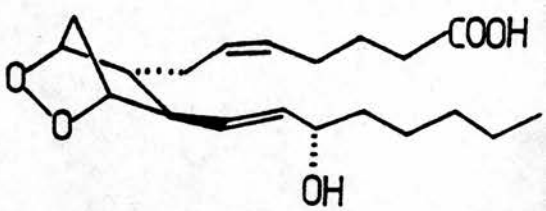
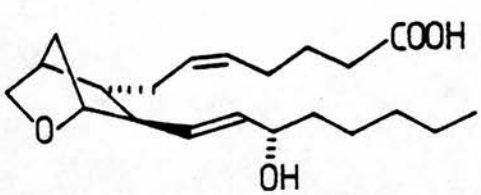
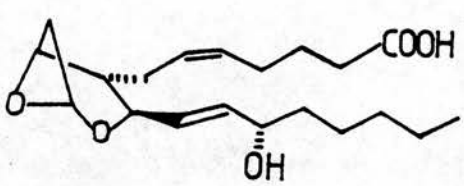


FIGURE B



11,9-epoxymethano PGH_2



TXA_2

ABSTRACT OF THESIS

Thromboxane A_2 (TXA_2) is a potent platelet aggregating agent and constrictor of coronary, vascular, and bronchial smooth muscle. This Thesis is concerned with the actions on human platelets of 5 endoperoxide analogues, EP035, EP037, EP043, EP045 and EP092. These analogues show competitive receptor blockade of TXA_2 action on smooth muscle: it was of particular interest to determine whether additional modes of action contributed to their anti-aggregatory effects in platelets.

The purification of a binding protein from sheep muscle and the development of a protein binding assay for the measurement of platelet cAMP levels are described. EP035 increases platelet cAMP levels markedly; this is thought to be due to a partial agonist effect on the PGI_2 receptor of human platelets. EP092 is the only other analogue to raise basal cAMP levels. Although this effect is very weak, it may be sufficient to augment the action of EP092 when high concentrations are used.

The development of a GC-MS assay for the measurement of platelet TXB_2 levels is described. EP043 was found to inhibit the biosynthesis of TXB_2 from arachidonic acid (AA) added exogenously to platelets. EP092 shows a slight inhibitory effect but at ten-fold higher concentrations. The inhibition of AA metabolism by EP035 is suggested to result from its action to increase platelet cAMP levels.

The development of a binding assay for ^3H 9,11-epoxymethano PGH_2 to whole platelets is described. Total binding appears to be the sum of binding to a non-specific saturable site, uptake of the lipophilic ligand and binding to a stereospecific, saturable site. The specific saturable component of binding can be displaced by TXA_2

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mimics and TXA_2 antagonists but not other PGs thought to act on different receptors. Displacement results correlate well with the pharmacological activity of these compounds.

The in-vivo activity of these analogues was studied. EP035, EP045 and EP092 (1-5 mg/kg) all protected against AA-induced death in rabbits. EP037 showed weak agonist activity and offered no protection. Of the five analogues tested, only EP045 is a specific TXA_2 receptor antagonist in the concentrations tested.

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CHAPTER I

General Introduction

Platelet aggregation is perhaps the most important of the processes which contribute to protection of the vasculature in the event of injury. When platelets adhere to the damaged endothelium, they are stimulated to aggregate and secrete the contents of intracellular granules in a process called the 'release reaction'. The secreted materials include coagulation factors, vasoconstrictors and aggregating agents. These substances promote recruitment of additional platelets to the primary haemostatic plug. As well as this physiological role, platelets may play a pathological role in the formation of intravascular aggregates responsible for various forms of arterial thrombosis.

Collagen lies beneath the endothelial cells that form the innermost layer of the vessel wall, and contact between platelets and collagen exposed in areas of damaged vessel may stimulate platelets to adhere and aggregate. Certainly platelets are deposited and platelet thrombi form where vessels are experimentally injured (1) or where the endothelial layer has been experimentally removed (2). Recent results have suggested that the arachidonic acid (AA) metabolite prostaglandin I_2 (PGI_2/prostacyclin [3,4,5,6]) produced by endothelial cells is responsible for keeping platelets refractory to aggregatory stimuli. The enzyme which converts prostaglandin endoperoxides into PGI_2-(prostacyclin synthetase) is most highly concentrated in the intimal surface and progressively decreases in activity towards the adventitial surface (7). It can be inhibited by 15-hydroperoxyarachidonic acid (15-HPAA) or 13-hydroperoxylinoleic acid (13-HPLA), both of which can abolish virtually all the anti-aggregatory activity of endothelial cells (8). (The reason that platelets do not aggregate to undamaged endothelium was previously unexplained, though thought to be connected to the presence of apyrase in the vessel wall. This enzyme metabolises adenosine-di-phosphate (ADP), a potent aggregating agent [9,10].)

Damage to the endothelium will expose platelets to a surface with a considerably reduced capacity for PGI_2 production, allowing platelet deposition and aggregation to occur. This thrombus formation can be prevented if PGI_2 is infused at the site of injury (11,12,13,14). However, PGI_2 is reported not to circulate in concentrations which affect platelet function (15,16,17) suggesting that a fall in PGI_2 production at the site of injury is not alone sufficient to instigate platelet aggregation, though it may be a contributory factor.

Perhaps no single agent is responsible for the initiation of platelet aggregation *in vivo*. Indeed ADP released from red blood cells under conditions of altered flow, such as may occur at the site of injury or when an atheromatous plaque partly obstructs a vessel, can act as another important stimulus (18). Also, the presence of fibrin in the thrombus indicates that thrombin is formed at the site of injury. Thrombin is also a potent aggregating agent.

When stimulated to aggregate, platelets undergo distinct morphological and biochemical changes. Calcium ions are intimately involved in the regulation of these changes. The evidence for this is partly circumstantial, based on comparisons with other cells, and partly experimental, where the effects of calcium ionophores, chelating agents and antagonists have been studied. Aggregation of human platelets *in vitro* can occur in two phases: primary and secondary aggregation (19). Primary aggregation is due to the direct interaction of the aggregating agent with a receptor, thought to be on the platelet surface (20,21). Secondary aggregation is associated with both the secretion of dense granule constituents and the activation of a pathway for conversion of AA to prostaglandins (PGs). The precise way in which platelets respond depends on the nature of the agent, on its concentration and on the experimental conditions.

It is probable that the first step in the activation of platelets by most agents involves the interaction with a specific receptor on the platelet surface. Surface receptors for ADP (22), thrombin (23,24,25), adrenaline (26) and 5-hydroxytryptamine (5HT) (27) have been identified. Although adrenaline and 5-HT are actively taken up into platelets, this does not appear to be associated with aggregation (28,29). The first manifestation of platelet aggregation is a shape change and an increase in the adhesive properties of the platelet membrane. So far it has not been possible to associate any of the surface receptors with an activity that might indicate how platelets do this, but it is probable that a contractile process is involved (30). Although platelet shape change does not require the presence of external Ca^{2+} ions, since it occurs in the presence of high external concentrations of a chelating agent EDTA (31), intracellular calcium may be involved. Indeed, shape change induced by ADP causes an increase in the internal free Ca^{2+} concentration, measured spectrophotometrically using the Ca^{2+}-indicator Murexide, although no $^{45}Ca^{2+}$ is taken up from outside (32). By employing low concentrations of chlortetracycline as a fluorescent probe for membrane-bound calcium, a redistribution of Ca^{2+} away from membrane sites during shape change was demonstrated (33). It is proposed that the local increase in calcium ions restricted to the submembranous region initiates the breakdown of the circumferential ring of microtubules (34) and cause local activation of the contractile system giving rise to the pseudopods and spikes observed in the course of shape change. For this reason it is assumed that the membrane release of Ca^{2+} ions is causal rather than a result of shape change.

Platelet shape change can be followed by primary aggregation if the platelets are not stimulated sufficiently to release their granule

constituents. This may require a lower cytosol calcium concentration than the induction of the release reaction and may involve a different pool of bound calcium ions (35,36,37). Primary aggregation is followed by disaggregation, and eventually the platelets regain their discoid form (38). Platelets also become refractory to a particular stimulant (39) suggesting that they have a compensatory mechanism to neutralise the effects of stimulatory agents. This is consistent with the involvement of a contractile process.

When further stimulated, platelets release stored substances which act to facilitate aggregation, promote clot formation and to constrict blood vessels. Platelet dense granules containing ADP, ATP, 5-HT, anti-plasmin, calcium and inorganic phosphate are released during this process. Platelet lysosomes containing acid hydrolases and platelet α granules containing cell growth factor, fibrinogen, β thromboglobulin and platelet factor 4 (anti-heparin factor) are also released, but to a limited degree (21,40,41). Although these are all secretory mechanisms, these processes can be distinguished by differences in time course and metabolic requirement for ATP as well as differential release by certain aggregating agents (21).

The release reaction is calcium-dependent (42) but, since the dependency on external calcium is variable, it is thought that internal stores must be available for mobilisation. A vesicular fraction of platelet homogenates which actively accumulates Ca^{2+} has been described (43,44) and is the most likely source of calcium released at this stage. It is thought to be analogous to the sarcoplasmic reticulum of muscle and is called the dense tubular system (45,46). However, other sources such as mitochondria (47) and α granules (48) have been postulated.

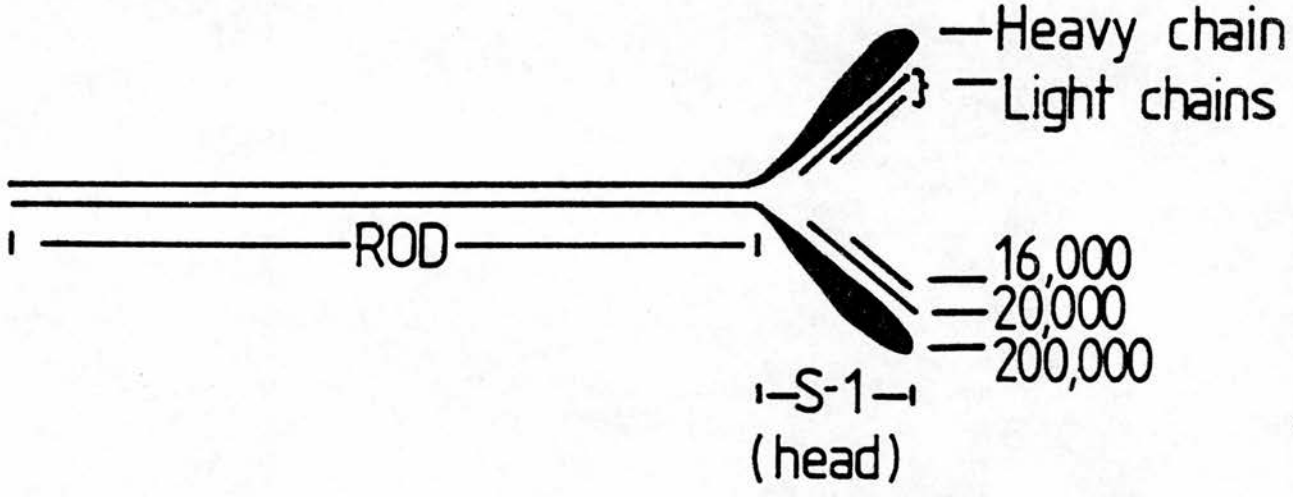


FIGURE 1.1: A schematic representation of platelet myosin.

The mobilisation of intracellular calcium between cell compartments is thought to be important because calcium ionophores, which facilitate the movement of calcium across membranes, can induce the release reaction (42,49,50,51,52,53) and secretion can be blocked by calcium antagonists (36). The movement of calcium into the cytoplasm may exert an effect on the microtubules, which normally lie between the circumferential edge of platelets, but which during the release reaction move towards the centre of the cell in a contractile wave (54) involving Ca^{2+}-activated platelet actomyosin (55,56,57,58).

Platelet myosin (Fig. 1.1) has a molecular weight of 460,000 and is composed of six polypeptide chains (59). Each of the two heads contains a binding site for actin as well as enzymatic sites for the hydrolysis of ATP. Platelet myosin light chain kinase is an enzyme of 83,000 molecular weight and catalyses the transfer of the γ-phosphate of ATP to the 20,000 dalton light chain of the myosin molecule (60). This phosphorylation of platelet myosin increases its actin-activated myosin ATPase activity. Dephosphorylation by platelet myosin phosphatase (61) decreases this activity (62).

A selective increase in the ^{32}P-labelling of polypeptides of molecular weight 48,000-40,000 and 25,000-19,000 reaches a maximum before completion of aggregation and is followed by slow dephosphorylation (55,56,57,58). No increase in phosphorylation is observed when aggregation without secretion is induced. The smaller protein is thought to be the myosin light chain and it seems that its phosphorylation is required before secretion is initiated (58).

During the process of release, the membrane of the dense bodies fuse with the plasma membrane or with the membrane of the surface-connected cannalicular system (63) thereby forming an opening through

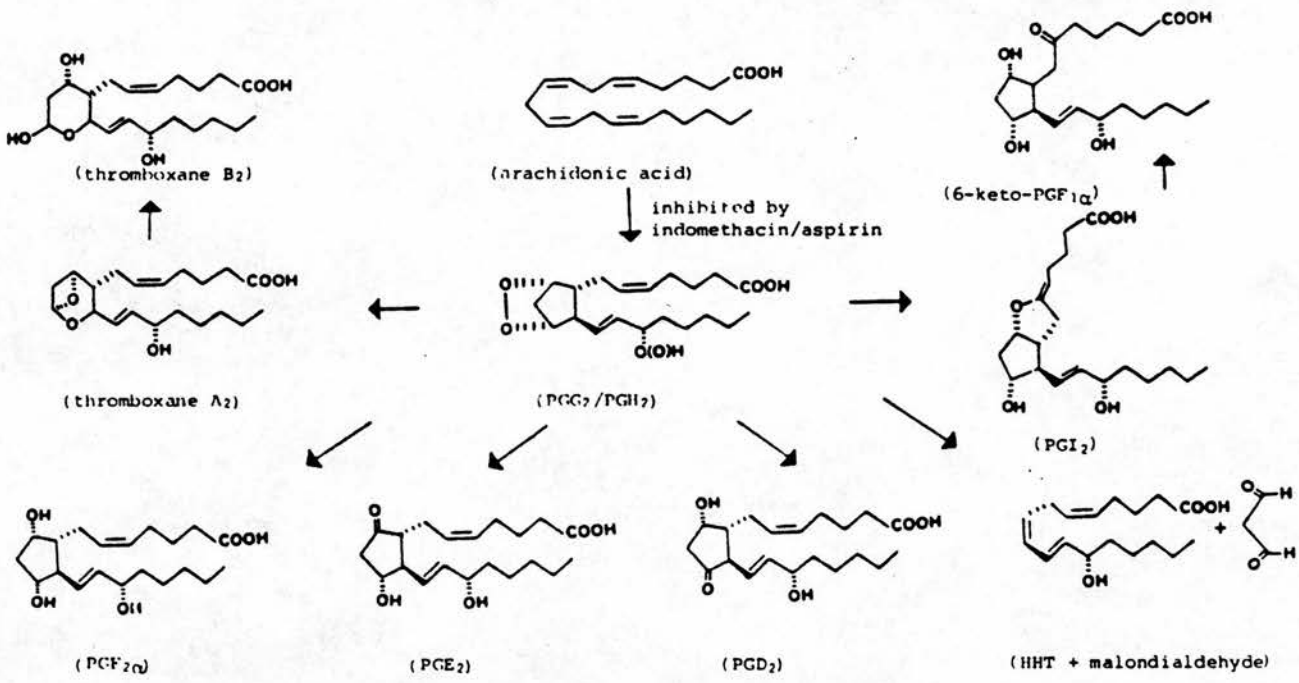


FIGURE 1.2: The major metabolites of the prostaglandin endoperoxides PGG_2/H_2.

which the contents are expelled. Calcium ions play a crucial role in the fusion of membranes (64) which may partly explain why a rise in the cytoplasmic Ca^{2+} concentration will initiate the release reaction.

The secondary wave of aggregation is associated not only with the release reaction, but with the stimulus-induced release of AA from endogenous phospholipids and its rapid transformation into prostaglandins and thromboxane A_2 (TXA_2). Prostaglandins are a family of cyclopentane fatty acids which are biosynthesised from polyunsaturated fatty acids. These acids include dihomo-γ-linolenic acid ($C_{20}: 3\omega6$), arachidonic acid ($C_{20}: 4\omega6$) and eicosapentaenoic acid ($C_{20}: 5\omega3$) which give rise to the mono, bis and tri-enoic prostaglandins respectively. AA is the most common PG precursor found in membrane phospholipids and is metabolised by two types of enzyme:

1. lipoxygenase which catalyses the formation of hydroxy acids and leukotrienes (65,66,67);
2. prostaglandin endoperoxide synthetase (cyclo-oxygenase) which catalyses the formation of PGG_2. This enzyme is then irreversibly deactivated, either by radicals formed during the reduction of PGG_2 to PGH_2 (68,69,70) or by a hydroperoxy product of the lipoxygenase pathway (71).

The major metabolites of PGH_2 are prostaglandins D_2, E_2, $F_{2\alpha}$ and I_2 as well as a non-prostanoid derivative TXA_2 (Fig. 1.2). TXA_2 has a strained acetal structure and is very acid labile: under physiological conditions it has a half-life of about 30 seconds. It is a potent inducer of platelet aggregation, platelet release reaction and smooth muscle contraction (72,73) and is the predominant metabolite of AA in platelets (65,72,74).

The discovery that non-steroidal anti-inflammatory drugs (NSAIDS), such as aspirin or indomethacin, inhibit cyclo-oxygenase (75,76,77) has led to the use of these drugs to elucidate the role of PG endoperoxides and TXA_2 in platelet aggregation. Indomethacin was found to inhibit the release reaction induced by collagen, ADP, thrombin and adrenaline, but not PGH_2 (78,79,80). This suggested that induction of the release reaction relied on the release of AA from membrane phospholipids and its conversion to TXA_2. It was further suggested that TXA_2 did not itself aggregate platelets but, by inducing the release reaction, the ADP released would mediate the second wave of aggregation (81,82, 83,84).

The major evidence for an involvement of secreted ADP is the enzyme-catalysed removal of ADP or antagonism of ADP by frusemide and both these experimental procedures have been criticised (85,86). Furthermore it has been demonstrated that low concentrations of the thromboxane mimic 11,9-epoxymethano PGH_2, the natural endoperoxides and TXA_2 can induce aggregation without secretion (87) suggesting a direct effect. Indeed, this primary aggregation is not affected by the ADP antagonist 2-n-amylthiro-5AMP, although the second wave is inhibited (88). AA can also induce aggregation in washed platelets that have been previously degranulated by repeated thrombin treatment (83,89) and in platelets with storage pool deficiency (90). It seems probable that PGH_2/TXA_2 can aggregate platelets by direct activation, and only in high concentrations can part of the effect be attributed to ADP release.

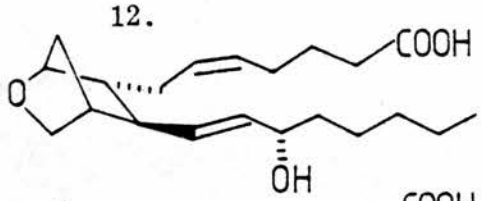
The original suggestion that TXA_2 mediated the second wave of aggregation, regardless of the particular stimulant used, has proved to be an oversimplification. Two different ways of inducing secretion

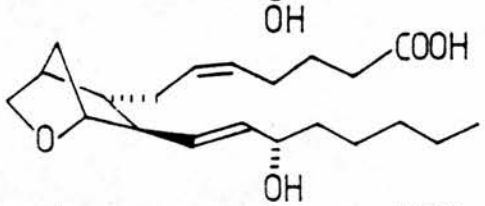
have been demonstrated (21,91,92,93,94). One is dependent on aggregation and is sensitive to indomethacin. The other is independent of aggregation, insensitive to indomethacin and shows simultaneous aggregation and secretion. ADP and adrenaline are thought to be capable only of inducing aggregation-dependent secretion whereas thrombin and the calcium ionophore A23187 are capable of inducing dense granule secretion, independently of aggregation. Since thrombin and A23187 can aggregate platelets which have been degranulated and aspirinised, a third pathway distinct from ADP release and TXA_2 formation has been postulated. Recently it has been suggested that 1-0-alkyl-2-acetyl-glyceryl-3-phosphorylcholine (platelet activating factor, PAF) may mediate this third pathway for platelet aggregation (95,96,97). Platelet aggregation induced by PAF does not require ADP secretion or thromboxane biosynthesis (98). Indeed, radioactive acetate is incorporated into PAF when platelets are stimulated by A23187 (99) and platelets desensitised to PAF lose their ability to respond to thrombin (100). Although TXA_2 is not essential for platelet aggregation its importance particularly in pathological conditions should not be underestimated.

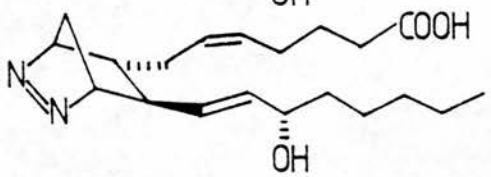
Since prostaglandins are capable of producing both aggregation and secretion, their role must somehow be related to that of calcium. The enzyme phospholipase A_2 (PLA_2) occupies a strategic position in the pathway for generation of PG endoperoxides and thromboxanes since there is little free AA present intracellularly and neither PG endoperoxides nor their by-products are stored in cells (101). Ca^{2+} ions are an absolute requirement for PLA_2, the enzyme being completely inactive in the absence of calcium (102,103). This effect of calcium is mediated through calmodulin, a ubiquitous Ca^{2+}-binding protein (104).

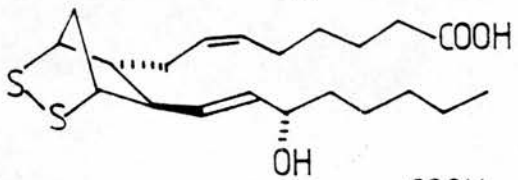
The release of intracellular Ca^{2+} by platelet-stimulating agents will result in activation of PLA_2 with the resultant formation of PG endoperoxides and TXA_2 (105). This suggests that there is a prostaglandin component associated with aggregation, even if the aggregating agent itself does not specifically stimulate PLA_2.

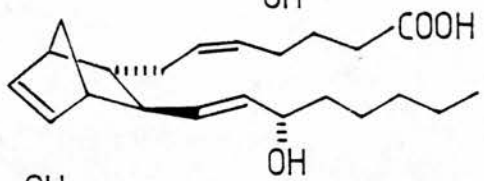
The mechanism by which PG endoperoxides and TXA_2 trigger platelet activation is largely unknown. The most marked action of these intermediates is to stimulate internal platelet contraction (106) and the aggregation induced is reported to mimic that of the established Ca^{2+} ionophore A23187 (107,108). The inner membrane of the dense tubular system is rich in phosphatidylcholine and inositol (109,110) as well as being the site of the PG endoperoxide synthetase system (111, 112,113). It has therefore been proposed that TXA_2 acts as a calcium ionophore to transport calcium directly from the dense tubular system of platelets to the cytoplasm, to activate the contractile proteins (108,114). Ionophores are usually molecules with backbones of diverse structures that contain strategically placed oxygen atoms (115). The backbone is capable of assuming conformations which focus these oxygen atoms about a ring into which an ion of suitable size may fit. It has been suggested that oxygens of two or possibly more prostaglandins could form a hydrophilic cavity around a dehydrated calcium ion, with the hydrocarbon backbones affording some degree of protection for the charge (116,117). TXA_2 has been shown to have properties which would allow it to take calcium from a site within the lipid bilayer to the cytoplasm: it can transport calcium from a water phase into diethyl ether at physiological pH (114) and shows a higher affinity for calcium ions in this respect than for Mg^{++}, K^+ or Na^+ ions (118). Alternatively, PGs may alter cell permeability by displacing Ca^{++} ions from superficial membrane-binding

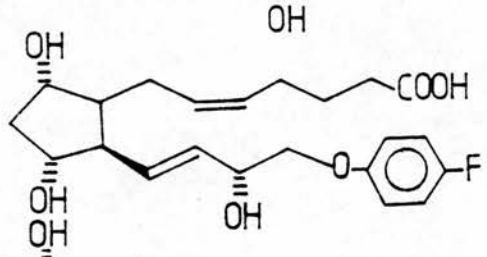
9,11-epoxymethano PGH_2  120

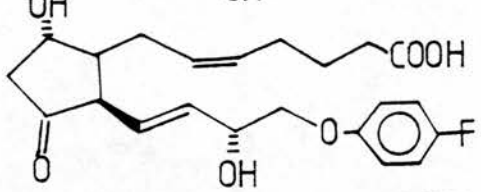
11,9-epoxymethano PGH_2  120,121

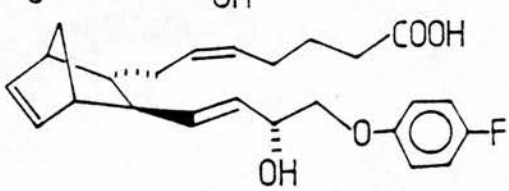
9,11-azo PGH_2  122

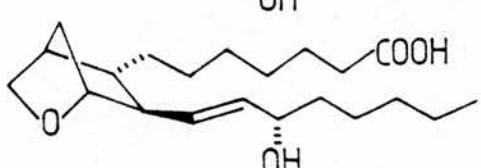
endo disulfide analogue of PGH_2  123,124

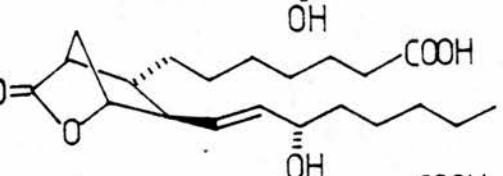
9,11-ethano PGH_2  125

rac 17,18,19,20-tetranor-16-p-fluorophenoxy $PGF_{2\alpha}$ (ICI 79,939)  126

rac 17,18,19,20-tetranor-16-p-fluorophenoxy PGD_2  127

17,18,19,20-tetranor-16-p-fluorophenoxy-9,11-etheno analogue of PGH_2 (EP011)  127

11,9-epoxymethano PGH_1  128

11,9-epoxycarbonyl PGH_1  128

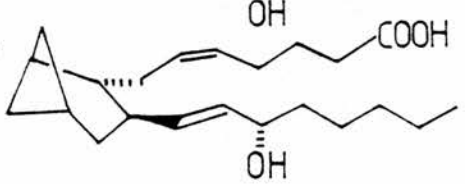
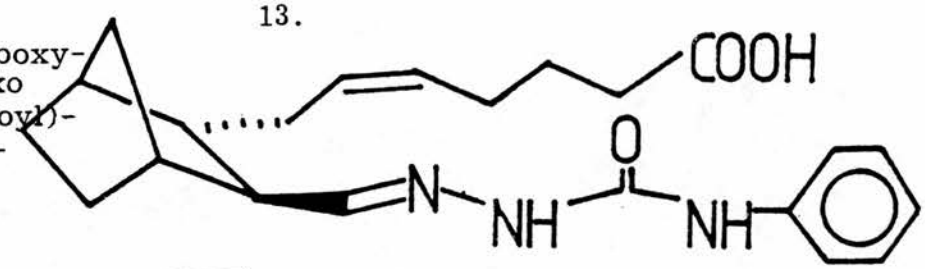
rac-(9,11),(11,12)-dideoxa-(9,11),(11,12)-dimethylene TXA_2 (CTA_2)  129

FIGURE 1.3a: TXA_2 mimics

rac-5-endo(6[1]-carboxy-hex-2[1]2-enyl)-6-exo [N-(phenylcarbamoyl)-hydrazanomethyl]-bicyclo[2,2,1] heptane (EP045)



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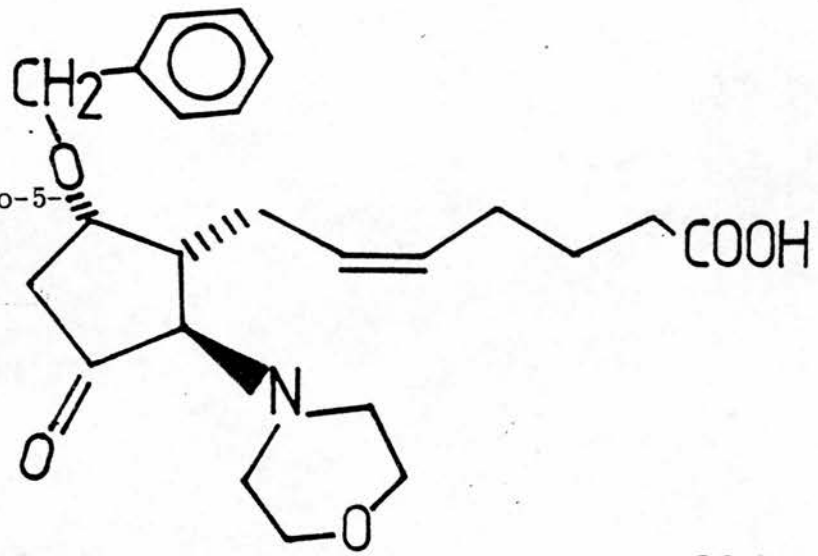
[1α(2),2β,5α] methyl 7-[2-(4-morpholinyl)-3-oxo-5-(phenyl-methoxy) cyclopentyl]-5-heptenoate (AH 19437)



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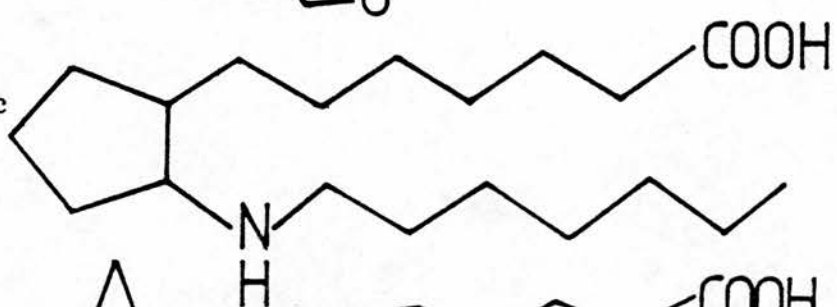
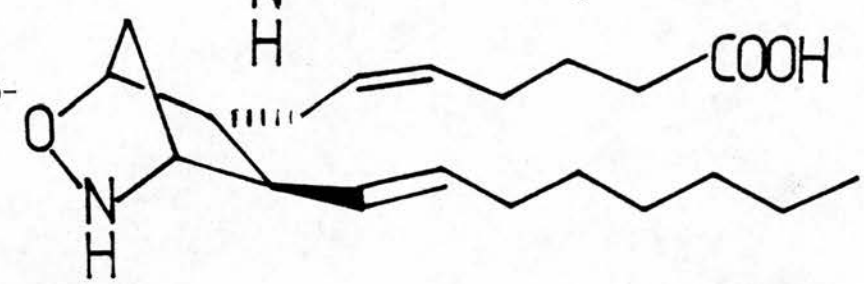
13-azo prostanoic acid



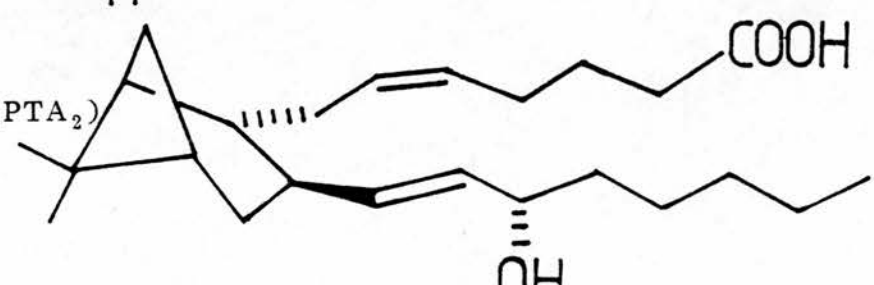
132

9,11-epoxyimino-prosta-5,13-dienoic acid



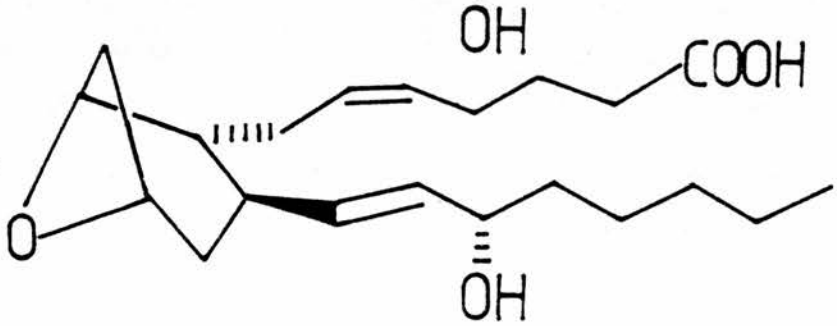
133

pinane TXA_2 (PTA_2)



134

11α-carba TXA_2



135

FIGURE 1.3b: TXA_2 antagonists.

sites (119) or by initiating a conformational change in a membrane protein, thus allowing entry of calcium into the cytoplasm.

The suggestion that TXA_2 acts on discrete receptor sites has been supported by the synthesis of structurally similar analogues (Fig. 1.3) which have afforded a range of receptor agonists, partial agonists and antagonists (120-135). The discovery of receptor antagonists has been put forward as a major objection to the proposed action of TXA_2 as an ionophore (136) although it is possible that these antagonists may interfere at the site where TXA_2 first picks up the calcium, at the site of transport, or at the site of release (137). In fact these two proposals, that TXA_2 acts on discrete receptor sites and that TXA_2 acts as a calcium ionophore, need not be mutually exclusive.

At present, very little is known about the TXA_2 receptor of platelets. Since platelets aggregate to exogenously added PG endoperoxides, TXA_2 or thromboxane mimics, it suggests that receptors for these agonists are present on the surface of the platelet membrane. However, when AA aggregates platelets, prostaglandin endoperoxides and TXA_2 are formed intracellularly suggesting that intracellular receptors may also be present.

This situation is further complicated by the uncertainty as to the true biological activities of the PG endoperoxides. It appears that TXA_2 is largely responsible for AA-induced aggregation of human platelets:

1. maximal aggregation is observed 40-60 seconds after AA is incubated with platelets, at a time when the PG endoperoxide concentration has decreased to low levels after an initial surge, highest at 20 seconds;

2. this aggregation factor disappears with a half life of 30-40 seconds, similar to that reported for TXA_2;

3. the concentrations of endoperoxides detected are 100 times lower than would be required for the aggregation observed;

4. thromboxane synthetase inhibitors can abolish virtually all the aggregatory activity of AA (138,139).

However, exogenous PGG_2 added to platelets *in vitro* induces very rapid aggregation and release within 2 seconds (140). Also, exogenous PGH_2 is degraded in PRP predominantly to PGD_2 and PGE_2 with less than 1% conversion to TXA_2 (141). The direct action of the endoperoxides suggested by these experiments is further supported by the finding that thromboxane synthetase inhibitors cannot abolish the aggregation induced by exogenously added PGG_2/H_2 (142,143).

The available evidence suggests that although AA induced aggregation is primarily dependent on TXA_2 formation, the direct action of the endoperoxides might not be evident, in experiments where AA induced aggregation is blocked by thromboxane synthetase inhibitors, for two reasons:

1. the endoperoxides are considerably less potent than TXA_2 in inducing aggregation, and the concentration of free endoperoxides might not be sufficient for aggregation;

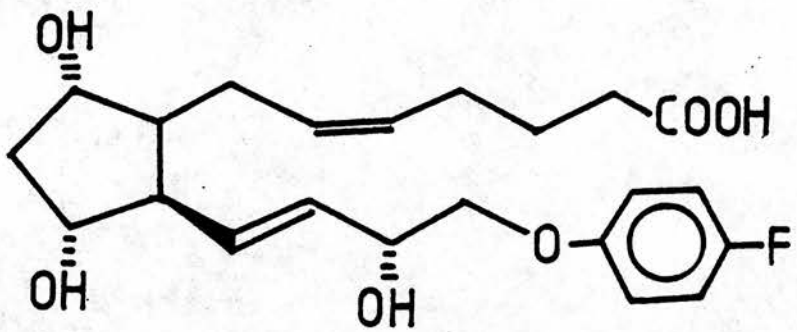
2. the direct action of the endoperoxides may be masked by their rapid transformation into PGD_2, a potent inhibitor of platelet aggregation (144).

Indeed, the finding that PGG_2 is approximately three times more potent than PGH_2 as an aggregating agent (145) may be related to the fact

that PGG_2 will be transformed to a lesser extent than PGH_2 into PGD_2. It is possible that the primary wave of aggregation, observed when PGH_2 is added exogenously to platelets pretreated with a thromboxane synthetase inhibitor, is reversed by PGD_2 formed from the PGH_2 added. If the effects of PGD_2 could be pharmacologically removed the direct actions of PGG_2 and PGH_2 on platelet function may become clearer.

It is generally assumed that if the endoperoxides have a direct action, they induce aggregation by acting on the TXA_2 receptor. Certainly many of the potent thromboxane mimics are endoperoxide-like in structure (120,122,123,125,128) rather than thromboxane-like. In addition, endoperoxide-like antagonists (131,134) are capable of blocking the aggregatory effects of both PGH_2 and AA which suggests that endoperoxide-like antagonists act on the thromboxane receptor. However, as yet the possibility that separate endoperoxide receptors exist cannot be dismissed.

Although pharmacologists have classically identified receptors in terms of the relative affinities of agonists, antagonists are more useful for this purpose. Comparison of receptors in different tissues which are activated by the same agonist, can be made by comparing affinity constant (K_B) values for a common antagonist. By using a dose-ratio method no assumption about the relationship between receptor occupancy and response need be made. At the start of this thesis no antagonists were available, or referred to in the literature, to aid such a classification. Also, no receptor-specific radiolabelled ligands were available to enable binding to receptor sites to be studied directly. This thesis is therefore concerned with the actions of certain novel endoperoxide analogues on the platelet system. It was hoped that if these compounds proved to be specific receptor antagonists, they might aid classification of the thromboxane receptor of platelets.



ICI 79,939

FIGURE 1.4: The structure of ICI 79,939.

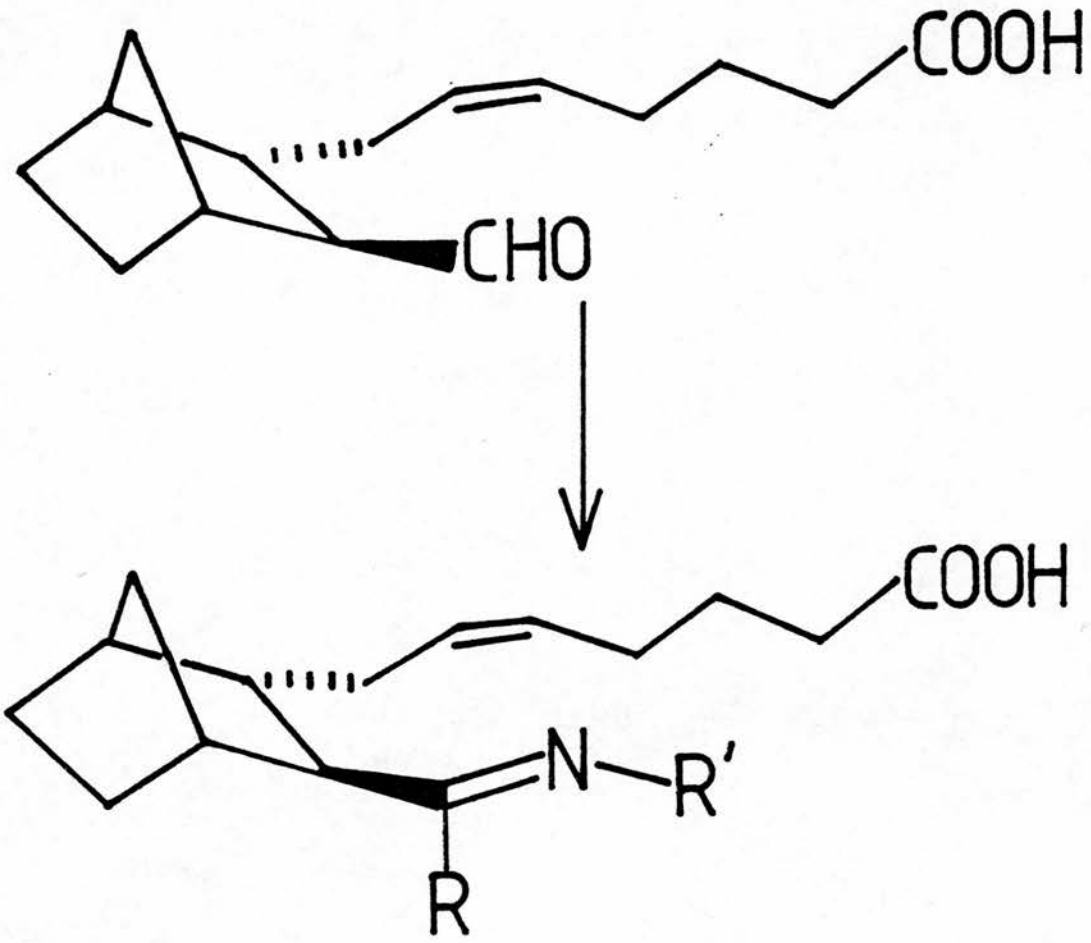
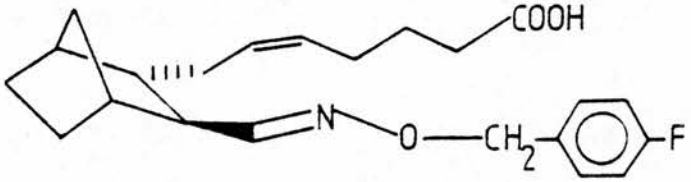


FIGURE 1.5: The antagonists are all made from the aldehyde precursor and have this common structure.

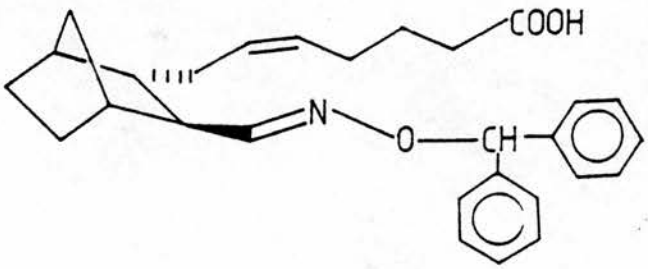
In 1975, Dr. R.L. Jones and Dr. N.H. Wilson of the Department of Pharmacology, University of Edinburgh, found that the toxicity of IC179,939 (Fig. 1.4), a $PGF_{2\alpha}$ analogue, appeared to be due to its surprisingly high thromboxane-like activity (127). The 16-p-fluorophenoxy analogue of PGD_2 was found to aggregate platelets unlike PGD_2 itself which inhibits platelet aggregation (128). It was discovered that the 9,11-ethano analogue of PGH_2 behaves as a partial agonist on thromboxane-sensitive preparations, whereas other analogues such as 11,9- and 9,11-epoxymethano PGH_2 are full agonists. This led to the development of 9,11-ethano analogues with modified ω side chains. One of these, the 16-p-fluorophenoxy analogue of 9,11-ethano PGH_2 - EP011 (128) - is a potent TXA_2 mimic, which is lethal to laboratory animals. Other analogues with N-substituted iminomethyl side chains are, however, potent competitive antagonists. These antagonists have a common structure (Fig. 1.5) in which the ring has now been changed to the bicycloheptane form.

The analogues were screened for antagonist activity on thromboxane-sensitive preparations including the rabbit aorta, dog saphenous vein, guinea pig trachea and human platelets. Of the vast number of analogues prepared, only the actions of five: EPO35, EPO37, EPO43, EPO45 and EPO92 were investigated specifically (Fig. 1.6). These analogues all inhibited platelet aggregation induced by AA and 11,9-epoxymethano PGH_2. Affinity constants for these analogues on the thromboxane-sensitive tissues mentioned above have been estimated by Dr. R.L. Jones (Table 1.1), using 11,9-epoxymethano PGH_2 as the standard agonist.

OXIMES

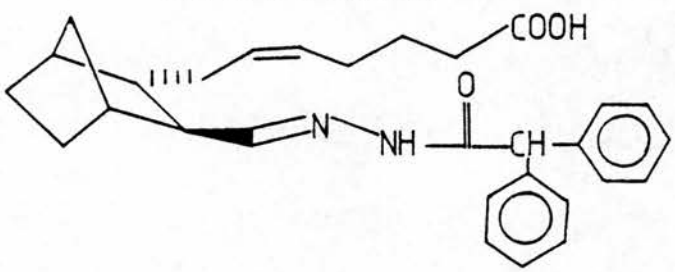


EP 037



EP 035

HYDRAZONES



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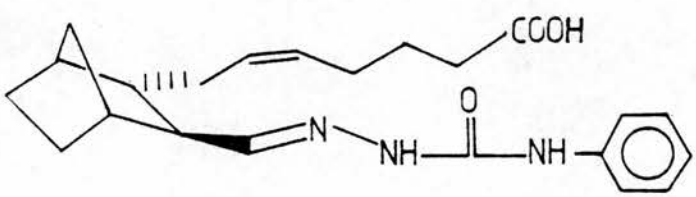

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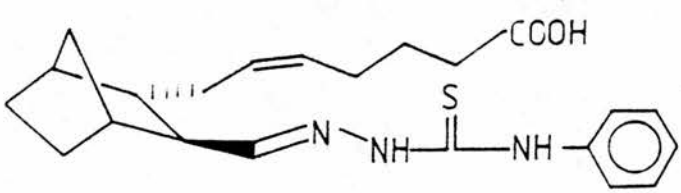


EP 092

FIGURE 1.6: The structures of the five antagonists studied.

TABLE 1.1: Estimates of the affinity constants for the five analogues tested (all values x $10^6 M^{-1}$)

	RA	DSV	GT	HP
EPO35	0.46	13.0	3.7	*
EPO37	5.3	3.8	2.5	0.46
EPO43	3.4	21.0	20.0	1.20
EPO45	2.0	22.0	33.0	0.87
EPO92	2.1	93.0	84.0	20.00

RA - Rabbit aorta
DSV - Dog saphenous vein
GT - Guinea pig trachea
HP - Human platelets

*The affinity constant cannot be determined as the dose-response curves in the presence and absence of EPO35 are not parallel. This analogue is, however, the most active inhibitor.

In the following sections the actions of these analogues on the cyclic AMP system of platelets, on the metabolism of AA by platelets and on the binding of a tritiated thromboxane mimic to platelets is described. These analogues have also been tested for biological activity *in vivo*, in rabbits.

CHAPTER II

Stimulation of platelet cAMP levels

INTRODUCTION

As well as being stimulated to aggregate, platelets also respond in a negative way to inhibit and even reverse aggregation. Such inhibitors of aggregation include PGI_2, PGD_2, PGE_1, adenosine and β adrenergic agonists. Substantial evidence now exists that an increase in platelet adenosine $3^1,5^1$-cyclic phosphate (cAMP) levels plays a role in mediating this inhibition of aggregation (146,147), although alternative mechanisms have been proposed (148,149,150). Indeed the available evidence satisfies Sutherland's criteria for the identification of cAMP as the second messenger of hormonal action (151). Firstly, it has been shown that PGI_2 (146,152), PGD_2 (153), PGE_1 (146,154,155,156), adenosine (157,158) and isoprenaline (159) can activate adenyl cyclase both in platelet homogenates and in intact platelets. Secondly, the increase in platelet cAMP precedes the inhibition of aggregation (160) and both the inhibitory action on aggregation and the associated rise in cAMP are greatly potentiated by phosphodiesterase inhibitors (161). Thirdly, exogenous N^6,2 - O-dibutyryl cyclic AMP can inhibit platelet aggregation (155, 159, 162) and finally, inhibitors of adenyl cyclase such as $2^1,5^1$-dideoxy-adenosine (DDA) and 9-(tetrahydro-2-furyl) adenine (SQ 22536) reduce the inhibitory effect of PGE_1 (163).

It has been further suggested that aggregation may result from a fall in platelet cAMP levels (164,165) and that a balance between the cAMP - inhibiting activity of TXA_2 and the cAMP-stimulating activity of PGI_2 could control human platelet aggregation *in vivo* (166). However, since considerable aggregation can occur in the presence of high cAMP levels it seems that a fall in platelet cAMP is not required for aggregation, although it may have a potentiating effect (167).

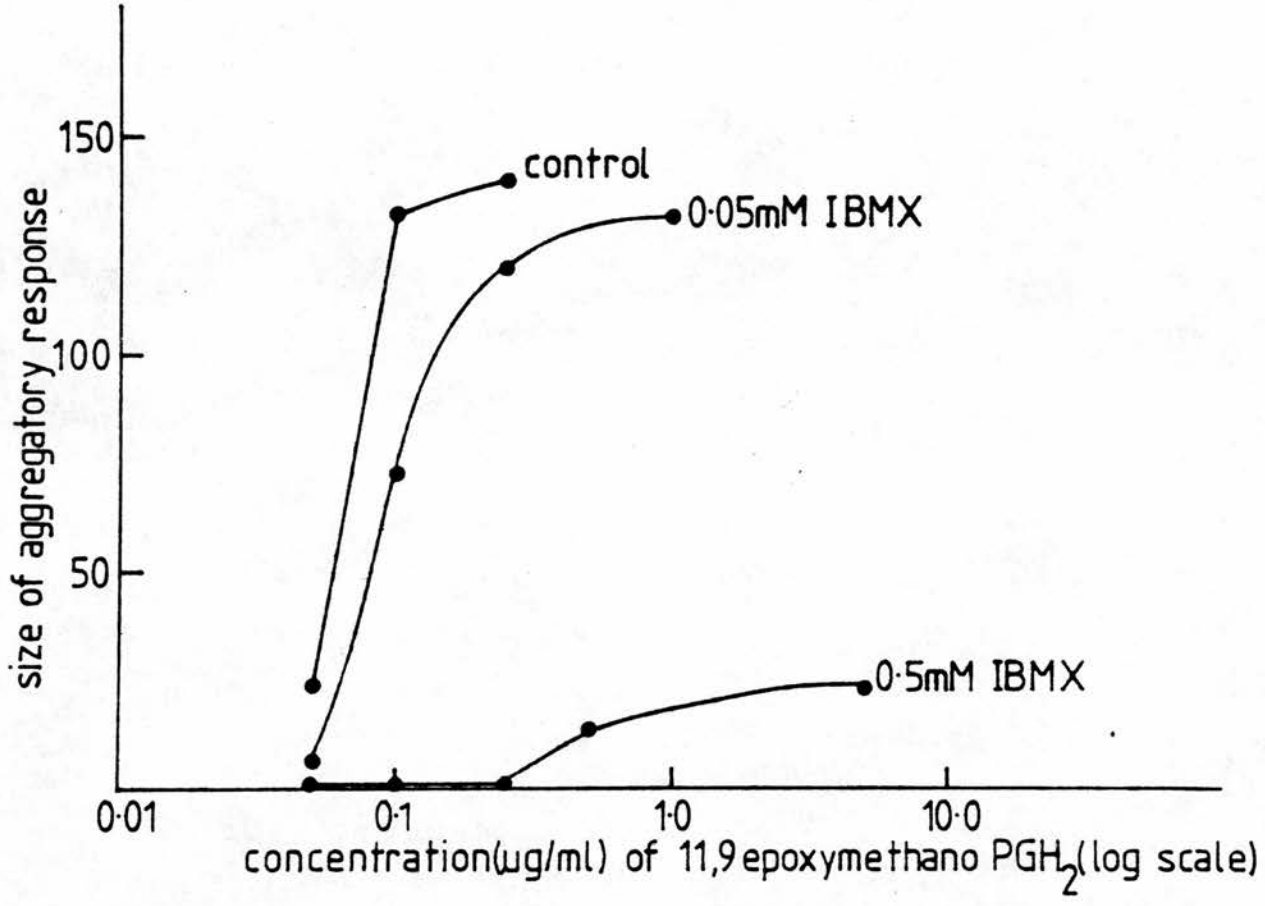
Indeed the only aggregating agent found to inhibit basal adenyl cyclase levels is ADP (168), the other aggregating agents only reducing cAMP levels once they have been elevated by PGE_1 (169, 170). Unlike ADP, A23187, PGH_2 and TXA_2 have no direct effect on adenyl cyclase levels of platelet homogenates (154,173). Since PGE_1-stimulated adenyl cyclase activity is inhibited by Ca^{2+} (171,172), it is possible that Ca^{2+} that has been mobilised by aggregating agents may be responsible for the inhibition observed. It has also been suggested that aggregating agents can activate a platelet membrane-bound phosphodiesterase (174) which, along with exposure of platelet cAMP phosphodiesterase during the release reaction (163), may contribute to the fall in cAMP levels. Perhaps the most convincing evidence against bidirectional control by cAMP is the recent finding that inhibitors of adenyl cyclase are not aggregating agents (163,175). This makes it unlikely that the basal level of cAMP in platelets exerts a significant tonic inhibitory effect, which suggests that cAMP has an undirectional role in the regulation of platelet function.

The observed inhibition of aggregation is most closely related to the concentration of cAMP about 15 seconds before the point of inhibition. This is consistent with the action of cAMP involving a time-consuming phosphorylation of a protein substrate, which can be dephosphorylated rapidly if the cAMP concentration falls (176). Myosin light-chain kinase is a substrate for the catalytic unit of a cAMP-dependent protein kinase (177). This phosphorylation of myosin kinase decreases its activity and increases its requirement for calmodulin, and thus Ca^{2+} (178). The resultant decrease in the relative amount of phosphorylated myosin (179) will decrease platelet contractile activity, since unphosphorylated myosin cannot interact with actin (180).

This may partly explain the inhibition by cAMP of the release reaction induced by aggregating agents (181,182,183,184). Certainly an increase in cAMP reduces the phosphorylation of polypeptides of molecular weight 40000 and 20000, which is associated with the secretion of dense granules induced by thrombin (185), collagen or A23187 (55,56,57,58,184). A reduction in this phosphorylation is also found with inhibitors of the release reaction, such as verapamil and tetracaine which inhibit Ca^{2+} movement.

cAMP also increases the phosphorylation of specific polypeptides of 24000 and 22000 daltons. P24 is found in the fractions containing highest specific activities of microsomal markers, glucose-6-phosphatase and cytochrome P-450, and which possess a capacity for ATP-dependent uptake of Ca^{2+} ions (186). Uptake of Ca^{2+} by a similar fraction can be stimulated by cAMP in the presence of a cAMP-dependent protein kinase (187) suggesting that phosphorylation of P24 represents part of the mechanism for activation of a Ca^{2+} ion pump situated in the membranes of the dense tubular system (43,44,188,189). It is uncertain whether the effect of cAMP is to increase Ca^{2+} binding to the membranes or to facilitate uptake of Ca^{2+} into the interior.

It may be possible to explain many of the effects of cAMP in terms of this stimulation of the active transport of Ca^{2+} ions out of the cytosol. Since calcium ions are essential for aggregation, agents which increase cAMP will inhibit aggregation irrespective of the aggregating agent used. Although a shift to the right in the dose-response curve for the aggregating agent can be obtained with low concentrations of such an inhibitor, with higher concentrations the dose-response curve is flattened so that irreversible aggregation can no longer be achieved, and with even higher concentrations the dose-response curve is virtually



IBMX (isobutyl methyl xanthine) increases platelet cAMP levels by inhibiting phosphodiesterase, the enzyme which catalyses the breakdown of cAMP. Each result is the mean of three observations.

FIGURE 2.1: Inhibition of platelet aggregation by isobutyl methylxanthine.

abolished (Fig. 2.1). Presumably at this point the platelet calcium stores are effectively immobilised.

In this section, the effects of analogues EPO35, EPO37, EPO43, EPO45 and EPO92 on platelet cAMP levels have been investigated. The extraction of cAMP from platelet-rich plasma and the development of a competitive protein-binding assay for the measurement of cAMP is described. The effect of EPO35 in increasing cAMP levels in the platelet is compared with that of the classical prostaglandin inhibitors, PGI_2, PGD_2 and PGE_1. The possibility that EPO35 acts as a partial agonist on the prostacyclin receptor of platelets is discussed.

MATERIALS AND METHODS

Preparation of a binding protein for cAMP

Reagents used: all prepared in glass-distilled water unless otherwise specified.

1. 4 mM Na EDTA adjusted to pH 7 with a few drops 1M NaOH.
2. 1M acetic acid.
3. 1M potassium phosphate buffer pH 7.2.
   For 500 ml: 31.3 g dipotassium hydrogen phosphate
   9.5 g potassium dihydrogen phosphate
   0.4 g sodium EDTA
4. 5 mM phosphate buffer with 2 mM EDTA.
   For 100 ml: 6.3 g dipotassium hydrogen phosphate
   1.9 g potassium dihydrogen phosphate
   7.45 g sodium EDTA
   This is diluted to 10 litres with 9.9 litres of distilled water.
5. 0.3 M phosphate buffer.
   For 400 ml: 6.4 g dipotassium hydrogen phosphate
   3.2 g potassium dihydrogen phosphate
   0.3 g sodium EDTA

6. 0.1 M phosphate buffer.

For 500 ml: 2.7 g dipotassium hydrogen phosphate
1.3 g potassium dihydrogen phosphate
0.4 g sodium EDTA

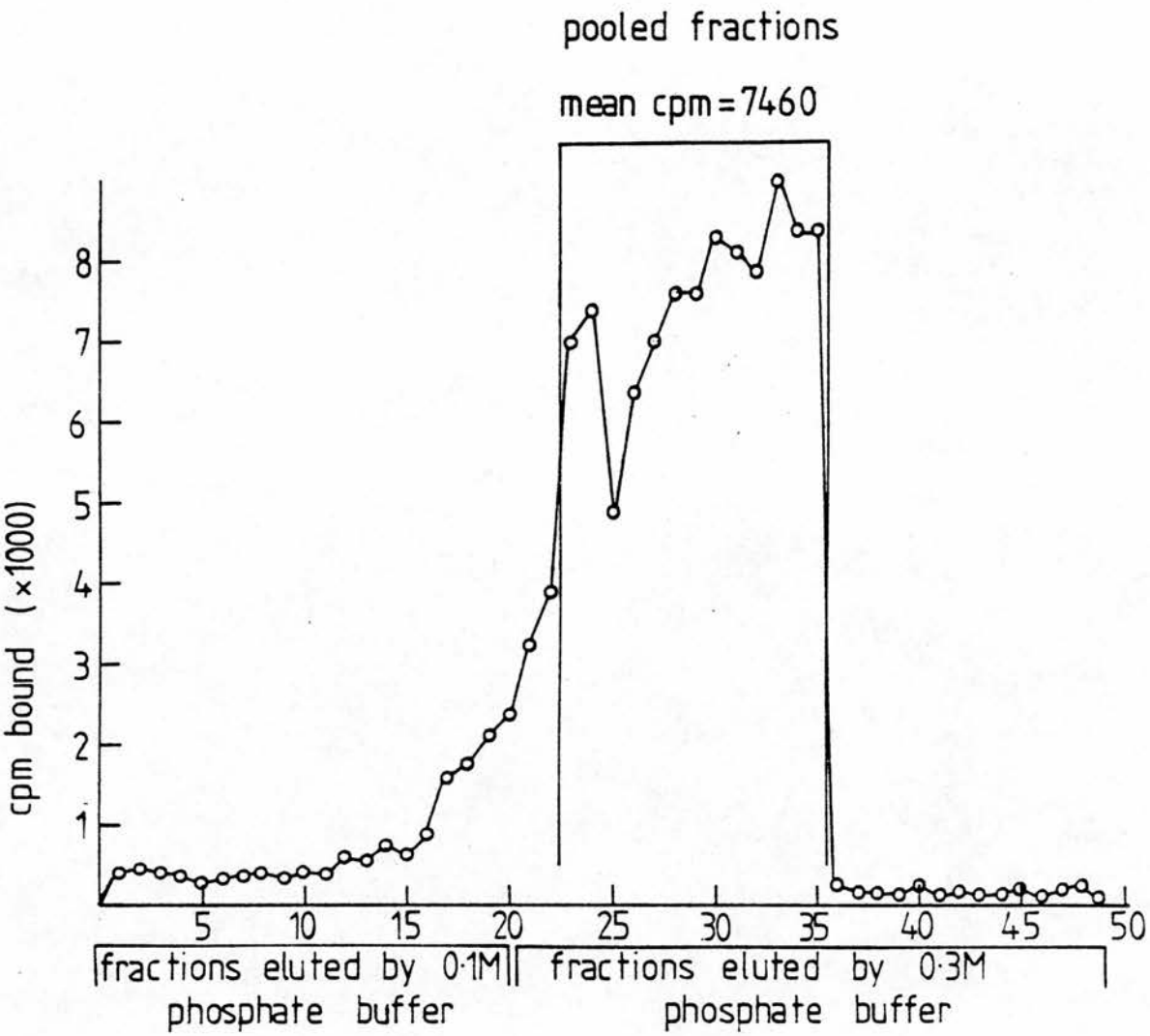
Preparation for DEAE - cellulose chromatography

A glass column (15 x 4 cm), plugged with glass wool at the bottom and fitted with a mariotte reservoir, was packed with an equilibrated slurry of DEAE sephadex (dry weight 14 g) and 5 mM phosphate buffer pH 7. The mariotte reservoir was then removed and a Watson and Marlow pump set up to deliver buffer to the head of the column. The pump speed was set to match a flow of 1.5 ml/min buffer through the column. The height of the column was 12 cm, giving a bed volume of 150 ml.

Purification of the binding protein

The method used has been adapted from others previously described (190,191). All steps were carried out at 0-4°C.

Fresh sheep skeletal muscle (flank) was obtained on ice from the slaughterhouse. The fat was removed and 500 g of meat was cut into small pieces, minced and homogenised in three volumes of 4 mM Na EDTA (pH 7), using a polytron blender. The homogenate was centrifuged at 2300 rev/min, to bring down the particulate matter, and the supernatant was further centrifuged at 15000 g for 30 minutes using the MSE 6 x 100 ml rotar. This required three runs. The precipitate was discarded and the supernatant adjusted to pH 4.8 by addition of drops of 1 N acetic acid, stirring constantly. This was allowed to settle before being centrifuged at 15000 g for a further 30 minutes. The precipitate was again discarded and the supernatant adjusted to pH 6.8 with 1 M phosphate buffer pH 7.2. Solid

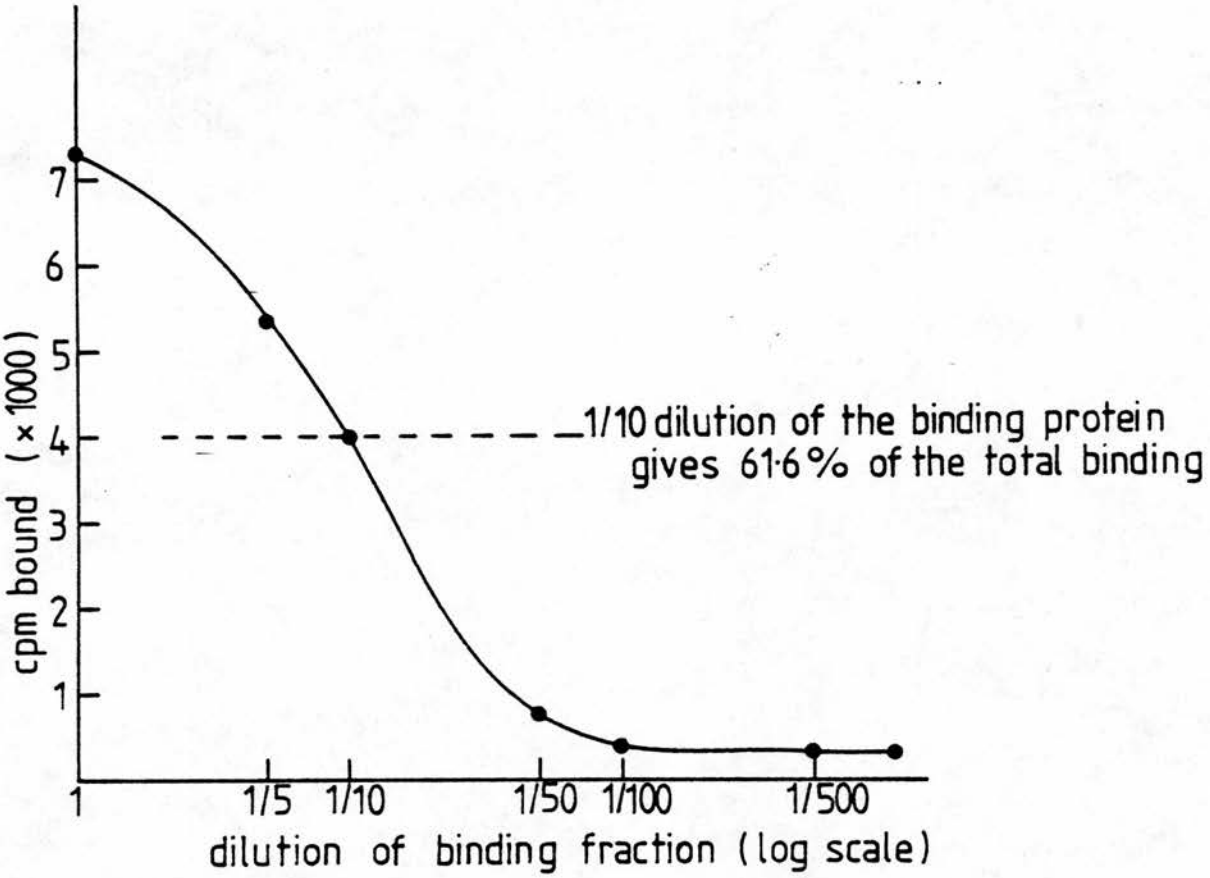


The cpm bound give a measure of the ^{3}H cAMP bound by each fraction.

FIGURE 2.2: The capacity of the fractions, eluted from the DEAE cellulose column, to bind cAMP.

ammonium sulphate, 33 g/100 ml, was added slowly, stirring constantly on ice and the solution was left for 30 minutes before being centrifuged again at 15000 g for 30 minutes. The supernatant was discarded and the precipitate was dissolved in 8% of the crude extract volume of 5 mM potassium phosphate buffer pH 7. This was dialysed against 20 times the volume of buffer for 14 hours, changing the buffer after 1 and 13 hours. The solution was filtered, then applied to the DEAE-cellulose column. The column was washed with two bed volumes of 5 mM phosphate buffer and the entire fraction eluted and collected in a beaker. The column was then washed with two bed volumes of 0.1 mM phosphate buffer, followed by two bed volumes of 0.3 mM phosphate buffer. 10 ml fractions were collected throughout using the drop-counting head of an LKB Ultrorac fraction collector. Each fraction was assayed for binding of cAMP. This was done by substituting 100 µl of the fraction for 100 µl of the binding protein supplied by the Amersham cAMP assay kit (192). Fig. 2.2 shows the cAMP binding activity of each fraction. Two peaks of activity were observed but the separation was not as good as that reported elsewhere (190). Fractions 23-35 were pooled and dialysed against five buffer changes of 5 mM phosphate buffer. The binding protein was dispensed into 3 ml ampules which were capped and stored at -40°C. They were defrosted and diluted as necessary immediately before use.

A dilution curve was obtained by measuring the ^{3}H cAMP bound (counts per minute, cpm) by increasing dilutions of the binding protein (Fig. 2.3). A $^{1}/_{10}$ dilution of the protein bound 61.6% of the total counts. This dilution was chosen for the standard curve and showed suitable displacement of binding within the 0.3-10 pmol cAMP range. The protein concentration of this dilution of the binding



The cpm bound give a measure of the ^{3}H cAMP bound by the protein.

FIGURE 2.3: Dilution of the cAMP binding protein.

protein was estimated, using a spectrophotometer, to be 0.13 mg/ml. This estimation is crude as there will be other proteins present which do not bind cAMP. The value of 0.021 pmol cAMP/µg protein which was estimated as the specific activity of this fraction will therefore be less than the true specific activity of the cAMP binding protein. Indeed, values of 0.1 (192,193) and 0.3 (190) pmol cAMP/µg protein have been reported for the specific activity of a similar binding protein, isolated from bovine muscle.

Determination of the specific activity

The protein concentration of the fraction used was 0.13 mg/ml. 100 µl of this fraction bound ≃ 4900 cpm (^{3}H cAMP). The efficiency of the scintillant (10.5 g PPO to 1.5 l toluene + 900 ml 2-ethoxy ethanol) to count tritium in the scintillation counter used is approximately 30%.

100 µl of the binding protein binds $4900 \times \frac{100}{30}$ (disintegrations per minute, dpm)

$$= 4900 \times \frac{100}{30} \times \frac{1}{2.2 \times 10^6} \ \mu Ci$$

The specific activity of the ^{3}H cAMP is 5 µCi/180 pmol
i.e. 1 µCi ≃ 36 pmol cAMP.

Therefore 100 µl of the binding protein binds $\frac{4900 \times 100 \times 36}{30 \times 2.2 \times 10^6}$ pmol cAMP

$$= 0.27 \text{ pmol cAMP}$$

100 µl of the binding protein is equivalent to approximately 0.013 mg protein.

Therefore 0.013 mg protein binds 0.27 pmol cAMP.

Specific activity of the protein is 0.021 pmol cAMP/µg protein.

cAMP assay procedure

The assay is based on competition between unlabelled cAMP and a fixed quantity of ^3H labelled compound for binding to a protein which has a high specificity and affinity for cAMP. Separation of protein-bound cAMP from the unbound nucleotide is achieved by adsorption of the free nucleotide onto coated charcoal, followed by centrifugation. An aliquot of the supernatant is removed for liquid scintillation counting; this gives an estimate of the bound ^3H cAMP. The concentration of unlabelled cAMP in the sample is then determined from a standard curve.

The assay protocol is given in Table 2.1

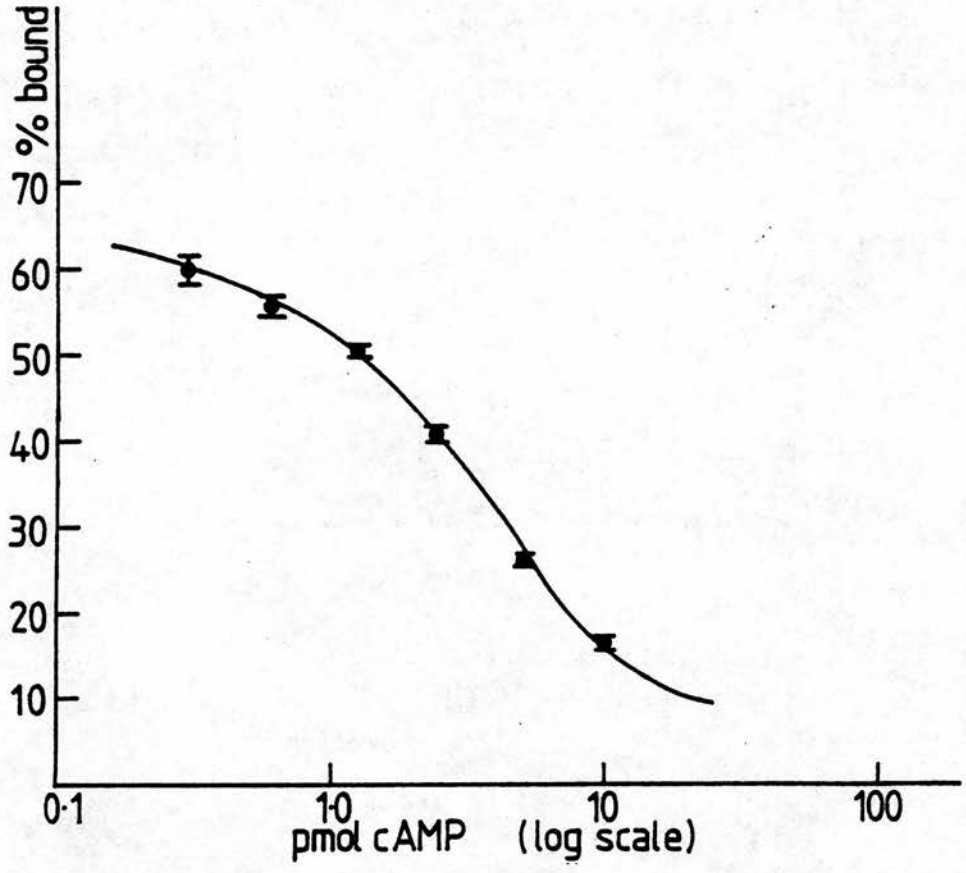
TABLE 2.1: Assay protocol

Tube number and description	Volume (µl) of: buffer	standard	unknown	^3H cAMP	binding protein
1/ 2/ 3 charcoal blank	150	-	-	50	-
4/ 5/ 6 0 pmol cAMP	50	-	-	50	100
7/ 8/ 9 0.3 "	-	50	-	50	100
10/11/12 0.6 "	-	50	-	50	100
13/14/15 1.25 "	-	50	-	50	100
16/17/18 2.5 "	-	50	-	50	100
19/20/21 5.0 "	-	50	-	50	100
22/23/24 10.0 "	-	50	-	50	100
25/26 total counts	250	-	-	50	-
27/28 unknowns	-	-	50	50	100
29/etc unknowns	-	-	50	50	100

Assay buffer is 0.05 M Tris pH 7.5 containing 4 mM Na EDTA. (EDTA is a phosphodiesterase inhibitor (195) and will prevent the breakdown of cAMP.) 8-^3H adenosine $3^1,5^1$-cyclic phosphate (cAMP) ammonium

salt was obtained from Amersham Radiochemical Centre (specific activity of 30 Ci/mmol) and diluted 2000-fold in the assay buffer so that 50 µl is equivalent to 0.9 pmol cAMP (0.025 µCi). Adenosine $3^1,5^1$-cyclic phosphate standard was obtained as the free acid from Sigma. The binding protein was diluted 10-fold with assay buffer containing 0.1% bovine serum albumin (Sigma) which is reported to promote cAMP binding by protein kinase (194). Tubes 1-3 determine the cpm bound in the absence of binding protein and the mean value is subtracted from the cpm counted for the remaining tubes, to give the true cpm bound to the binding protein. Tubes 4-24 are for determination of binding over the range of the standard curve, i.e. 0-10 pmol cAMP. Tubes 25 and 26 give a value for the total counts; two 200µl aliquots are counted.

The tubes were whirlimixed and left in ice in the cold room for two hours. At least 15 minutes before the end of the incubation period, the charcoal adsorbent was prepared by mixing 520 mg Norit GSX charcoal and 400 mg bovine serum albumin with 20 ml distilled water. This suspension was stirred constantly on ice, then 100 µl charcoal adsorbent was added to twelve tubes at a time. After whirlimixing these were centrifuged for 2 minutes at 12000 g in a refrigerated centrifuge. A 200 µl sample was withdrawn from each tube, added to 10 ml PPO scintillant (900 ml 2-ethoxy ethanol, 1.5 litres toluene and 10.5 g PPO) and counted on a Philips PW 4540 liquid scintillation analyser for 4 minutes. By doing the charcoal precipitation step on only twelve tubes at a time it ensured that the charcoal was present for a maximum of 6 minutes. During this period the charcoal will bind free cAMP, but with longer time intervals the charcoal will strip and bind protein-bound



Each result is the mean and standard error of 8 observations.

FIGURE 2.4: cAMP standard curve.

cAMP. This effect is minimised at 2-4°C and so the charcoal is kept on ice throughout the experiment.

TABLE 2.2: Displacement of ^{3}H cAMP binding over the range of the standard curve (0-10 pmol cAMP)

pmol cAMP	cpm bound	Standard error	Coefficient of variation	% bound	Standard error	Coefficient of variation
0	4872.4	± 60.4	3.5	65.3	±0.8	3.5
0.3	4468.0	±120.0	7.6	59.9	±1.6	7.6
0.6	4164.1	± 87.9	6.0	55.8	±1.2	6.0
1.25	3793.0	± 27.0	2.0	50.8	±0.4	2.0
2.5	3071.6	± 57.7	5.3	41.2	±0.8	5.3
5.0	2003.1	± 36.4	5.1	26.8	±0.5	5.1
10	1213.6	± 35.0	8.1	16.3	±0.5	8.1

n = 8

With a counting time of 4 minutes the detection limit equal to 2 standard deviations at zero dose is 0.3 pmol cAMP/50 µl sample (see Table 2.2, Fig. 2.4).

inter-assay coefficient of variation = $\frac{\text{s.d.}}{\text{mean}} \times 100$

for 5.0 pmol cAMP = 10.083% (n = 9)

intra-assay coefficient of variation = 6.09% (n = 66 from 3 different assays)

$$= \frac{\Sigma (d/x \times 100)^2}{2n}$$

where d = difference between duplicates

x = mean of duplicates

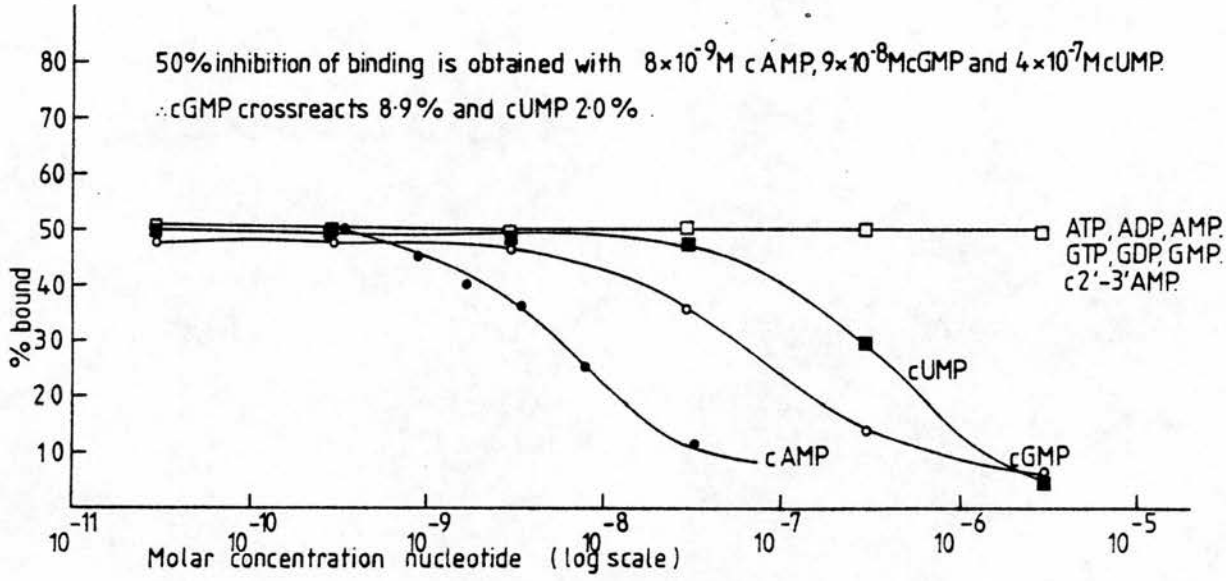


FIGURE 2.5: Cross reactivity of a variety of nucleotides.

A = adenine - nomenclature of nucleotides
G = guanine
U = uridine

Cross-reactivity

Competitive protein-binding assays are subject to interference from substances present in crude biological samples. The substances most likely to interfere are other nucleotides. The cross-reactivity of a variety of nucleotides was studied (Fig. 2.5). Of those studied, cGMP crossreacts 8.9% and cUMP 2.0%. Only cGMP occurs naturally in platelets and is unlikely to interfere in this assay at the concentrations of cGMP which have been found in platelets: the basal level of cAMP in platelets is 15 times greater than that of cGMP (170). Although the role of cGMP in platelets is uncertain, it is reported that aggregating agents increase cGMP levels in the platelet (196,197). Since the compounds tested here all inhibit aggregation, cGMP levels are unlikely to be affected by these agents. ATP and ADP are nucleotides present in platelets in high concentrations and these do not crossreact in concentrations up to 3×10^{-5} M, which represent over a 50000-fold excess over the tritium-labelled cAMP present.

Extraction procedures

CPD, for 100 ml: 0.33 g citric acid
2.63 g sodium citrate
0.22 g monosodium phosphate
2.55 g dextrose

Blood (1 pint) was taken from healthy donors by the Blood Transfusion Unit and collected into CPD (70 ml CPD/500 ml blood). Platelet-rich plasma (PRP) was collected after centrifugation at 160 g for 20 minutes. This PRP was 6-18 hours old when available for use; all storage and manipulation was at room temperature. Storage for up to 48 hours is reported to increase slightly the maximum capacity of the platelet to produce cAMP while basal levels decline gradually

by a maximum of 20% (198). This effect is suggested to result from a loss of platelet microtubules with time (198) resulting in a loss of the constraints imposed by microtubules on adenyl cyclase stimulation (199). The age of the PRP is unlikely to affect the type of responses seen and similar results were obtained when fresh blood was used.

Two different extraction procedures were tested.

Extraction 1: (153)

PRP was incubated at 37°C. Then 10-100 µl PG were added to 1.5 ml PRP and mixed rapidly. This was incubated at 37°C for 30 seconds before the addition of 375 µl 2 M perchloric acid which quenched the reaction. After centrifugation at 1000 g for 5 minutes, the supernatant was decanted and added to 375 µl 2 M potassium bicarbonate ($KHCO_3$). This was cooled in ice to achieve maximum precipitation, before the supernatant was withdrawn, frozen and freeze dried. The freeze-dried solid was extracted with 1 ml of ethanol, then centrifuged at 2000 rev/min for 20 minutes. The supernatant was evaporated to dryness at 55°C under a stream of nitrogen, then redissolved in 150 µl assay buffer. Duplicate 50 µl samples were assayed.

Extraction 2:

1 ml of PRP was added to a 10 ml silinised test tube and incubated for 30 seconds at 37°C. Then 10-100 µl PG were added to the PRP before the incubation and whirlimixed. The reaction was quenched by the addition of 2 ml ethanol, the tubes were whirlimixed and left to stand for 5 minutes at room temperature. The supernatant was decanted after centrifugation at maximum speed on a bench centrifuge. The precipitate was washed with 1 ml of ethanol/water (2 : 1), then centrifuged as before. The supernatants were combined and evaporated to

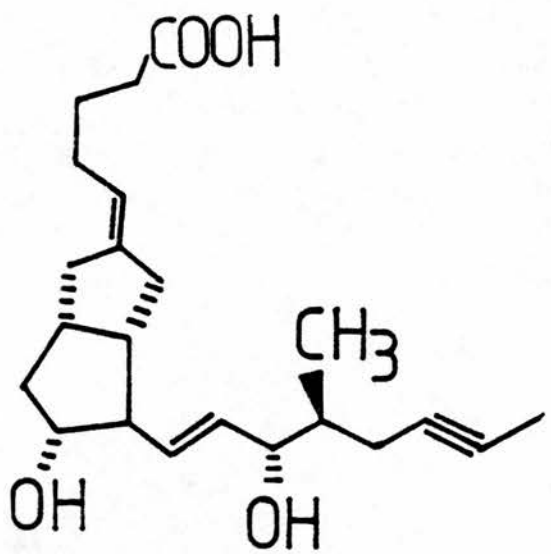
dryness at 55°C under a stream of nitrogen. The residue was dissolved in 0.5 ml assay buffer and centrifuged at 12000 g for 30 minutes to remove insoluble material. Two 50 µl samples of the supernatant were assayed directly, or the supernatant diluted in buffer if required, then assayed.

Use of SQ 22536: an inhibitor of adenyl cyclase

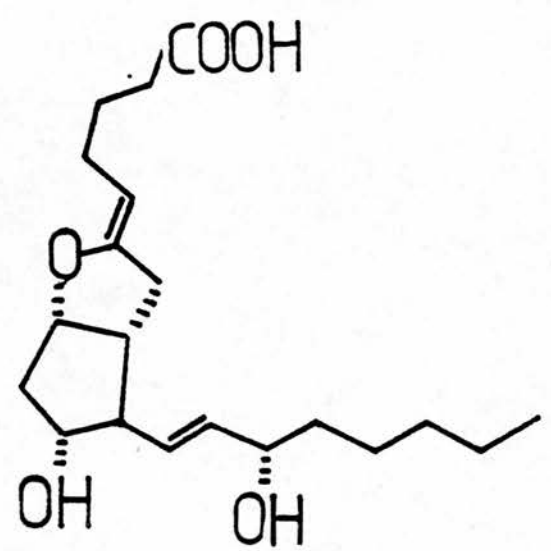
SQ 22536 (9-(tetrahydro-2-furyl) adenine) is an inhibitor of adenyl cyclase (175). Although the IC_{50} value of SQ 22536 has been quoted as 13 µM for the inhibition of PGE_1-activated adenyl cyclase activity of a platelet particulate fraction, the inhibitory action showed negative co-operactivity with increasing concentrations of the inhibitor, so that concentrations of 100 µM are required to inhibit adenyl cyclase in intact platelets (163).

Freshly prepared PRP was used for the experiments, and platelet aggregation was measured by the method of Born (200). 1 ml PRP was diluted with Krebs (1 ml) and saline (0.4 ml), and stirred at 37°C for 1 minute before the addition of an aggregating agent (50-100 µl). If an inhibitor was added to the platelet mixture two minutes before the aggregating agent it was added within the 0.4 ml volume of saline. This ensures that the total volume is kept constant and so the calcium concentration is unchanged.

11,9-epoxymethano PGH_2 (a gift from UpJohn Company, Kalamazoo) and ADP (free acid from Sigma, made into sodium salt) were used as standard aggregating agents. PGE_1, E_2 and D_2 (UpJohn Company, Kalamazoo) as well as the stable PGI_2 analogue ZK36374 (Fig. 2.6) (Schering AG, Berlin, 201,202,203) were used as standard inhibitors of aggregation and were always preincubated for 2 minutes before the



ZK 36 374



PGI_2

FIGURE 2.6: The structure of the stable PGI_2 mimic, ZK 36 374.

addition of an aggregating agent. 100 μM SQ22536 (Squibb) was added at this stage when required.

RESULTS

Of the two extraction procedures tested, the second was very much better, the mean recovery being 87.3 ± 1.36% (n = 16). The first extraction procedure had a poor recovery (10-20%) and was very variable. This may in part be due to the fact that the freeze drier available was not in very good order. Certainly the results of the actions of the classical prostaglandin inhibitors on cAMP, obtained using the second extraction procedure, compared very favourably with those published by Tateson, Moncada and Vane (153), who used the first extraction procedure. Although many of the compounds were tested using the first extraction procedure, and the results were qualitatively similar, all of the following results have been obtained using the second extraction procedure. Of the five analogues tested, only EPO35 showed a marked effect on platelet cAMP levels (Table 2.3). The cAMP concentrations are expressed as multiples of the basal level.

The effect of EPO35 was compared with that of the classical prostaglandin inhibitors PGE_1, PGD_2 and the stable PGI_2 analogue ZK 36374 (Table 2.4, Fig. 2.7).

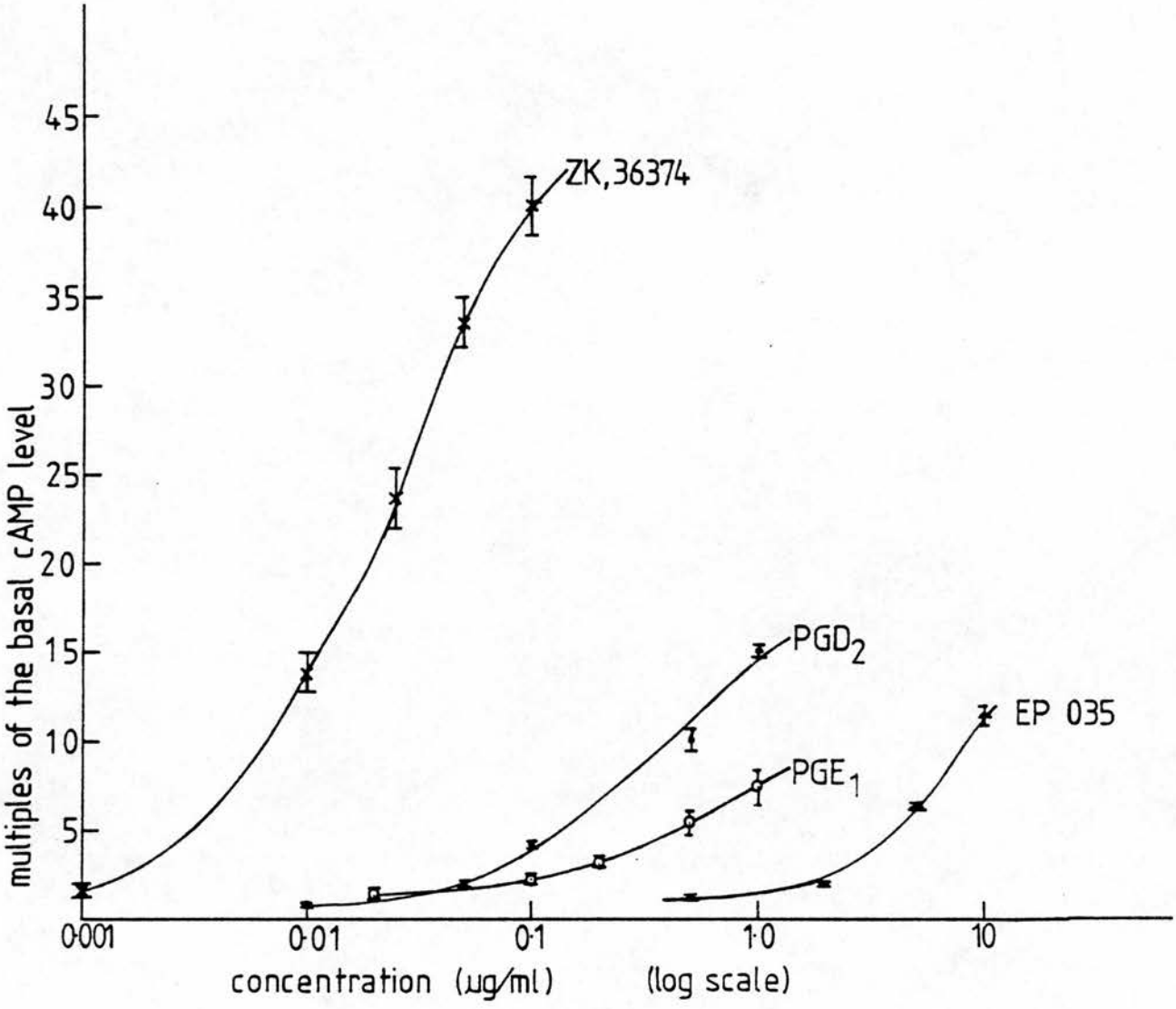
Although it requires higher concentrations of EP035 to produce an increase in cAMP levels, it is within this concentration range that EP035 inhibits platelet aggregation, presumably part of this inhibition being attributable to cAMP.

The effect of EPO35 could result either from stimulation of adenyl cyclase or by inhibition of phosphodiesterase, the enzyme which catalyses the breakdown of cAMP. SQ 22536 is a non-competitive

TABLE 2.3: Effects of the analogues tested on basal cAMP levels in human platelets

Concentration µg/ml	Multiples of the basal cAMP level induced by: EP035	EP037	EP043	EP045	EP092
0.5	1.21 ± 0.11	1.01 ± 0.05	0.96 ± 0.09	1.02 ± 0.13	0.89 ± 0.07
2.0	1.97 ± 0.09	1.03 ± 0.05	1.31 ± 0.25	0.73 ± 0.11	1.10 ± 0.04
5.0	5.35 ± 0.45	0.97 ± 0.05	0.60 ± 0.04	0.86 ± 0.12	1.19 ± 0.08
10.0	11.35 ± 0.63	0.94 ± 0.08	0.62 ± 0.03	0.60 ± 0.11	1.11 ± 0.08

Values represent the mean and standard error of 18 observations, 3 donors.



Each result is mean and standard error of 18 observations, 3 donors.

FIGURE 2.7: Comparison of the action of EP035 to increase platelet cAMP levels with ZK 36 374, PGE_1 and PGD_2

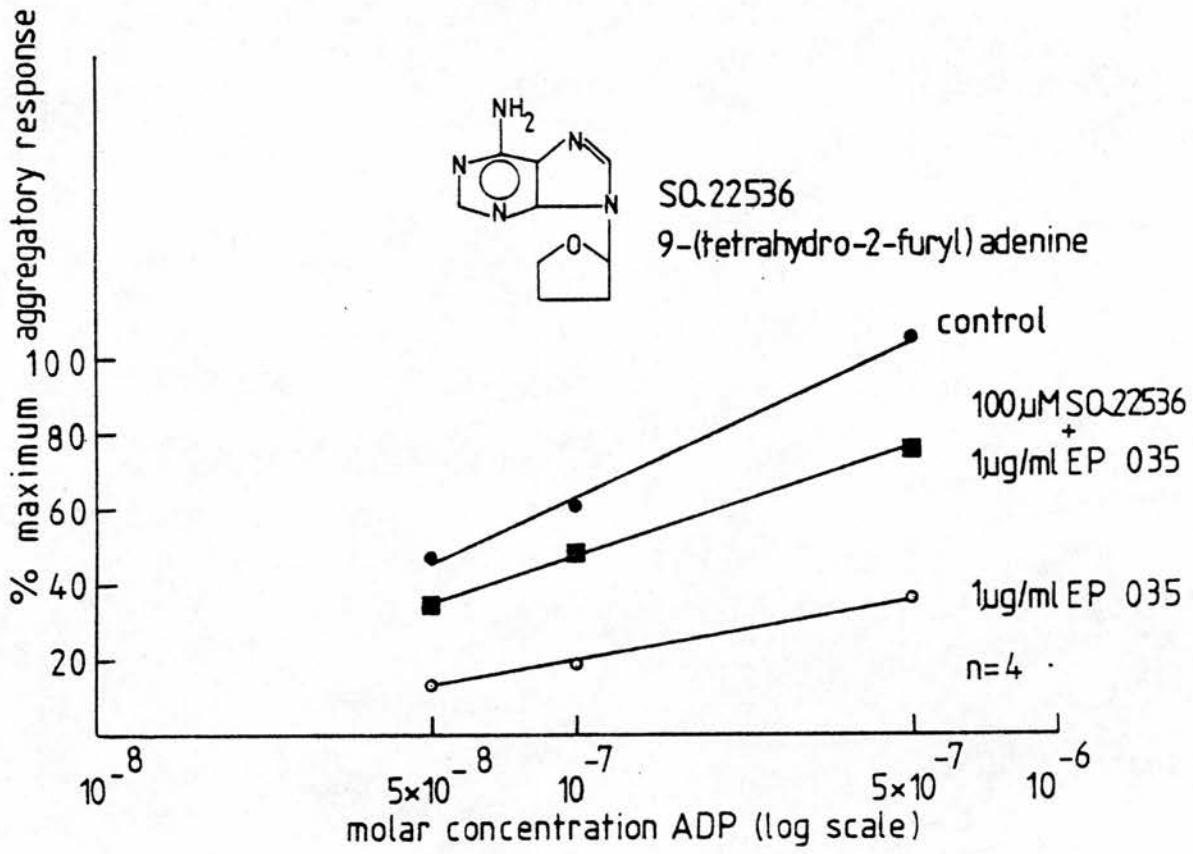
TABLE 2.4: Effects of ZK36374, PGD_2 and PGE_1 to raise cAMP levels in human platelets

Concentration ng/ml	Inhibitor: ZK36374	PGD_2	PGE_1
1	1.53 ± 0.2		
10	13.83 ± 2.15	0.77 ± 0.12	
20			1.35 ± 0.06
25	23.70 ± 1.70		
50	33.60 ± 1.41	1.97 ± 0.18	
100	39.78 ± 1.51	4.16 ± 0.19	2.23 ± 0.21
200			3.13 ± 0.36
500		10.21 ± 0.55	5.42 ± 0.79
1000		15.12 ± 0.35	7.42 ± 1.03

Mean and standard error of 18 observations, 3 donors.

inhibitor of adenyl cyclase and so will reduce the inhibitory action of a compound only if it raises cAMP levels by stimulating adenyl cyclase. In the presence of 100 μM SQ22536, the inhibition by EP035, of aggregation induced by ADP, is markedly reduced (Fig. 2.8). This suggests that EP035 stimulates adenyl cyclase to increase cAMP levels within the platelet. Similarly, PGE_1, PGD_2 and PGI_2 act by stimulation of adenyl cyclase (163).

When comparing the aggregatory response of ADP in the presence and absence of an inhibitor, it is important to preincubate the platelets for the same length of time before the addition of the aggregating agent. A portion of the ADP response appears to fade over the incubation period so that a greater degree of inhibition is seen if the effect of an inhibitor that has been preincubated for 2 minutes is compared with a control response that has been preincubated for only 1 minute (Table 2.5). This time difference is not important with other aggregating agents and it is not clear what is responsible for this effect.



Each result is the mean of 4 observations.

FIGURE 2.8: The effect of the adenyl cyclase inhibitor, SQ 22536, on the action of EP035 to inhibit platelet aggregation.

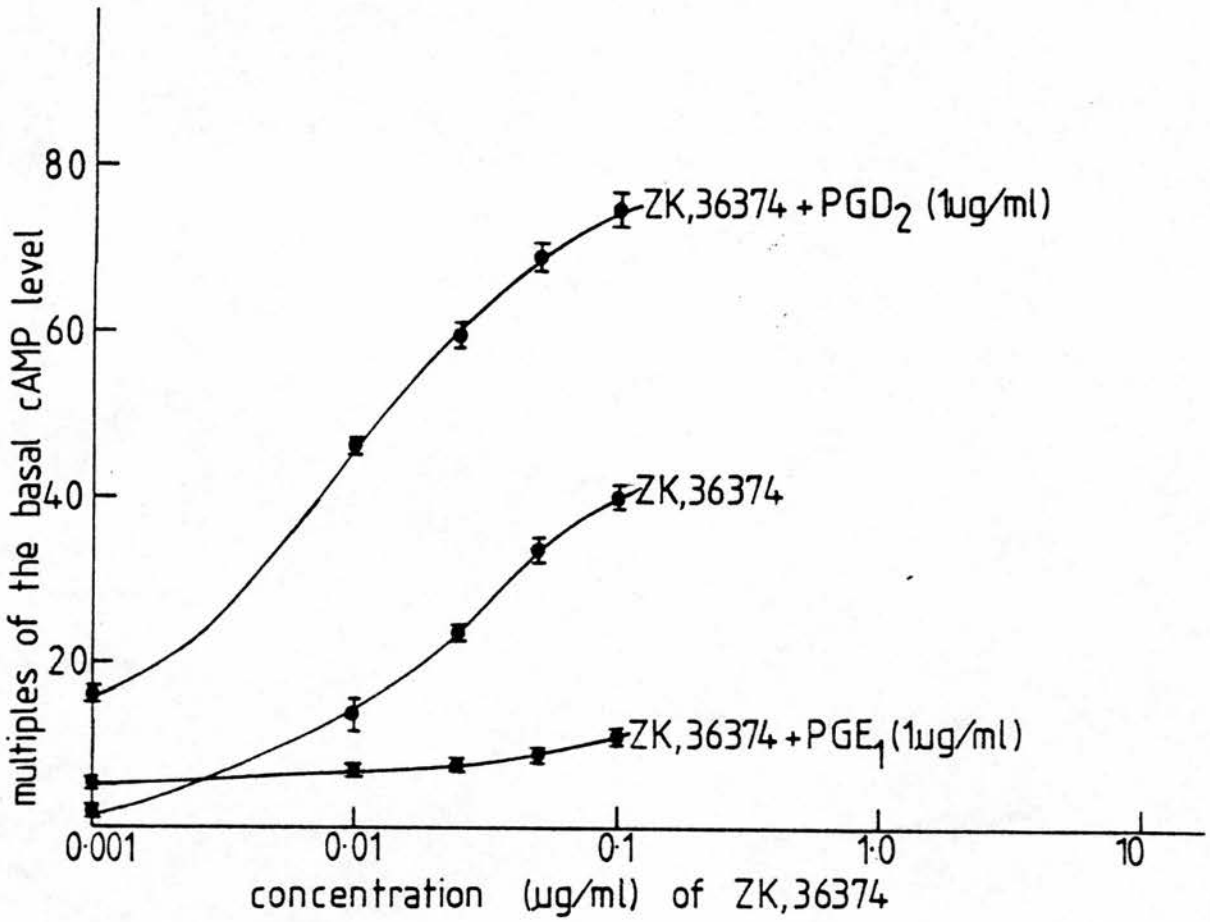
TABLE 2.5: Phenomenon of fade

Concentration of ADP	Preincubating time (min)	Size of response
10^{-6}M	0.5	125
	1.0	120
	2.0	117
	3.0	110
	4.0	105

Results are the mean of 3 observations.

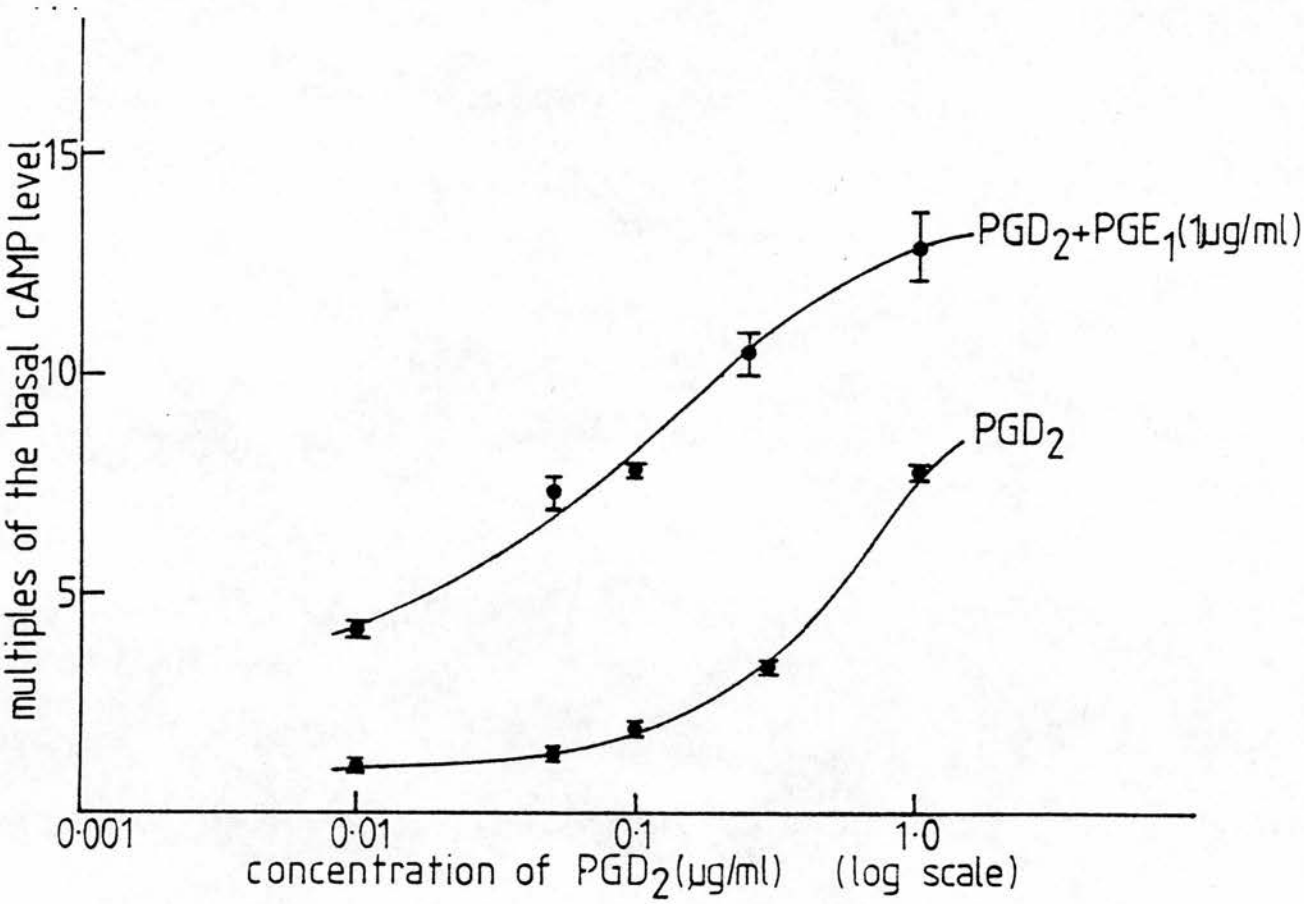
The response is taken as the height of the aggregation achieved 100 seconds after incubation of the PRP with ADP

The actions of PGI_2 and PGE_1 to raise platelet cAMP levels have been shown to differ from PGD_2 in both species specificity (204,205) and dose-response characteristics (143,206). This led to the suggestion that platelets have distinct receptors for PGD_2 and PGI_2 and that PGE_1 acts on the PGI_2 receptor. More recently binding studies of tritiated PGI_2, PGE_1 and PGD_2 to whole platelets and platelet fractions have supported this concept (207,208,209,210,211). If PGE_1 acts on the prostacyclin receptor, the dose-response characteristics (Fig. 2.7) suggest that PGE_1 is a partial agonist since it is incapable of producing a maximal increase in cAMP. To investigate this possibility, the dose-response curve for ZK36374 was determined in the presence and absence of 1 µg/ml PGE_1 (Fig. 2.9). The effect of ZK36374 to raise cAMP is severely depressed in the presence of 1 µg/ml PGE_1 whereas PGD_2 1 µg/ml acts additively with ZK36374. This does not appear to be a non-specific effect of PGE_1 on adenyl cyclase because PGE_1 has an additive effect on the dose-response curve for PGD_2 (Fig. 2.10). Dose-response curves for ZK36374 were carried out in the presence of



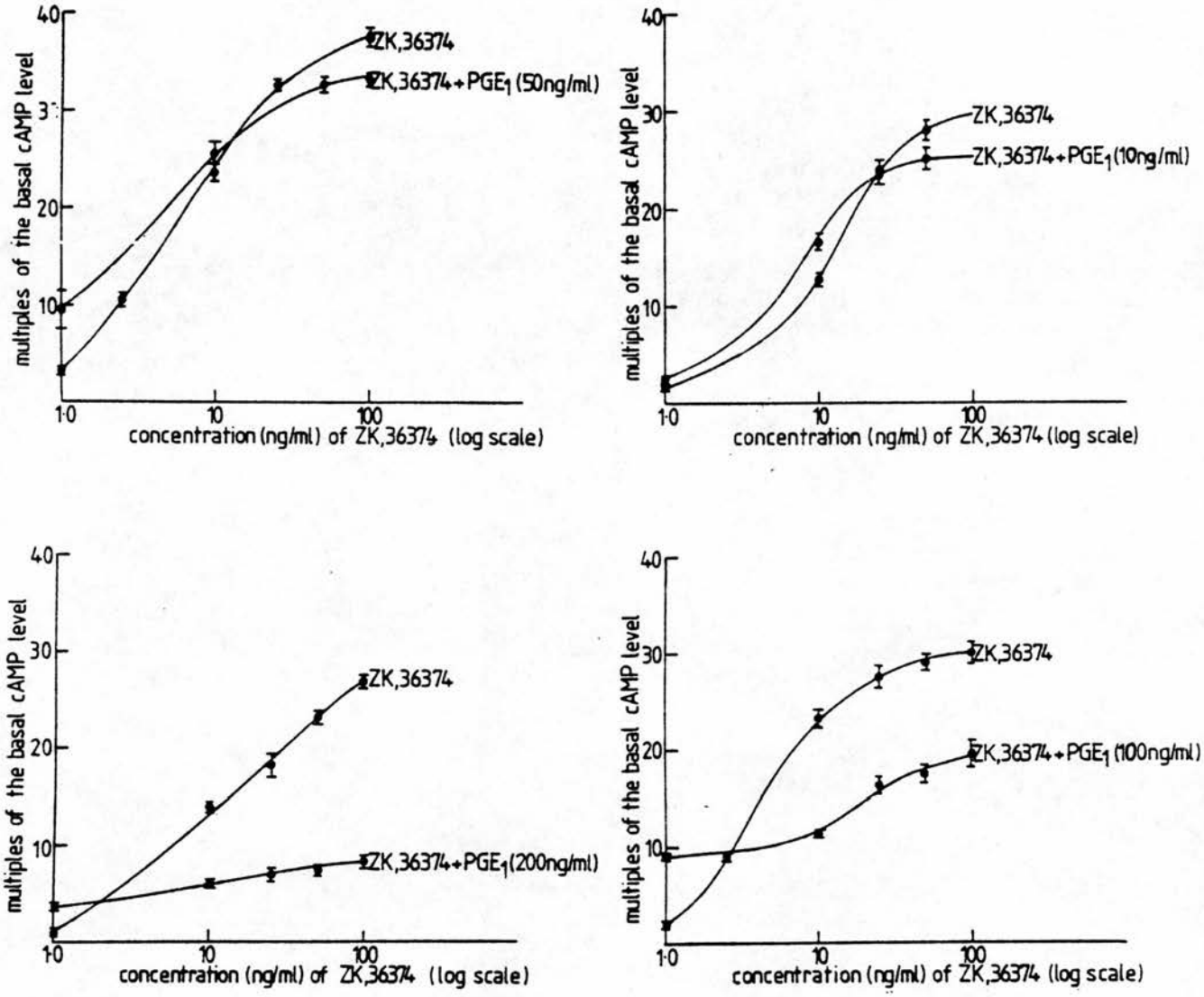
Each result is the mean and standard error of 18 observations, 2 donors.

FIGURE 2.9: Comparison of the effect of PGE_1 (1µg/ml) with PGD_2 (1 µg/ml) on the dose-response curve for ZK 36374.



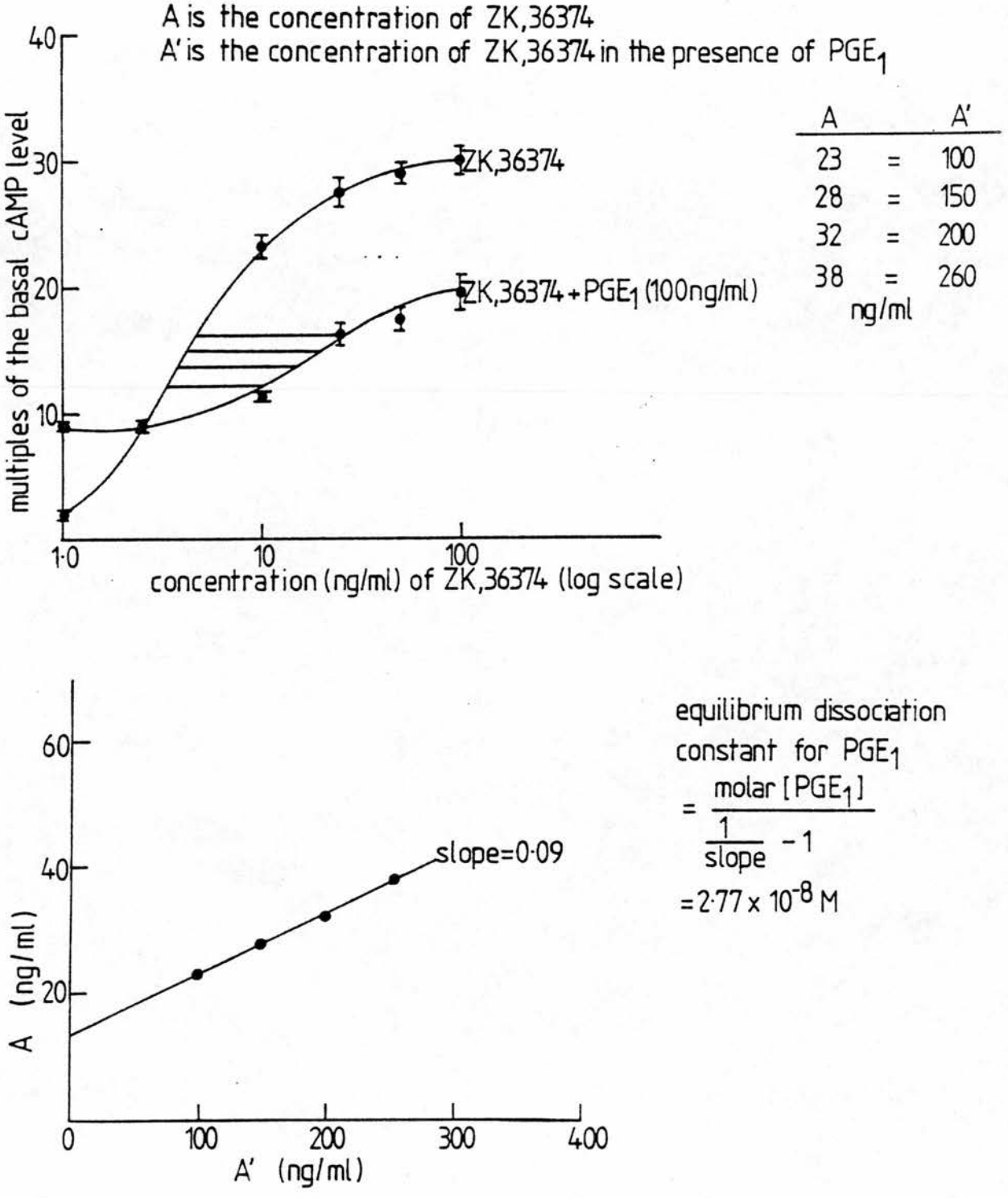
Each result is the mean and standard error of 18 observations, 2 donors.

FIGURE 2.10: The dose-response curve for PGD_2 in the presence of PGE_1 (1 µg/ml)



Each result is the mean of 9 observations, 1 donor.

FIGURE 2.11: The effect of various concentrations of PGE_1 on the dose-response curve for ZK 36374.



The method used to determine the affinity constant of PGE_1 assumes that PGE_1 is a partial agonist, acting on the PGI_2 platelet receptor.

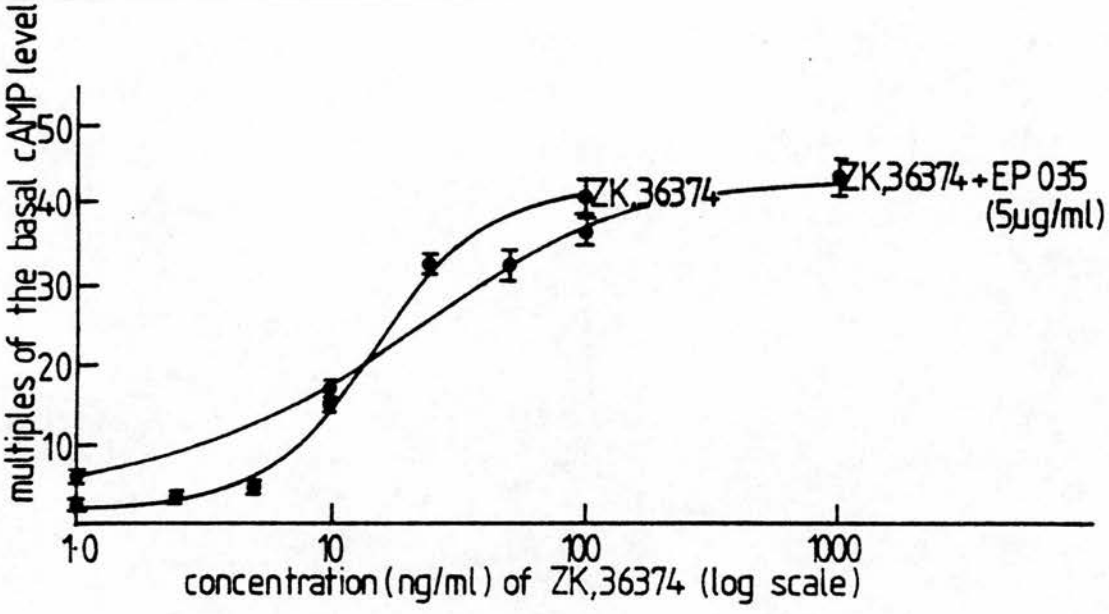
Each result is the mean and standard error of 9 observations, 1 donor.

FIGURE 2.12: Determination of the affinity constant of PGE_1 for the PGI_2 receptor of human platelets.

10 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml PGE_1 (Fig. 2.11). It was hoped to find a concentration of PGE_1 which would produce a shift in the dose-response curve which was parallel over a reasonable portion of the curve so that the affinity constant of PGE_1 for the PGI_2 receptor could be estimated. None of these curves is ideal for this purpose, the dose-response curve in the presence of 100 ng/ml PGE_1 being the most suitable. These data give a value of 2.77×10^{-8} M (Fig. 2.12) as the equilibrium dissociation constant (K_D) of PGE_1. Values of 3×10^{-9} and 6.6×10^{-9} for K_D have been reported from binding studies (211,217).

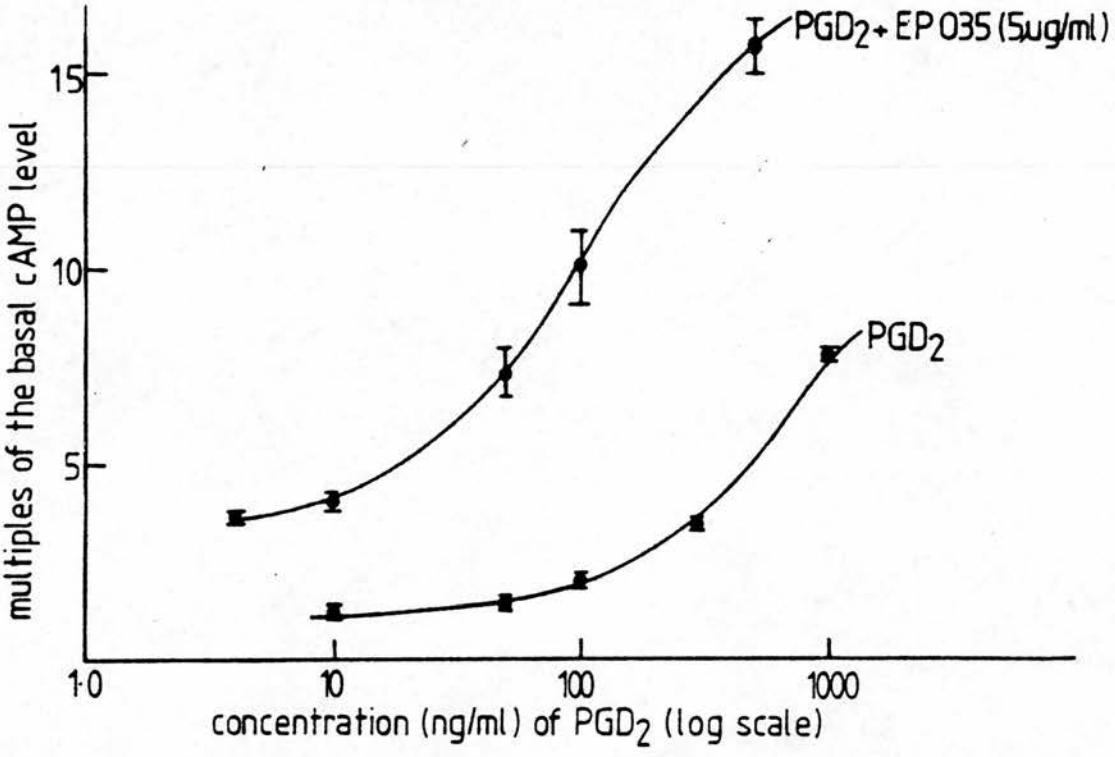
To investigate the possibility that EPO35 also acts on the PGI_2 receptor, the dose-response curve for ZK36374 was determined in the presence and absence of 5 µg/ml EP035 (Fig. 2.13). Although a shift to the right was not observed, the expected additive effect was inhibited. In contrast, the dose-response curve for PGD_2 was additive in the presence of 5 µg/ml EPO35 (Fig. 2.14). This suggests that EPO35 also acts as a partical agonist on the prostacyclin receptor, but has a lower affinity for the receptor than PGE_1.

However, it has been reported that the effect of PGE_1 to inhibit the response of PGI_2 is not due to a partial agonist action but is due to desensitisation of the prostacyclin adenyl cyclase receptor (212). The increase in cAMP in platelets exposed to PGE_1 has a characteristic time course, reaching a peak in 20-30 seconds and declining thereafter. This is typical of agonist-specific desensitisation which is characterised by a rapid synthesis of cAMP followed by a decrease in the level of cAMP, resulting from either a decrease in the rate of cAMP synthesis or an increase in its rate of degradation. Subsequent stimulation by the agonist, or by a different agonist active at the same receptor, are



Each result is the mean and standard error of 18 observations, 2 donors.

FIGURE 2.13: The dose-response curve for ZK 36374 in the presence of EP035 (5 µg/ml).



Each result is the mean and standard error of 18 observations, 2 donors.

FIGURE 2.14: The dose-response curve for PGD_2 in the presence of EP035 (5 µg/ml).

ineffective. Agonist-specific desensitisation has been studied in various adenyl cyclase systems and both mechanisms of reducing cAMP stimulation are thought to involve a time-consuming, protein-synthesising step (214,215,216). The mechanism operating in platelets is not known but is unlikely to involve increased degradation of cAMP as agonist-specific desensitisation is still evident in the presence of high concentrations of a phosphodiesterase inhibitor (212).

It is not known to what extent the depression of the ZK36374 response observed within 30 seconds of stimulation can be attributed to desensitisation by PGE_1. It is certainly possible that a protein could be synthesised within 30 seconds to switch off cAMP synthesis. Since the increase in cAMP induced by EP035 does not show a time course similar to that induced by PGE_1, it is less likely that this partial agonism could be attributed to agonist-specific desensitisation. Indeed, the increase in cAMP induced by EP035 increases with time, at least up to 120 seconds (Fig. 2.15). Moreover, PGI_2 is reported to increase cAMP rapidly for the first 60 seconds, followed by a slow increase which is maintained for 60 minutes (even in the absence of a phosphodiesterase inhibitor) and does not show agonist-specific desensitisation (212).

Since complete inhibition of aggregation can be achieved with very low concentrations of ZK36374 (1 ng/ml), PGD_2 (10 ng/ml) and PGE_1 (20 ng/ml), only small increases in cAMP must be required to produce this inhibition. Indeed, PGE_2, which is a weak inhibitor of platelet aggregation (217), can only raise basal levels of cAMP by a maximum of 1.30 ± 0.07 (Table 2.6). In agreement with these results, it is reported that PGI_2 can cause a marked shift in the aggregation induced by AA when basal levels are raised 1.38 times, and abolish aggregation when raised 1.96 times (153).

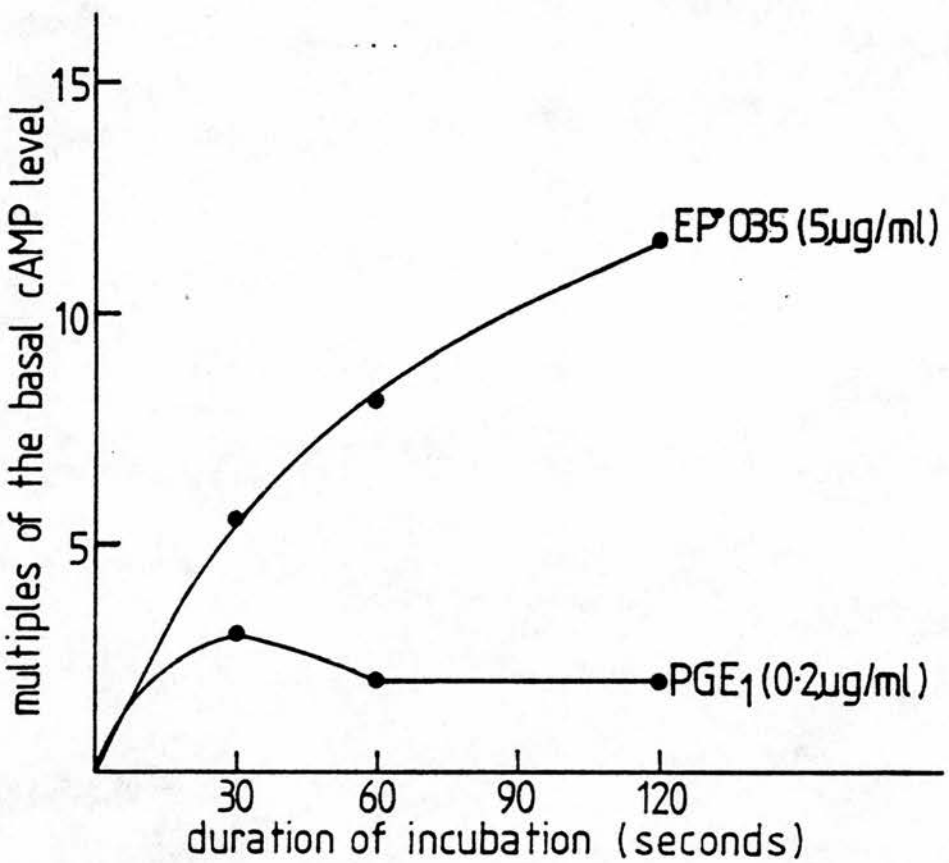


FIGURE 2.15: Time course of the action of EP035 to increase platelet cAMP levels.

TABLE 2.6: Effect of PGE_2 on cAMP levels of human platelets

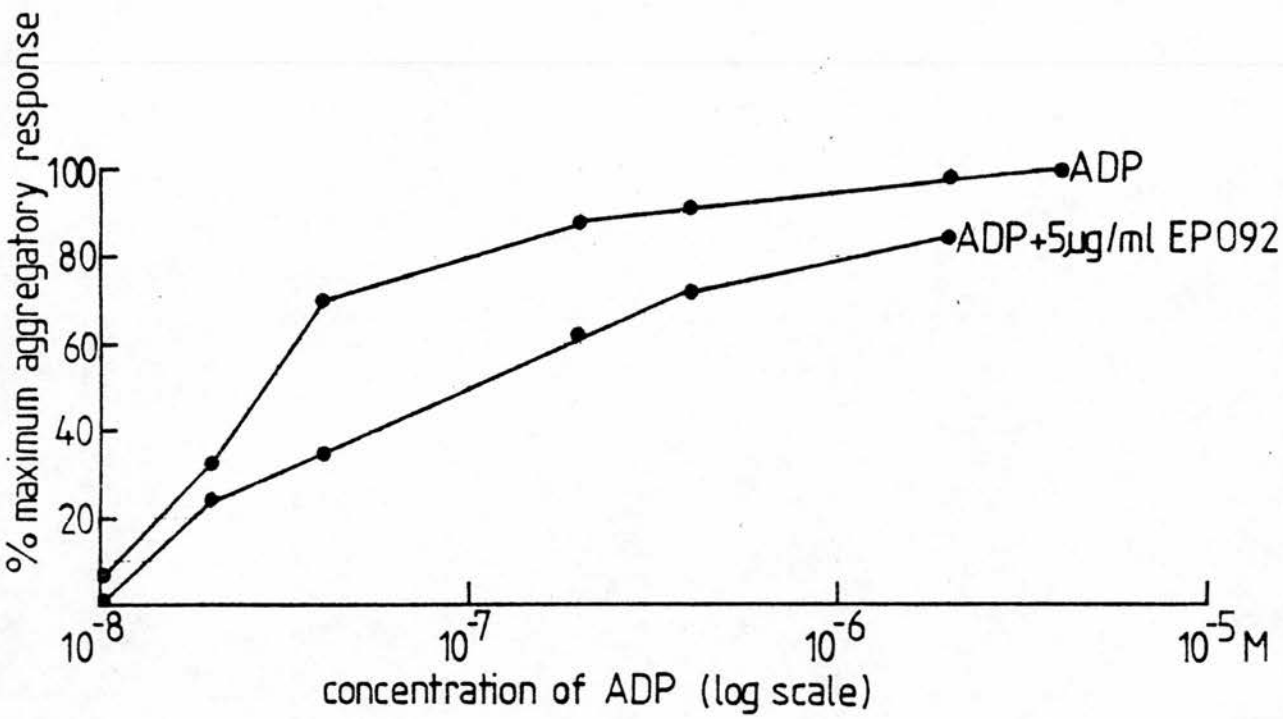
Concentration µg/ml	Multiples of the basal cAMP level induced by PGE_2
0.01	1.00 ± 0.06
0.05	1.07 ± 0.09
0.10	1.15 ± 0.08
0.50	1.20 ± 0.09
1.00	1.25 ± 0.06
5.00	1.28 ± 0.05
10.00	1.30 ± 0.07

Mean and standard error of 18 observations, 3 donors.

Although EP035 was the only analogue to cause a marked increase in cAMP levels (Table 2.3), it is possible that the very slight increase of basal levels induced by EP092 is sufficient to contribute to its inhibitory effect. Indeed with high concentrations of EP092, aggregation induced by ADP can be inhibited (Fig. 2.16). The effect of EP035 to raise cAMP levels enables this analogue to inhibit aggregation induced by ADP or thrombin in the same concentrations as it inhibits AA and 11,9-epoxymethano PGH_2 (Fig. 2.17).

DISCUSSION

The effect of EP035 to increase platelet cAMP levels appears to result from stimulation of platelet adenyl cyclase. This increase in cAMP does not show a time course characteristic of PGE_1 where there is a rapid peak followed by a fall in cAMP levels. Both PGD_2 (212,218) and PGE_1 show this time course of response and exhibit the phenomenon of agonist-specific desensitisation, where subsequent challenge with the agonist is ineffective. Since the increase in cAMP induced by PGE_1



This concentration of EP092 is more than ten times higher than that required to inhibit aggregation induced by 11,9-epoxymethano PGH_2. Each result is the mean of 4 observations.

FIGURE 2.16: Inhibition of ADP-induced aggregation by EP092 (5 µg/ml).

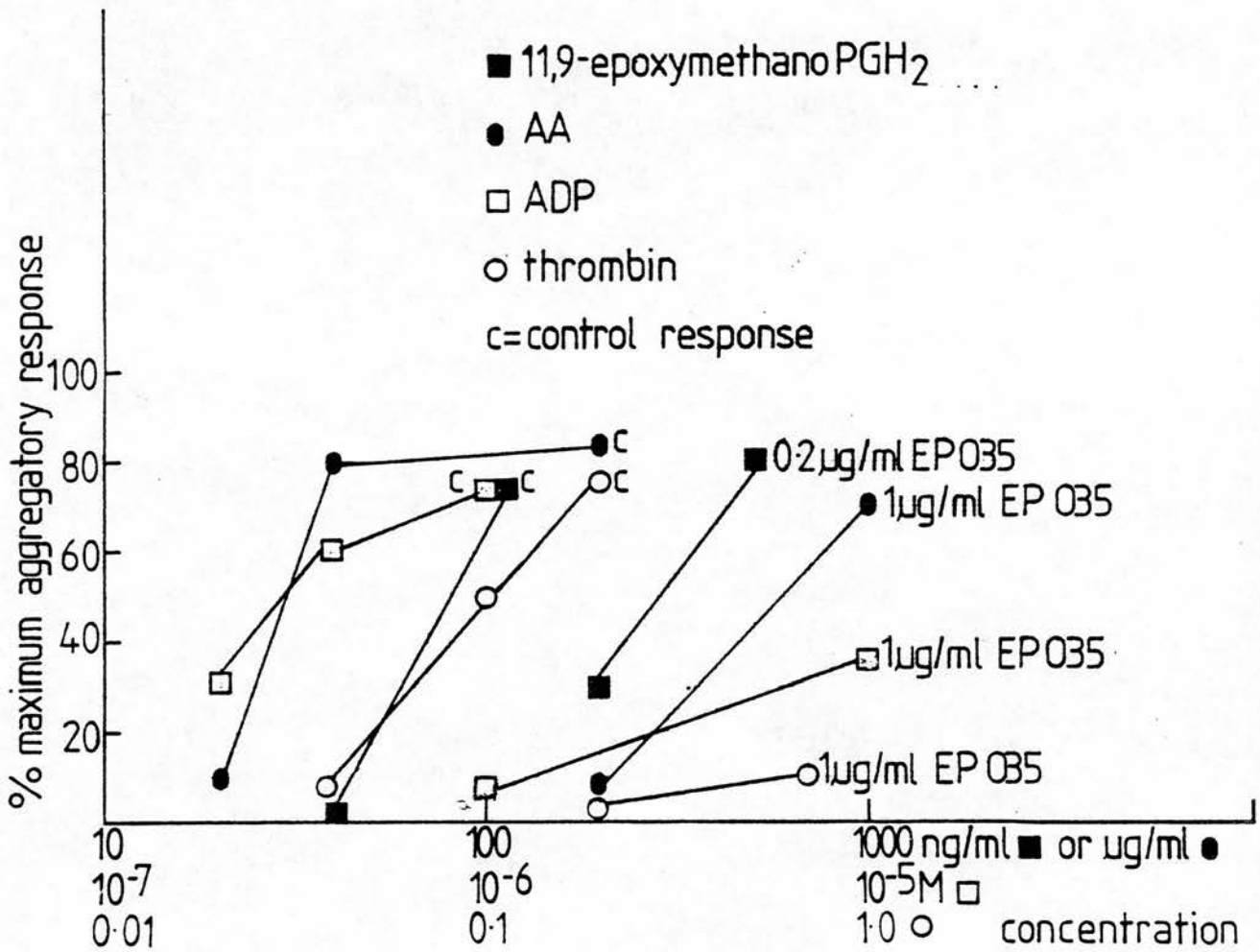


FIGURE 2.17: EP035 inhibition of aggregation induced by AA, 11,9-epoxymethano PGH_2, ADP and thrombin.

reverses after 20-30 seconds, it suggests that synthesis of the protein suggested to inhibit adenyl cyclase, can occur within this short period of time. However, stimulation of adenyl cyclase by EP035 shows a time course more similar to PGI_2, as the cAMP levels are still rising 120 seconds after stimulation. This suggests that EP035 does not switch on synthesis of the inhibitory protein, at least within 120 seconds of stimulation. The ability of EP035 (5 µg/ml) to suppress the dose-response curve for stimulation of cAMP by ZK36374, when incubated simultaneously for 30 seconds, is therefore best explained by a partial agonist action of EP035. Since EP035 has an additive effect with PGD_2, the effect on ZK36374 cannot be attributed to a non-specific action of EP035, an adenyl cyclase.

Similarly, PGE_1 shows an additive effect with PGD_2 but depresses the dose-response curve for ZK36374. It is possible that agonist-specific desensitisation is responsible for, or contributes to, the partial agonist action of PGE_1. Agonist-specific desensitisation has been characterised for various adenyl cyclase systems (but not platelets) and the fall in cAMP levels is thought to arise from either increased degradation of cAMP, or decreased synthesis of cAMP, both mechanisms involving a time-consuming, protein-synthesising step (214,215,216). Since this phenomenon is evident in platelets in the presence of a phosphodiesterase inhibitor, it is unlikely that increased degradation of cAMP is responsible (212). The reason why only some agonists show agonist-specific desensitisation is not known. A possible explanation is that synthesis of the putative inhibitory protein is switched on only when a large number of receptors are occupied. Since PGI_2 is exceptionally active at very low concentrations (1-10 ng/ml) it will only occupy a fraction of the receptors available. However, neither PGE_1 nor PGD_2

can elicit a maximum increase in cAMP levels which suggests that even when all the receptors are occupied, the efficacy of these compounds is still too low to achieve a maximum response. (Since the effect of PGE_1 and PGD_2 to increase cAMP levels is measured within 30 seconds of stimulation it is unlikely that a maximal increase in cAMP is masked by their ability to desensitise their own response.) The ability of EP035 to increase cAMP is evident with higher concentrations of this compound (1-10 µg/ml) than required with PGE_1 or PGD_2 (0.02-1.0 µg/ml). EP035 is particularly insoluble so it is impossible to get sufficiently high concentrations of EP035 into solution to see if it exhibits agonist-specific desensitisation when it occupies a larger number of receptors.

PGE_1 can completely flatten the dose-response curve for ZK36374 in concentrations from 0.2-1.0 µg/ml, but even with concentrations as low as 10 ng/ml, PGE_1 inhibits the expected additive effect of PGE_1 and ZK36374, and suppresses the maximum response. EP035 does not flatten the dose-response curve for ZK36374, but in a concentration of 5 µg/ml the additive effect is severely reduced and the maximum response depressed. It is suggested that both PGE_1 and EP035 act on the prostacyclin receptor of human platelets and are partial agonists. The ability of PGE_1 to induce agonist-specific desensitisation may contribute to the partial agonist effects seen here.

This effect of EP035 reduces its usefulness as a pharmacological tool because its action as a TXA_2 receptor antagonist is no longer specific. But it opens up an interesting concept of drugs with both TXA_2 antagonist and prostacyclin-like activity. There has been considerable interest in the development of thromboxane synthetase inhibitors since the demonstration that the vessel wall can synthesise prostacyclin from PG endoperoxides released by the platelets (219,220).

When platelets are pretreated with the thromboxane synthetase inhibitor imidazole (221) or 9,11-iminoepoxyprosta-5,13-dienoic acid (222) endoperoxides are made available for the vessel wall. In fact in the presence of a thromboxane synthetase inhibitor, AA added to whole blood *in vitro* leads to the formation of 6-oxo $PGF_{1\alpha}$ suggesting that prostacyclin has been synthesised by some cell other than platelets (223). Thromboxane synthetase inhibitors are thought to be potentially useful anti-thrombotic agents since they not only protect against the noxious effects of TXA_2 but may result in a redirection of synthesis of platelet PG endoperoxides to prostacyclin. However, *in vivo*, the vessel wall could be damaged or partially obstructed by an atheromatous plaque so that the prostacyclin synthetase enzyme would not be available to utilise the platelet endoperoxides. Under these conditions EP035, which itself has antagonist activity against TXA_2 as well as prostacyclin-like activity, would be more useful.

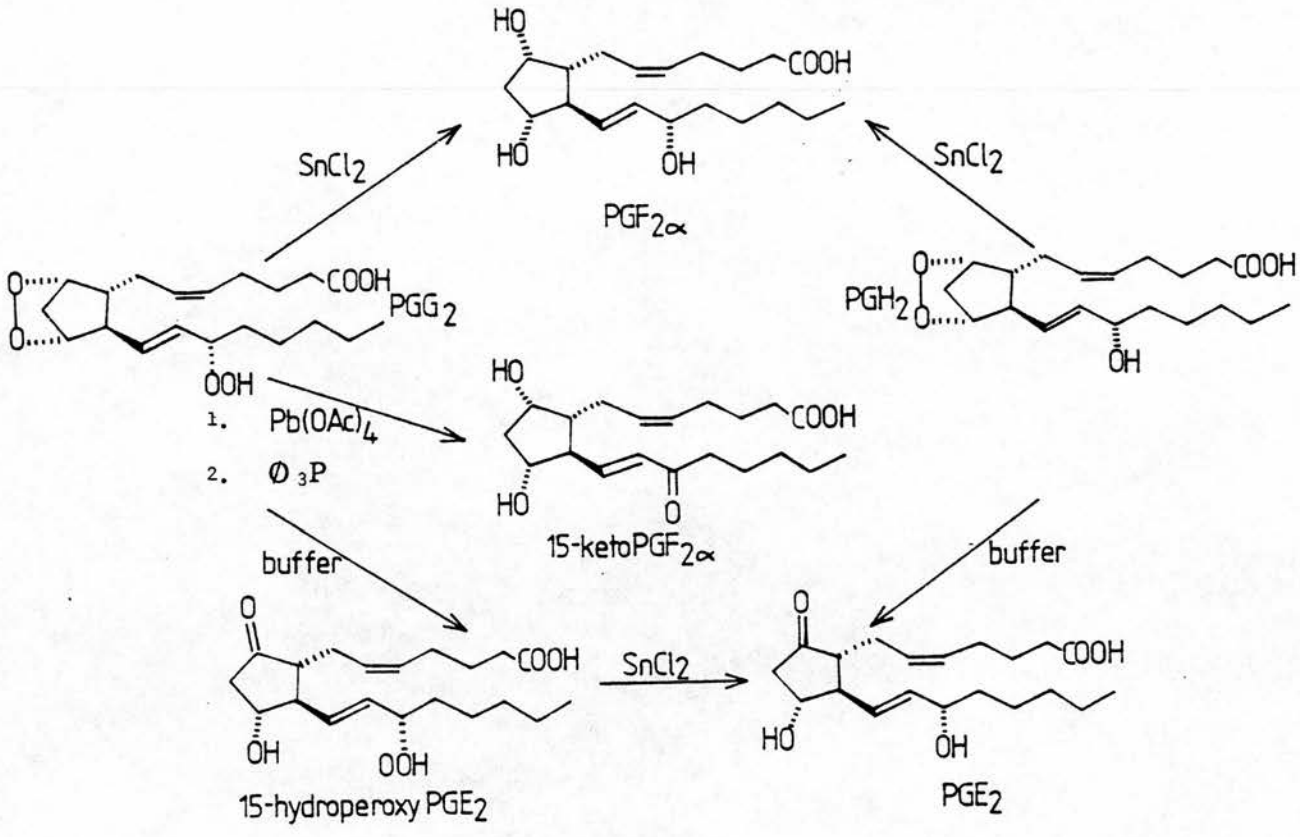
CHAPTER III

Metabolism of AA to TXB_2 by Human Platelets

INTRODUCTION

The existence of endoperoxide intermediates in prostaglandin biosynthesis was postulated after experiments with mixtures of $^{16}O_2$ and $^{18}O_2$ gas which demonstrated that when dihomo-γ-linoleic acid was incubated with sheep vesicular gland microsomes, the oxygen atoms at C-9 and C-11 of PGE_1 were derived from the same molecule of O_2 (224,225,226). However, it was not until 1973 that two such intermediates, PGG_2 and PGH_2 were isolated after short incubations of AA with these microsomes (145). PGH_2 was the more polar compound, and its structure was established by treatment with stannous chloride or triphenyl phosphine, resulting in its conversion to $PGF_{2\alpha}$. Dehydration of PGG_2 with lead tetraacetate, followed by reduction with triphenyl phosphine, resulted in the formation of 15-keto-$PGF_{2\alpha}$, indicating that a hydroperoxy group was present at C-15 (Fig. 3.1). Both compounds are unstable in aqueous solution ($t_{\frac{1}{2}}$ = 4-5 minutes at 37°, pH 7.4) and were shown to induce platelet aggregation, platelet release reaction and smooth muscle contraction.

The PG endoperoxides are rapidly transformed by most tissues into other prostaglandins (PGE_2, D_2, $F_{2\alpha}$ or I_2) which usually have biological activity distinct from the endoperoxides themselves. Platelets, however, metabolise the AA-derived endoperoxides predominantly to non-prostanoid derivatives - malanaldehyde (MDA), HHT and TXB_2 (65,72,74). TXA_2 was later detected, as a short-lived intermediate in the conversion of PGH_2 to TXB_2, by trapping experiments where methanol, ethanol or sodium azide acted as nucleophilic agents (65) (Fig. 3.2). TXA_2 was considerably more active as an inducer of platelet aggregation and release than the parent endoperoxides.



$SnCl_2$ = stannous chloride

$Pb(OAc)_4$ = lead tetra acetate

FIGURE 3.1: Determination of the structure of the PG endoperoxides PGG_2 and PGH_2

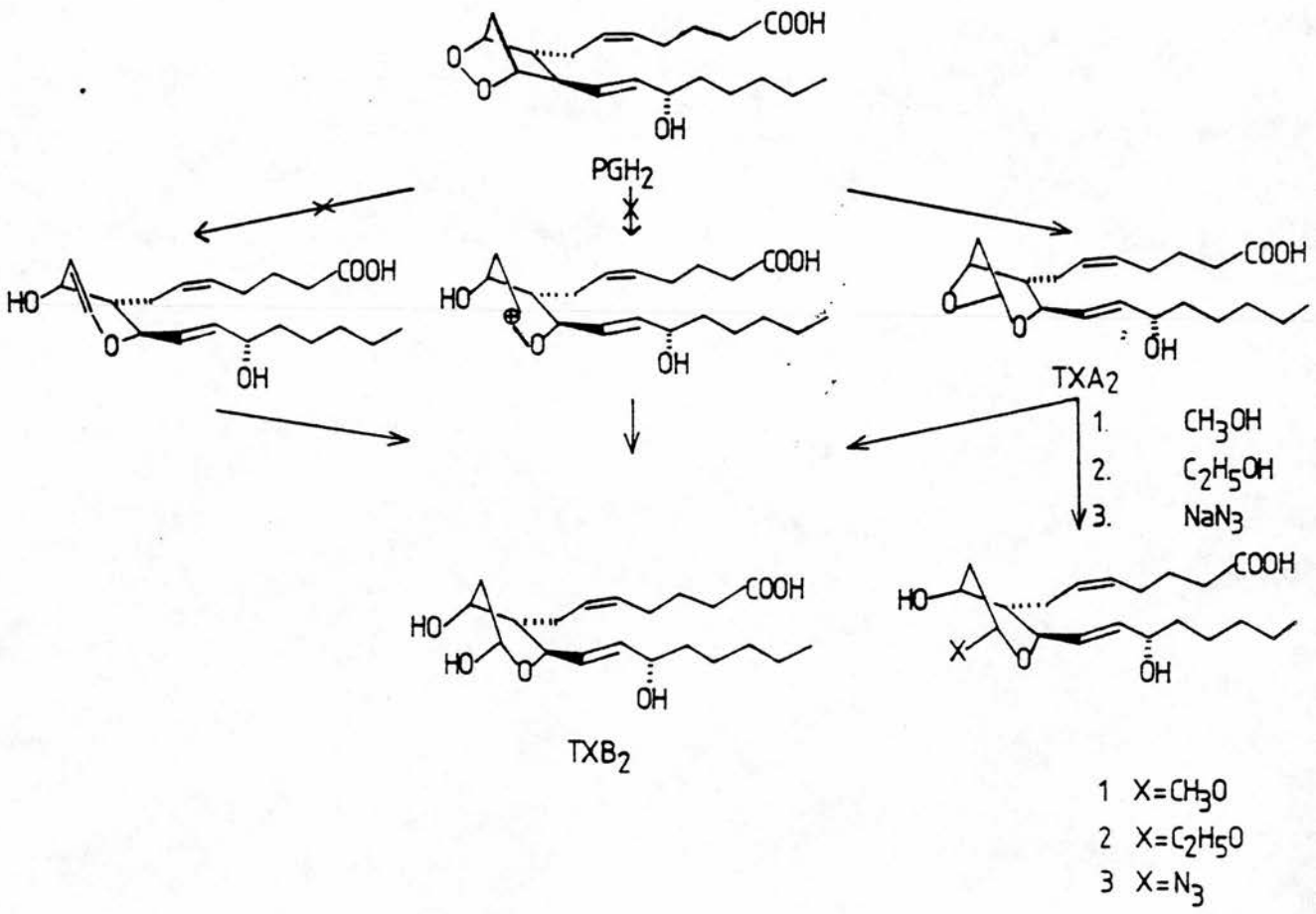


FIGURE 3.2: Determination of the structure of TXA_2

The discovery of PG endoperoxides and TXA_2 as intermediates responsible for AA induced aggregation of human platelets resolved a previous dilemma that aspirin-like drugs, known to inhibit PG biosynthesis from AA (75,76,77), could inhibit the second wave of aggregation yet neither $PGF_{2\alpha}$ nor PGE_2 (the previously known AA-derived prostaglandins formed by platelets in small amounts) showed significant proaggregatory activity. Furthermore, previously uncharacterised biological principles, labile aggregation stimulating substance (LASS) and rabbit aorta contracting substance (RCS) were found to be biologically indistinguishable from PGH_2 and TXA_2 (with residual PGG_2/H_2) respectively (227,228,229). The two-step synthesis of thromboxanes from arachidonic acid is catalysed by a microsomal enzyme complex. This complex sediments between 12000 and 100000 g and consists mainly of the dense tubular fraction of platelets (111,112, 113). This microsomal complex can be fractionated by DEAE cellulose chromatography after treatment with Triton X-100 (112,230) to separate the PG endoperoxide synthetase (cyclo-oxygenase) from the thromboxane synthetase. Studies of the kinetics of both enzymes suggest that PG endoperoxide formation is the rate-limiting step in the biosynthesis of TXA_2 from AA (231).

Stoichiometrically, TXA_2 can be regarded as a rearrangement of PGH_2 and so thromboxane synthetase is often called an isomerase. However, kinetic analysis suggests that this is not the case (232) and a dismutase reaction has been proposed, in which one molecule of both TXA_2 and HHT (12L-hydroxy-5,8,10-heptadecatrienoic acid) are produced simultaneously from two molecules of PGH_2. This may explain earlier observations that TXB_2 and HHT are produced in approximately equal amounts from AA (233,234) and that inhibition of TXB_2 formation also inhibits the production of HHT (235,236).

Isolated enzyme preparations of thromboxane synthetase have been used extensively in the search for specific enzyme inhibitors. Determination of TXA_2 itself is hampered by its short half-life but can be achieved by biological assay using tissues which are highly sensitive to TXA_2 such as rabbit aorta, dog saphenous vein, in cascade with tissues which are sensitive to other prostaglandins such as rat stomach strip. Quantitation can be attempted by measuring the response induced by TXA_2 in units equivalent to responses induced by a stable thromboxane mimic. Validation that the response is due to TXA_2 relies on the decay of the biological activity with a half-life of 30-40 seconds at 37°C and inhibition of biosynthesis by both cyclo-oxygenase and thromboxane synthetase inhibitors (237).

It has become increasingly common to measure TXA_2 formation by assaying its stable metabolite TXB_2. This has a disadvantage in that other metabolic pathways of TXA_2 may exist, although there is no evidence for this at present. Several different types of assays for TXB_2 have been developed including radio-immuno assay (RIA) and gas liquid chromatography-mass spectrometry (GLC-MS) (238,239,240, 74). Another approach has been to trap TXA_2 with methanol (72) and assay the stable product mono-o-methyl TXB_2 (241).

Several inhibitors of thromboxane synthetase have been reported (Table 3.1). In general, these fall into two categories: imidazole derivatives and PG endoperoxide analogues. As can be seen from Table 3.1 overleaf, a number of compounds which are structurally similar to the PG endoperoxides inhibit thromboxane synthetase. This is not always a specific action of the compound since 9,11-azo PGH_2 and 9,11-epoxymethano PGH_2 are thromboxane mimics (253), 9,11-azo-prosta 5,13-dienoic acid is a partial agonist (254) and 9,11-azo-13 oxa-15-

TABLE 3.1: IC_{50} values (μM) quoted for inhibitors of microsomal preparations of thromboxane synthetase

Compound	Ref. No.	IC_{50} quoted for microsomal enzyme (μM)
Imidazole	242	1500
1-methyl imidazole	242	1000
Butyl imidazole	243	9.8
Butyl imidazole	139	40
Hexyl imidazole	243	7.6
Actyl imidazole	243	53
Carboxyl hexyl imidazole	243	0.5
Carboxy heptylimidazole	243	0.14
Carboxy actyl imidazole	243	0.22
UK-37,248-01 (4-(2-[IH-imidazol-1-Yl]ethoxybenzoic acid)	244	0.003
Dipyridamole	245	0.39
L-8027 (2-isopropyl-3-nicotinyl indole)	246	10
N-0164 (sodium p-benzyl-4-[1-oxo-2-(4-chloro-benzyl)-3-phenyl propyl)phenyl phosphate	247	24
Burimamide	248	25
9,11-azo ; 13-oxa-15 hydroxy prostanoic acid	249	1
9,11-azo PGH_2	250	2
9,11-epoxymethano PGH_2	250	20
9,11-iminoepoxy prosta-5,13-dienoic acid	251	
9,11-azo prosta-5,13-dienoic acid	252	
12-L-hydroperoxy 5,8,10,14-eicoscitetra-enoic acid	112	
Pinane TXA_2	135	50

hydroxyprostanoic acid and pinane TXA_2 are antagonists (249,135) at the PGH_2/TXA_2 receptor.

Many of these compounds appear considerably more active as thromboxane synthetase inhibitors when isolated enzyme preparations are used than they are when whole platelets are used. This has led to the development of much more water-soluble compounds. However, even with UK-37,248-01, relatively high concentrations (about $10^{-5}M$) are required for a 50% reduction in thromboxane synthesis, in contrast to the potent activity, $IC_{50} = 3 \times 10^{-9}M$, in a microsomal system.

In this section the effect of analogues EP035, EP037, EP043, EP045 and EP092 on the production of TXB_2 from AA added exogenously to platelets was investigated. This test cannot discriminate between an inhibitory action on cyclo-oxygenase and one on thromboxane synthetase, as is possible when using microsomal enzyme preparations. But by using whole platelets it is possible to determine whether an inhibitory action on TXA_2 production will contribute to the inhibition of aggregation induced by arachidonic acid, in the concentrations in which these compounds are effective in platelet-rich plasma. The extraction of TXB_2 from platelet-rich plasma and the development of a quantitative assay for the measurement of TXB_2 by GLC-MS are described. EP035 and EP043 both inhibit TXB_2 synthesis from AA. Their effects are compared with other inhibitors and the possibility that EP035 mediates this effect through a rise in cAMP levels is discussed.

MATERIALS AND METHODS

Principles of gas liquid chromatography (GLC)-mass spectrometry (MS)

Chromatography is used as a way of separating particular entities. With GLC, where a gas is the mobile phase and a non-volatile liquid the stationary phase, components can be separated on the basis of two criteria: temperature (and thus volatility) and polarity. The temperature of the column will determine the retention time of the compounds within the column, the more volatile components being eluted first. The packing of the column is non-polar so that the more polar components are not retained; the non-polar components partition into the packing.

Samples must be derivatised to make them both volatile and thermally stable. Here the carboxylic acid group of TXB_2 is made less polar by conversion to the methylester, the hydroxyl groups are protected by forming the trimethylsilyl (TMS) ether and the aldehyde group is stabilised by conversion to an oxime. Helium is used as the carrier gas to introduce the effluent from the GLC column into the mass spectrometer.

Ionisation of the sample molecules occurs by bombardment with electrons generated from a tungsten filament and accelerated by a trap electrode. Once ionised the fragments are accelerated into a mass analyser where the fragments pass through a magnetic field, the ion path bending according to the molecular weight.

Qualitative analysis

To obtain a complete mass spectrum, the magnetic field is continually raised while a constant number of sample molecules are entering the ion source. This causes ions of increasing mass to focus on the electron multiplier where the abundance of each ion is measured. The mass spectrum is similar to a finger print as it is characteristic of the original molecule.

Quantitative analysis (multiple ion detection, MID)

To assay samples quantitatively using the GLC-MS it is usual to monitor major ions formed when the molecule splits. The instrument must be focussed for a given time on each ion monitored. This is done by repeatedly switching the accelerating voltage to bring each ion in turn into focus on the detector. Identification of a compound relies on the detection of one or more representative ions appearing at the appropriate retention times. The more ions that are monitored, the more conclusive is the identification, but unfortunately at the same time the sensitivity of detection is reduced as the time allowed for each ion to be focussed on the detector is much shorter. The total number monitored must therefore be kept to a minimum.

The response of the sample is measured relative to an internal standard. This is frequently a deuterated analogue which when added in excess has the advantage of acting as a carrier to reduce extraction losses and adsorptive loss of the sample in gas chromatography. Octadeuterated TXB_2 has been used previously for this purpose (65). Since the sample and standard are chemically identical they are not distinguishable until final detection by the mass spectrometer where the deuterated standard will peak with the same retention time as the sample but will be several mass units heavier. The ratio of protium peak height to deuterium peak height is determined over the range of a standard curve and the concentration of TXB_2 in the sample is determined from the ratio of protium to deuterium in the sample. Unfortunately, no deuterated TXB_2 was available so a suitable analogue of TXB_2 was used instead to act as an internal standard.

The GLC-MS data reported in this section were obtained by using a Pye Unicam 204 gas chromatograph coupled to a VG micromass 70-70F

This mass spectrum gives the GC-MS background peaks. The prominent ions contain silicon and are derived from the septum, column stationery phase and separator.

FIGURE 3.3: Mass spectrum of column bleed peaks.

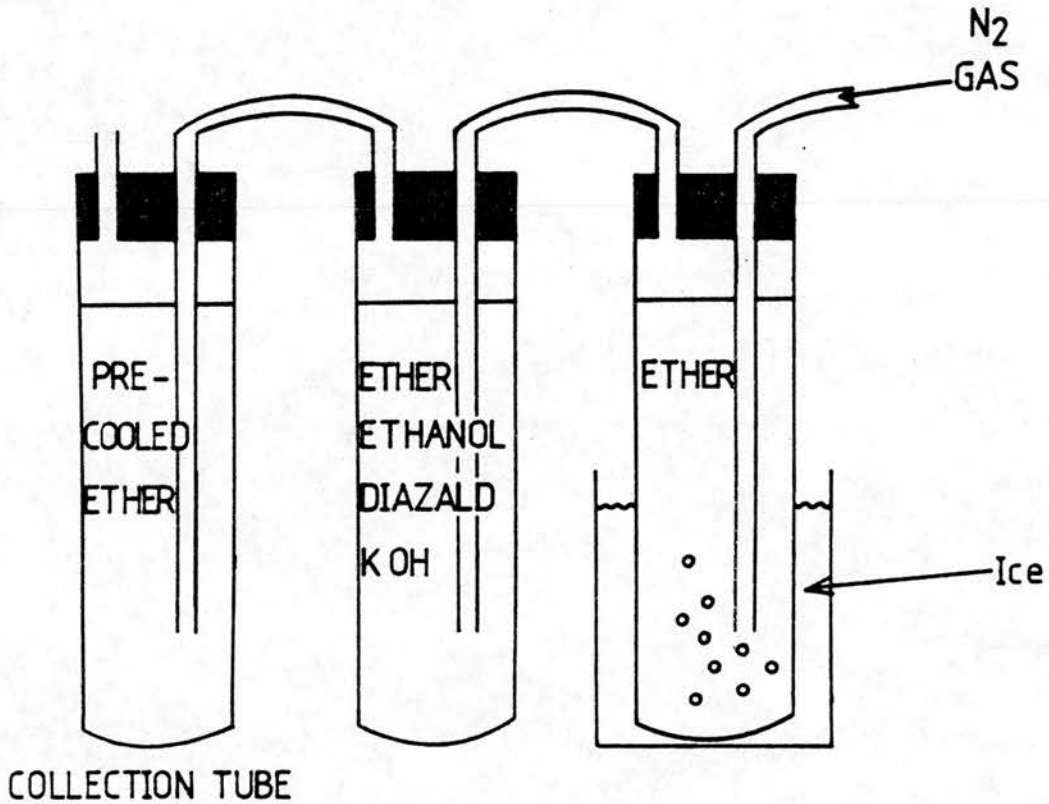


FIGURE 3.4: Preparation of diazomethane.

mass spectrometer. The gas chromatograph details are as follows. The gas chromatograph was equipped with a spiral column (either 1.5 m x 4 mm or 3 m x 4 mm) packed with 3% OVI in 100-120 mesh Supelcopart (Supelco, Inc.). The temperature of the column was fixed between 250-270°, the separator temperature was 250°, the ion source temperature 250°, the basic accelerating voltage 4 kV and the electron energy 22 or 70 eV. Helium was used as the carrier gas with a flow rate of 30 ml/min.

Derivatisation of TXB_2 is required to improve its chromatographic behaviour and to provide a suitable ion in the mass spectrum for detection and assay by GLC-MS. The ion must not coincide with a column bleed peak (Fig. 3.3), but should be reasonably close in mass to minimise the loss in sensitivity which occurs as the voltage is switched from the column bleed peak to the ion, throughout the assay. The column bleed peak is used as a reference to provide accurate switching between monitored peaks.

Preparation of TXB_2, Me, BuOX, TMS

10 µg TXB_2 standard (UpJohn Co., Kalamazoo) in ethylacetate were dispensed into an eppendorf tube and blown dry. Diazomethane was generated from diazald (N-methyl-N-nitroso-p-toluene-sulphonamide, Aldrich), ethanol and potassium hydroxide (KOH), and displaced with ether vapour saturated nitrogen into a tube containing cold diethyl ether (Fig. 3.4). The standard was taken up in a few drops of methanol (Rathburn Chemicals), 0.3-0.4 ml diazomethane added, and left for 5 minutes before being blown dry on a heating block and dessicated. Butoxyamine hydrochloride, 3-5 drops (5 mg/ml in pyridine) were then added. This was heated at 60°C for 90 minutes as a fast oximation procedure. The pyridine was blown off using a heating block and

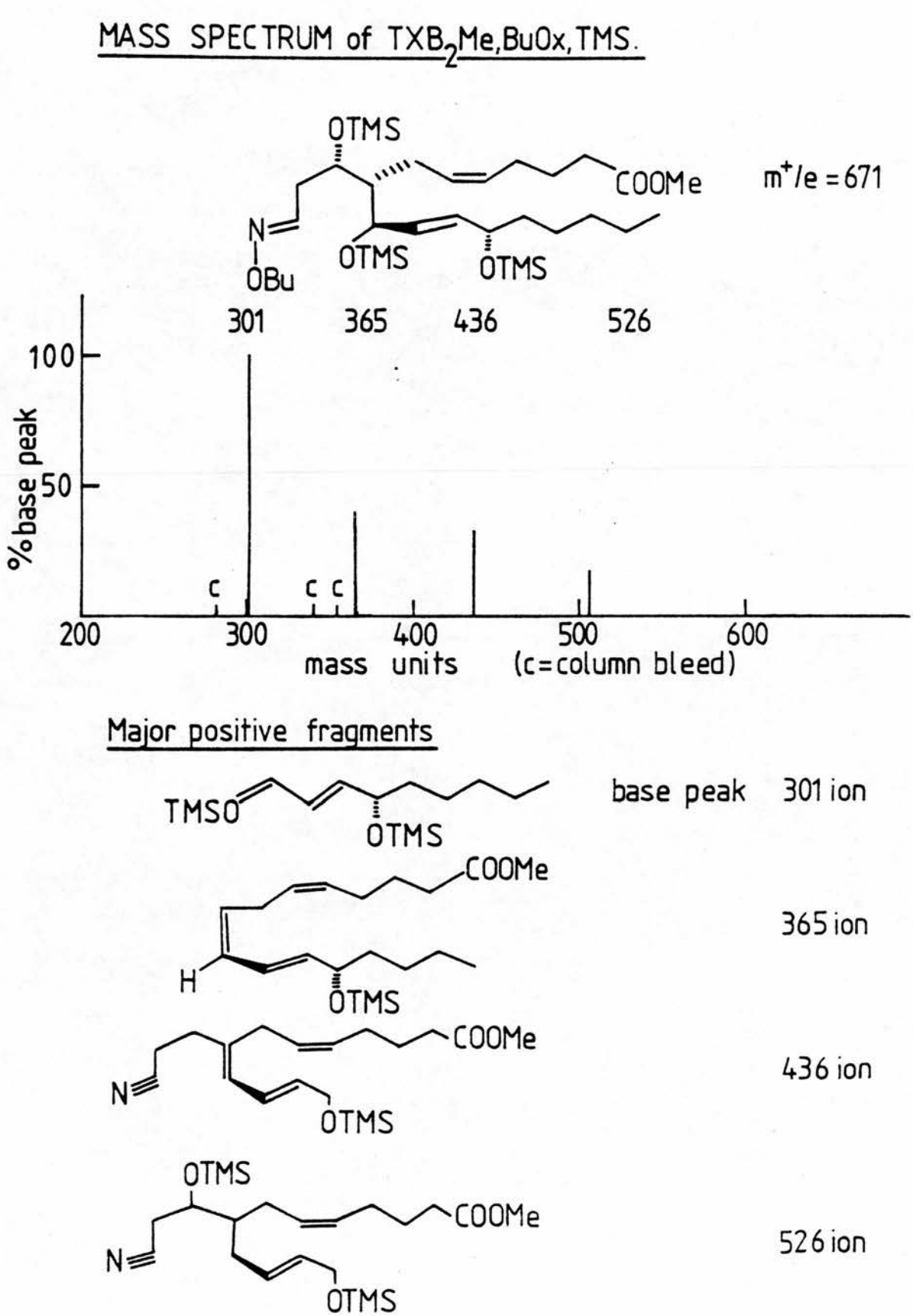


FIGURE 3.5: Fragmentation of TXB_2Me, BuOx, TMS by GC-MS.

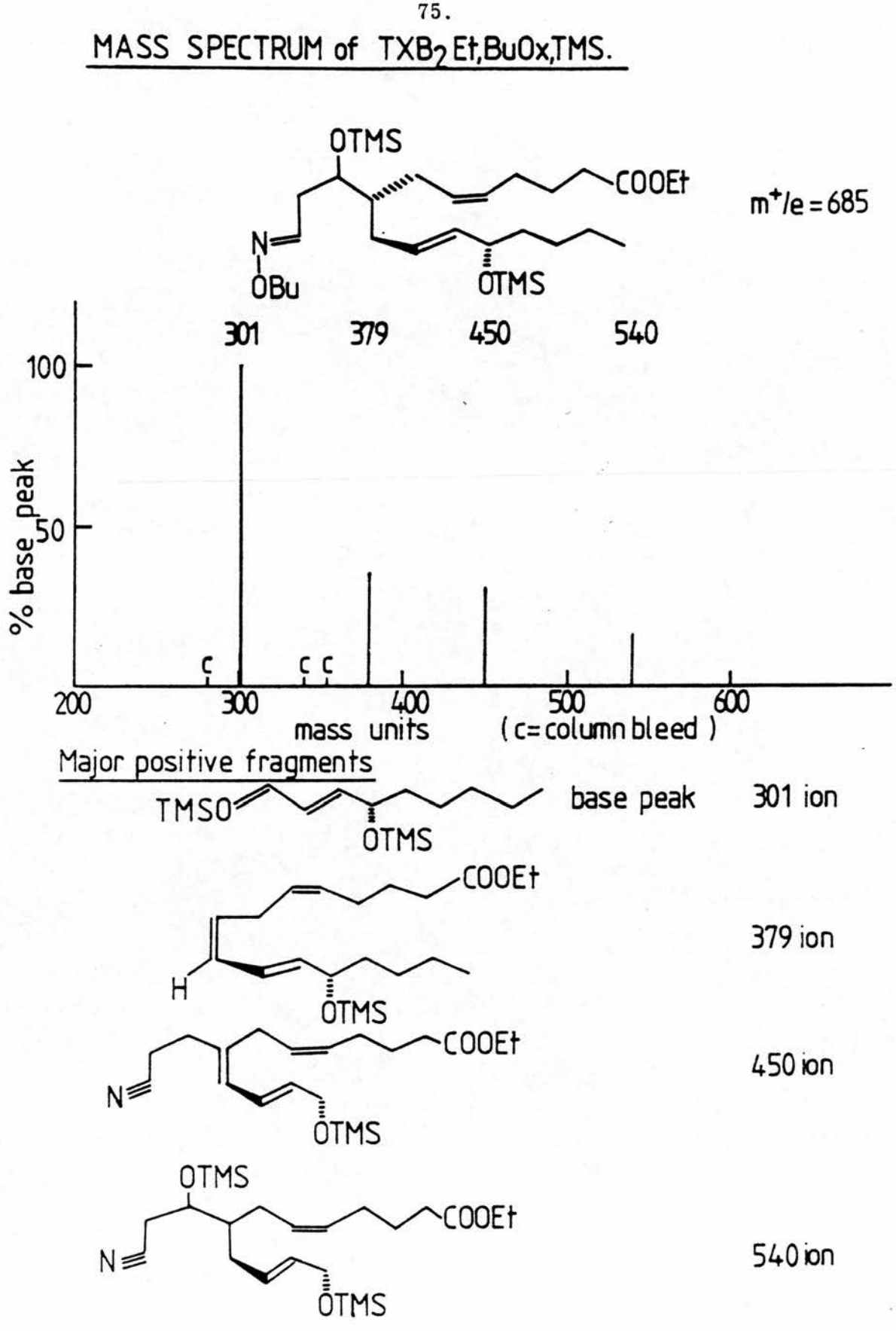


FIGURE 3.6: Fragmentation of TXB_2Et, BuOx, TMS by GC-MS.

the standard vacuum dessicated. The standard was taken up in 20 µl BSTFA (sigma-$N_1$0-bis(trimethylsilyl)trifluoroacetamide) and incubated at 60°C for 15 minutes. 5 µl of this TXB_2 methyl ester, butyloxime, TMS ether was injected in BSTFA into the GLC-MS and run total ion to obtain a mass spectrum of the ions formed as the molecule splits (Fig. 3.5).

The m/e = 301 ion is the base peak, i.e. the most abundant ion formed. Although it is usual to use the deuterated form of the molecule to act as both internal standard and carrier, since no deuterated TXB_2 was available, the ethyl ester of TXB_2 was tested as a suitable internal standard. TXB_2 ethyl ester, butyloxime, TMS ether was prepared as for the methyl ester, replacing diazomethane with diazoethane and increasing the reaction period to 15 minutes. Diazoethane was prepared from N-nitrosoethylurethane (fluka) and ethanolic KOH (saturated), rather than aqueous KOH, was used. The mass spectrum of TXB_2 ethylester, butyloxime, TMS ether is given in Fig. 3.6.

The m/e = 301 ion is the base peak for both methyl and ethyl esters and appears to have the same relative abundance in both. The two spectra can be distinguished by the ions which retain the α chain as these ions will be 14 mass units heavier for the ethyl ester. Since the major ions are formed in similar amounts it suggests that the different ester group has not affected the way in which the molecule fragments so that the ions are formed in the same proportions.

The m/e = 301 ion was chosen as a suitable ion to monitor since it is formed in large amounts, being the base peak, and is suitably close in mass to the 281 column bleed. Both of these factors help to increase the sensitivity of the assay. The m/e = 301 ion peaks will be separated by virtue of the longer retention time of the ethyl ester. The fact that

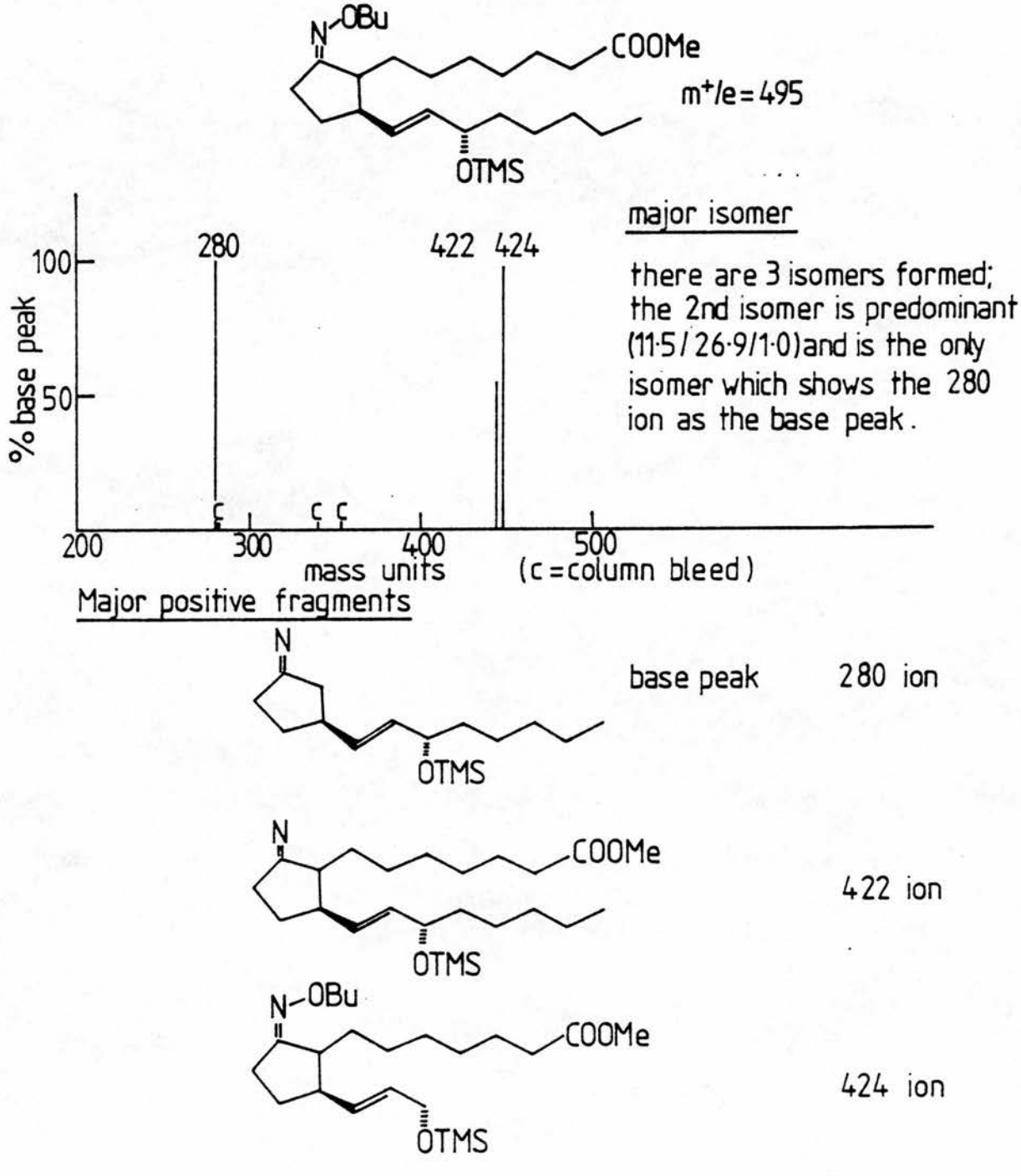


FIGURE 3.7: Fragmentation of 11 deoxy PGE_1Me, BuOx, TMS by GC-MS.

the 301 ion is not a major ion formed by the more classical prostaglandins, coupled with the characteristic retention pattern of the 301 ion, will improve the specificity of this assay. Although the ethyl ester can be used as a suitable internal standard, it has the disadvantage that it cannot act as a carrier as it is added during the final stage of derivatisation and so can only compensate for losses during the GLC-MS procedure.

A methyl ester was chosen as a second internal standard as this ensured that any problems encountered during methylation would be detected. 11-deoxy PGE_1 was selected for this purpose as the mass spectrum of 11-deoxy PGE_1 methyl ester, butyloxime, TMS ether showed the m/e = 280 ion to be the base peak (Fig. 3.7). Again this ion is close in mass to the 281 column bleed. The m/e = 280 ion could be formed by loss of the ω chain (-71) and the α chain (-144) or by the loss of the butyloxime (-73) and the α chain (-142). Since the m/e = 280 ion is also the base peak of 11-deoxy PGE_1, Me, Meox, TMS it must be achieved by loss of the oxime (here -31) and the α chain (-142).

Since it is of lower molecular weight than TXB_2, 11-deoxy PGE_1 runs with a comparatively short retention time.

MID assay

Amounts of TXB_2, 0 ng, 10 ng, 20 ng, 40 ng, 60 ng and 80 ng were dispensed into eppendorf tubes. 40 ng 11-deoxy PGE_1 were added to each tube. The tubes were methylated twice using diazomethane, as before. Once dry, 40 ng TXB_2 ethyl ester standard were added to each tube and the tubes were dessicated. The standards were oximated overnight at room temperature then taken to dryness on a heating block and dessicated. 20 µl BSTFA were added to each tube, the tubes were incubated at 60° for 15 minutes and 5 µl samples were used for injection.

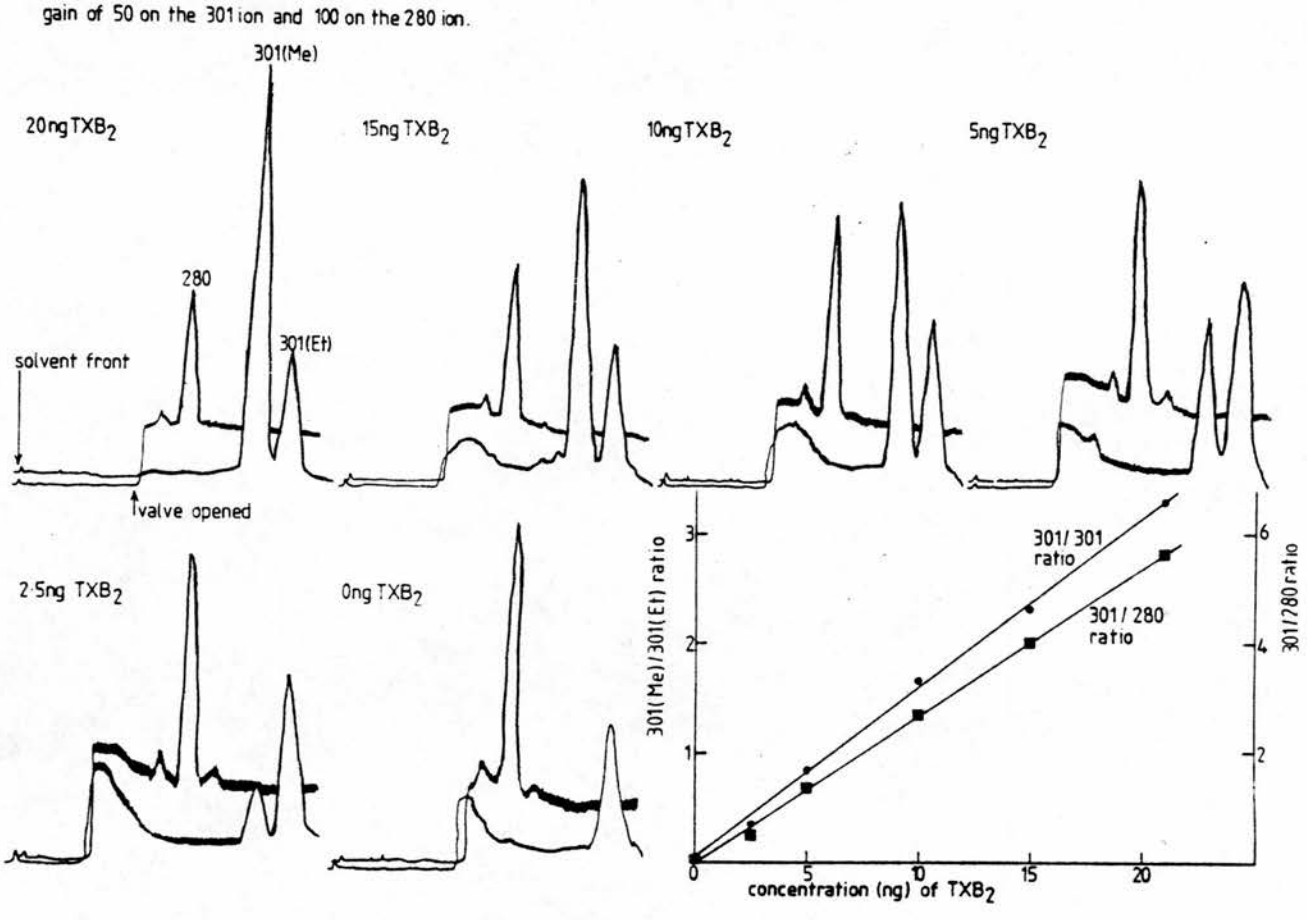


FIGURE 3.8: Typical GC-MS traces for the TXB_2 standard curve (0-20 ng).

This gives a standard curve of 0-20 ng TXB_2, and the samples were diluted to fall within this range.

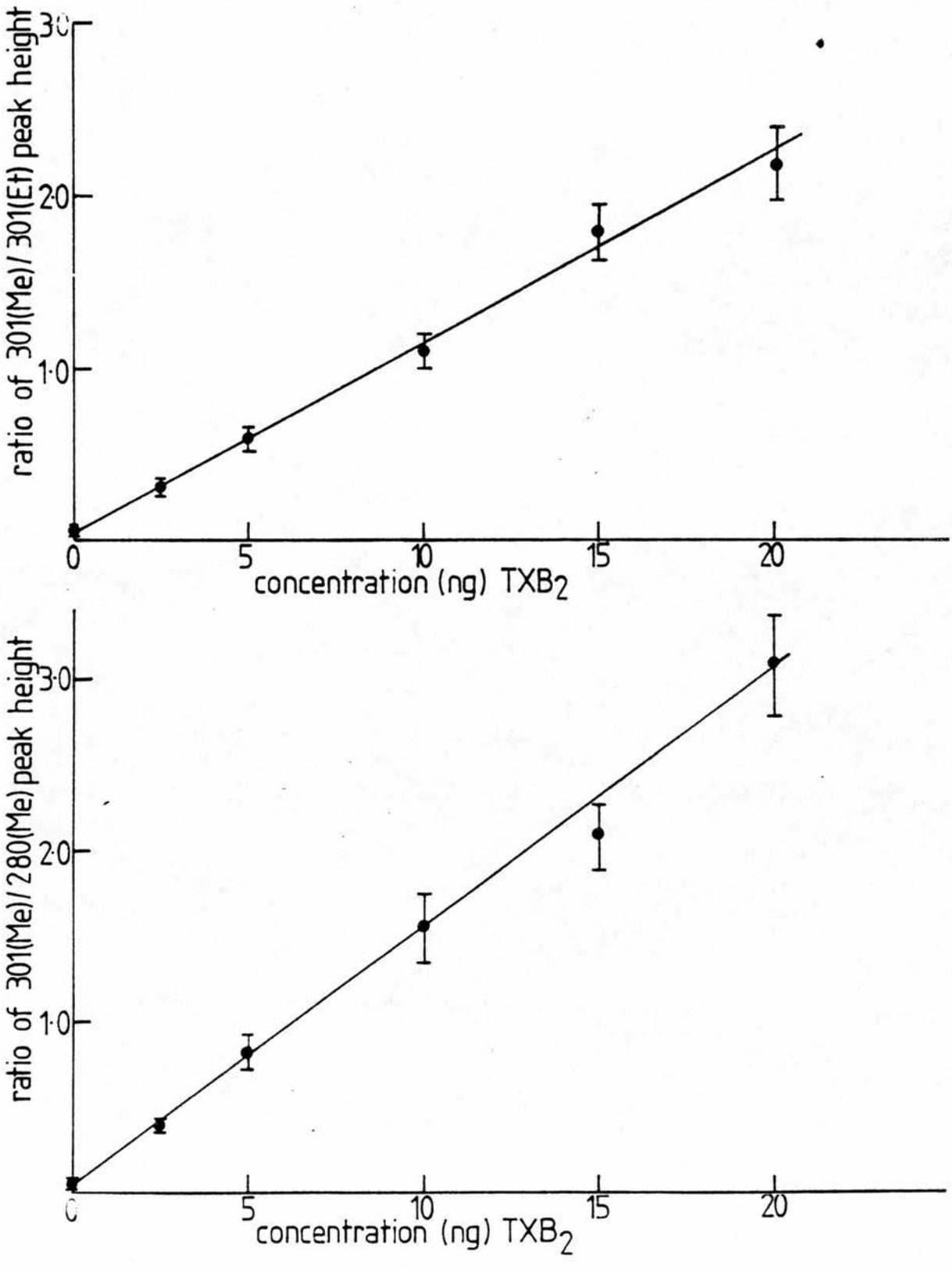
The GLC-MS was set up for multiple ion detection (MID). The program used is given in Table 3.2.

TABLE 3.2: GLC-MS program for detection of TXB_2 by MID

Channel	Ion	Exact mass (g)	MID voltage
1	280	280.20965	9.8000
2	281	281.05169	9.7706
3	301	301.2019	9.1170

The instrument was tuned into the 281 column bleed, and electronically locked on, to facilitate its use as a reference peak. The machine was focussed on each MID voltage for 0.1 seconds in turn, the MID voltage regulating the accelerating voltage. Each standard was run and the ratio of peak heights of the 301 (Me)/301 (Et) and of 301 (Me)/280 ions determined. Typical traces are shown in Fig. 3.8, along with the standard curve which these ratios produce. The mean of the estimates from the two ratios was taken as the TXB_2 content of the sample.

Fig. 3.9 and Table 3.3 show the variation of 20 standard curves, performed on different days. The variation observed was sufficiently great to make it necessary to use a standard curve at the start of each assay.



The variation is sufficient that a standard curve was always performed at the start of each assay.

FIGURE 3.9: Variation of 20 standard curves, performed on different days.

TABLE 3.3: Variation of 20 standard curves performed on different days

ng of TXB_2 injected	301 (Me)/301 (Et)	301 (Me)/280 (Me)
0	0.04 ± 0.01	0.04 ± 0.01
2.5	0.32 ± 0.04	0.37 ± 0.06
5.0	0.58 ± 0.08	0.83 ± 0.11
10.0	1.07 ± 0.13	1.54 ± 0.20
15.0	1.77 ± 0.15	2.07 ± 0.19
20.0	2.17 ± 0.21	3.07 ± 0.29

Preparation of arachidonic acid

Arachidonic acid (Sigma), grade 1, about 99% pure, was made into a stock solution of 50 mg/ml in methanol (Rathburn Chemicals). As a check of purity, samples were spotted onto a 5 cm silica gel plate and run in hexane : ether (50 : 50) containing 0.5% glacial acetic acid. The plate was removed from this solution, sprayed with phosphomolibdic acid solution and heated at 100-120°C for 15 minutes until the full colour developed. In both batches, the AA ran as one spot which was taken as being sufficiently pure. 3 ml aliquots of AA (10 mg/ml) were ampouled and stored at -40°C until use.

Extraction of TXB_2 from platelet-rich plasma

Fresh platelet-rich plasma (PRP) was prepared as before (see Chapter 2) and incubated in a waterbath at 37°C. 2.5 ml of PRP were incubated for 3 minutes at 37°C with 75 µg/ml palmitic acid, then 100 µg/ml AA was added to the PRP and allowed to act for 30 seconds before the reaction was quenched by the addition of 500 µl 2M HCL. This resulted in a pH of about 3. The sample was extracted twice with ethyl acetate (Rathburn Chemicals) and the ethyl acetate taken to dryness

using a rotary evaporator. The remaining samples were processed to this stage, each being extracted immediately after the reaction. Drugs to be tested were added at the same time as the palmitic acid, allowing 3 minutes for incubation. n-butyl imidazole (a gift from Wellcome Research Laboratory, Beckenham, Kent) was used as a standard thromboxane synthetase inhibitor.

Each sample was taken up in 2.5 ml absolute alcohol and transferred to a stoppered test tube. The pear-shaped flasks were washed out with 2.5 ml distilled water which was added to the alcohol. 5 ml of benzene (BDH Chemicals - analar quality) was added to each tube. This gives an ethanol : water : benzene (1 : 1 : 2) partition. The tubes were mixed, then spun in a bench centrifuge to ensure a clear separation between the two layers. The upper benzene layer was removed, using a pasteur pipette, and discarded. The ethanol layers were transferred back to the original pear-shaped flasks and the test tubes washed out with a further 2.5 ml ethanol. This was added to the ethanol in the pear-shaped flasks, to bring it up to 67% ethanol, and then taken to dryness using a rotary evaporator.

Each sample was taken up in 2 ml methanol and aliquots of 2-fold and 4-fold dilutions were derivatised as for the standards and assayed on the GLC-MS. The efficiency of extraction was determined by the addition of 0.125 μCi ^{3}H TXB_2 (New England Nuclear - TXB_2 [5,6,8,9, 11,12,14,15-$^{3}H(N)$] 150 ci/mmol) to the PRP before extraction and an aliquot of the sample (in methanol) was added to 10 ml PPO scintillant and counted for 4 minutes.

RESULTS

Palmitic acid (75 µg/ml) was incubated for 3 minutes with the PRP, before the addition of AA. It was hoped that the palmitic acid would saturate the plasma protein-binding sites. This is important for two reasons. Firstly, arachidonic acid binds strongly to albumin in the plasma (255,256) so that if the binding sites were saturated, more of the AA added exogenously would be available for transformation into TXA_2 by cyclo-oxygenase. Secondly, it has recently been shown that PGH_2 and TXA_2 themselves bind strongly to plasma proteins, and it is this action which serves to prolong the half-life of TXA_2 in plasma, as compared with aqueous solutions (257,258,259). The latter effect was minimised by using a short incubation time of 30 seconds (260) but would also be reduced if the binding sites were saturated with palmitic acid. This is particularly important when measuring the hydrolysis product TXB_2, as representative of TXA_2, because protein-bound TXA_2 will not be measured.

Basal levels of TXB_2 in PRP were low (2.62 ± 0.89 ng TXB_2/ml PRP, n = 10). However, when the PRP was stimulated with 100 µg/ml AA there was a surge of TXB_2 produced. The amount of TXB_2 produced within 30 seconds is dependent on the donor used, but the yield was increased when the PRP had been preincubated with palmitic acid, for example from 238.8 ± 8.2 ng TXB_2 to 406.8 ± 26.0 ng TXB_2. This effect can be partly attributed to the increase in extraction efficiency from 44.3 ± 2.90% to 66.7 ± 0.95% when preincubated with palmitic acid. When corrected for extraction efficiency, the yield of TXB_2 increases from 539.2 ± 18.4 to 601.6 ± 38.8 ng. This difference probably results from the increase in AA available for biosynthesis once the plasma proteins saturated with palmitic acid. The action of palmitic acid to increase the

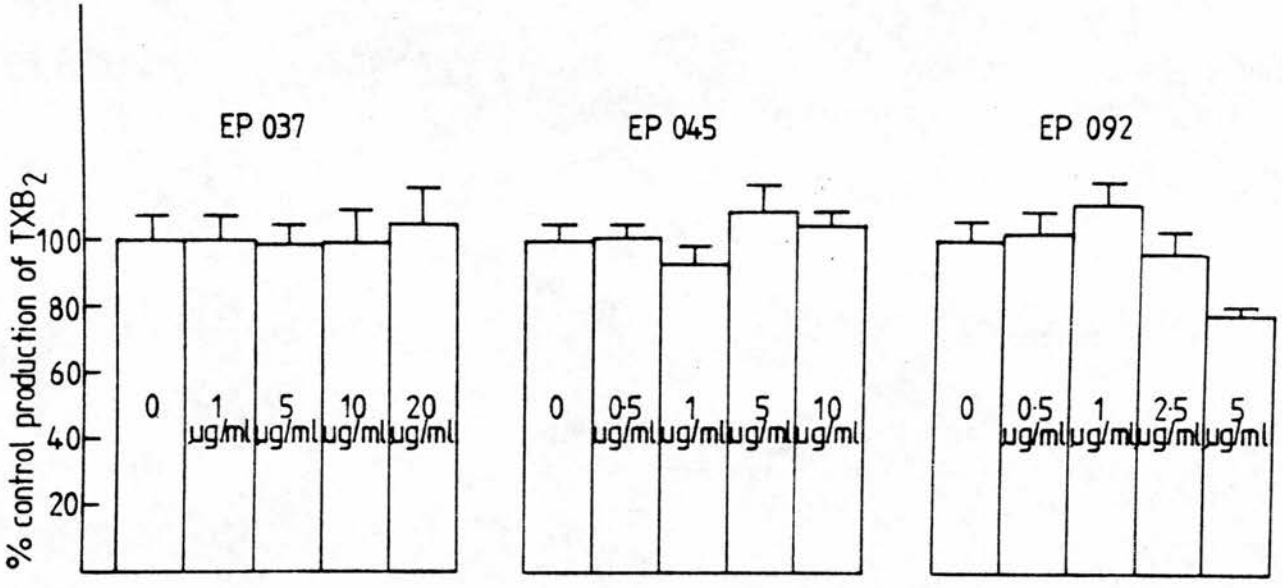
extraction efficiency is partly due to a carrier action. Palmitic acid added after the reaction, before extraction, increases the extraction efficiency from 44.3 ± 2.9% to 64.9 ± 1.9%, whereas palmitic acid present throughout the reaction only increases the efficiency slightly to 66.7 ± 0.95%.

The effect of the analogues on the control production of TXB_2 from AA is shown in Table 3.4 overleaf. EP037 and EP045 do not appear to inhibit thromboxane biosynthesis by whole platelets. EP092 may have a slight inhibitory effect in concentrations above 2.5 μg/ml (Fig. 3.10). EP043 and EP035 both inhibit thromboxane biosynthesis. Inhibition by EP043 is comparable with that induced by the thromboxane synthetase inhibitor butyl imidazole (Fig. 3.11). The possibility that the action of EP035 is related to the increase in cAMP was investigated by comparing the effect of EP035 with that of PGD_2 and ZK36374 (Fig. 3.12), both of which raise cAMP levels but are not reported to have a direct inhibitory effect on either cyclo-oxygenase or thromboxane synthetase. The effects of butyl imidazole, PGD_2 and ZK36374 on thromboxane production are given in Table 3.5.

TABLE 3.4: Effect of EP035, EP037, EP043, EP045 and EP092 on the production of TXB_2 30 seconds after the addition of 100 µg/ml AA to PRP

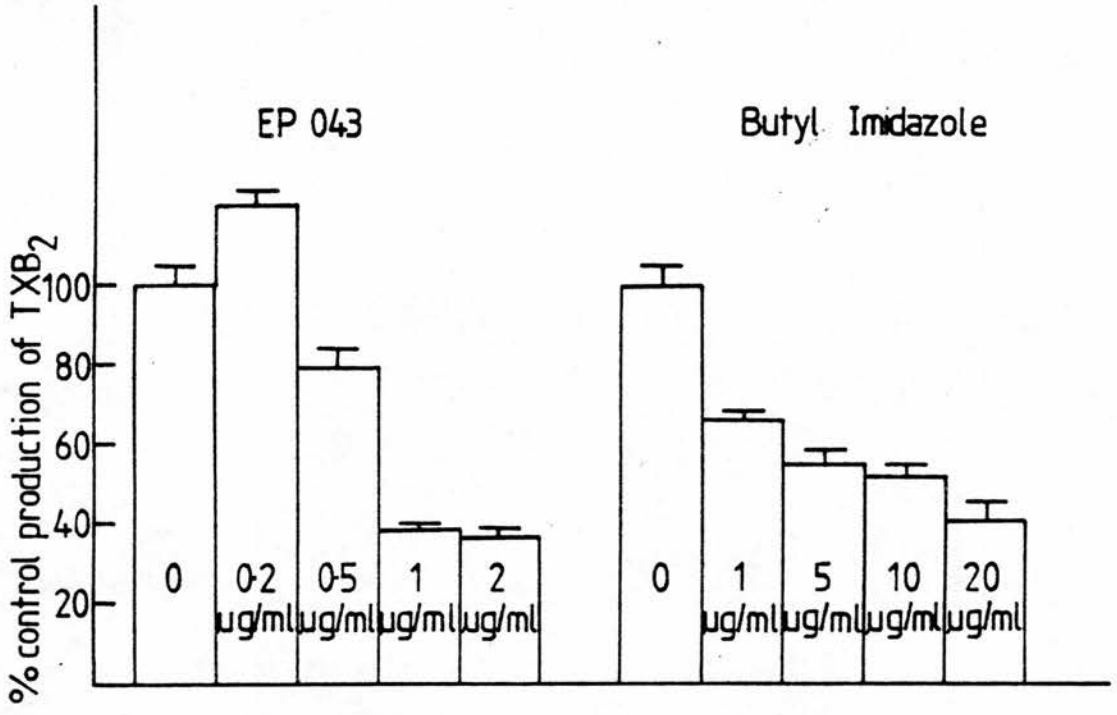
Concentration (µg/ml)	% control production of TXB_2 by: EP035	EP037	EP043	EP045	EP092
0	100.00 ± 5.50	100.00 ± 7.60	100.00 ± 5.03	100.00 ± 5.10	100.00 ± 6.30
0.1	74.75 ± 3.70				
0.2			120.30 ± 3.90		
0.5	67.23 ± 3.43		79.10 ± 4.50	101.06 ± 4.10	102.50 ± 7.20
1.0	61.70 ± 2.30	100.00 ± 7.40	38.57 ± 1.79	92.70 ± 5.60	111.31 ± 7.10
2.0			36.80 ± 2.13		
2.5					96.35 ± 6.84
5.0	48.70 ± 2.99	97.90 ± 7.50		109.15 ± 8.40	78.09 ± 3.10
10.0		99.40 ± 9.60		104.70 ± 4.10	
20.0	26.90 ± 2.60				

(Values represent the mean and standard error of 16 observations, 2 donors)



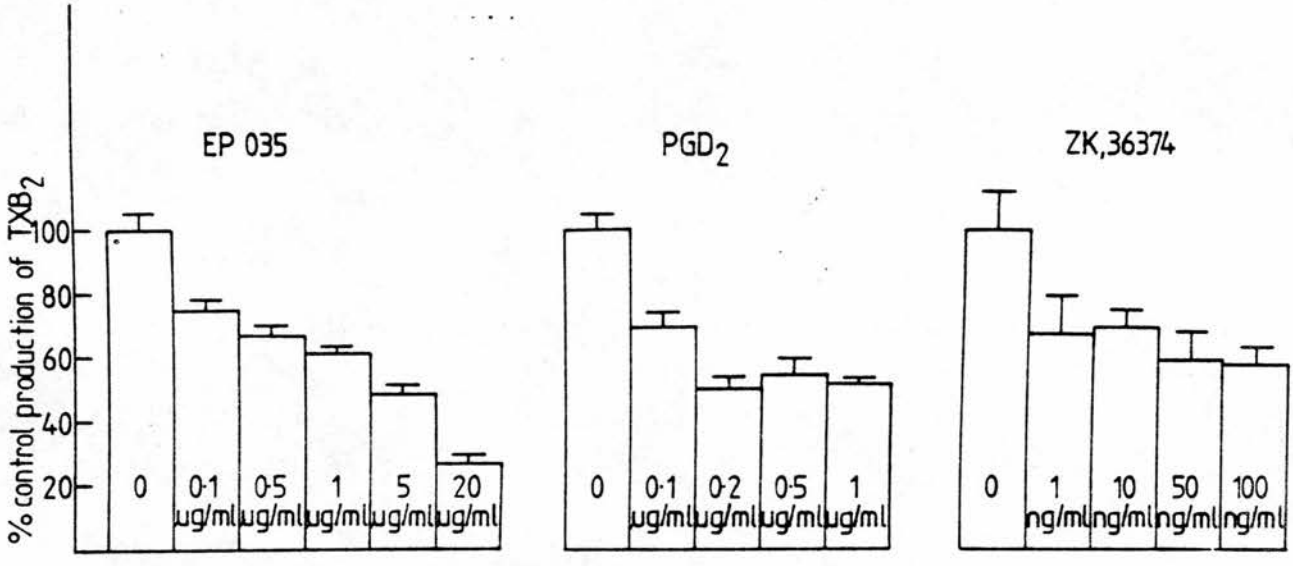
Each result is the mean and standard error of 16 observations, 2 donors.

FIGURE 3.10: The effect of EP037, EP045 and EP092 on TXB_2 production from AA added exogenously to platelets.



Each result is the mean and standard error of 16 observations, 2 donors.

FIGURE 3.11: Inhibition by EP043 and butyl imidazole of TXB_2 production from AA added exogenously to platelets.



Each result is the mean and standard error of 16 observations, 2 donors.

FIGURE 3.12: Inhibition by EP035, PGD_2 and ZK 36374 of TXB_2 production from AA added exogenously to platelets.

TABLE 3.5: Effect of butyl imidazole, PGD_2 and ZK36374 on the production of TXB_2 30 seconds after the addition of 100 µg/ml AA to PRP

Concentration	% control production of TXB_2 by: Butyl imidazole	PGD_2	ZK36374
(µg/ml)			
0	100.00 ± 5.2	100.00 ± 4.8	100.00 ± 11.7
0.1		69.40 ± 4.5	
0.2		50.40 ± 3.1	
0.5		54.70 ± 5.4	
1.0	66.20 ± 2.4	52.10 ± 1.7	
5.0	55.05 ± 3.4		
10.0	52.10 ± 3.0		
20.0	40.70 ± 4.3		
(ng/ml)			
1			67.50 ± 11.2
10			69.85 ± 5.3
50			59.52 ± 8.7
100			58.30 ± 5.6

Values represent the mean and standard error of 16 observations, 2 donors.

DISCUSSION

Whenever TXB_2 levels are measured, there is a problem as to how closely these levels reflect TXA_2 production. Since the basal levels of TXB_2 are very low, these have been neglected, so that the surge of TXB_2 measured must reflect the rapid biosynthesis of TXA_2 from AA. It is reported that the concentration of TXA_2 formed from AA reaches a maximum within 30 seconds and thereafter declines to zero by 2 minutes (232).

The concentration of TXB_2, however, continues to increase for 2 minutes, the increase after 30 seconds being concomitant with the decrease in the concentration of TXA_2.

The factor responsible for limiting the synthesis of TXA_2 is as yet unknown. Certainly it is not substrate depletion as less than 10% of the substrate is utilised (232,138). It has been suggested that cyclo-oxygenase may be deactived by free radicals (70), possibly hydroxyl radicals generated during the reduction of PGG_2 to PGH_2 (71). Similarly thromboxane synthetase may be inhibited by 12L-hydroperoxy-5,8,10,14-eicosa tetraenoic acid (HPETE), which is an intermediate in the lipoxygenase pathway (112). However, the importance of such mechanisms in platelet-rich plasma, rather than cell-free systems using microsomal enzyme fragments, is not certain.

Here, TXB_2 synthesised during the first 30 seconds of incubation with AA has been measured. A reduction in the level of TXB_2 measured has been taken to mean that the biosynthesis of TXA_2 from AA has been inhibited. It is, however, possible that such a reduction could result from interference in the rate of production of TXA_2 so that less TXB_2 was formed within 30 seconds.

The capacity of platelets from different individuals to synthesise TXB_2 from AA varied considerably, from 308.6 ng to 814.2 ng/ml PRP. This may partly result from the variation in age group and sex of the blood donors, but may also reflect differences in plasma protein binding. It is not known how seriously the protein binding of TXA_2 will interfere with this assay, even after palmitic acid saturation. Where the level of TXB_2 measured exceeds the control value, it is possible that by limiting the extent of platelet aggregation, through an antagonist action rather than an effect on TXB_2 synthesis, more TXB_2 is produced from the AA added.

The GLC-MS assay is fairly convenient for routine use but is too time consuming to handle a large number of samples at once (each sample

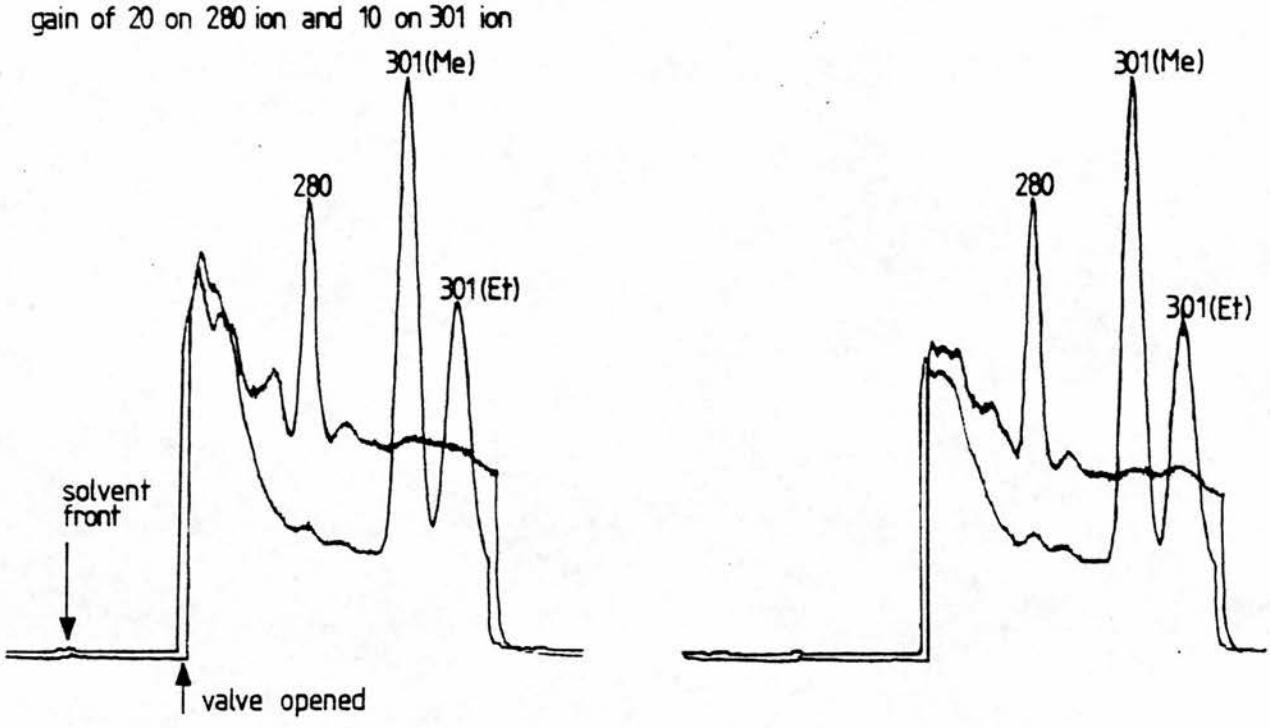
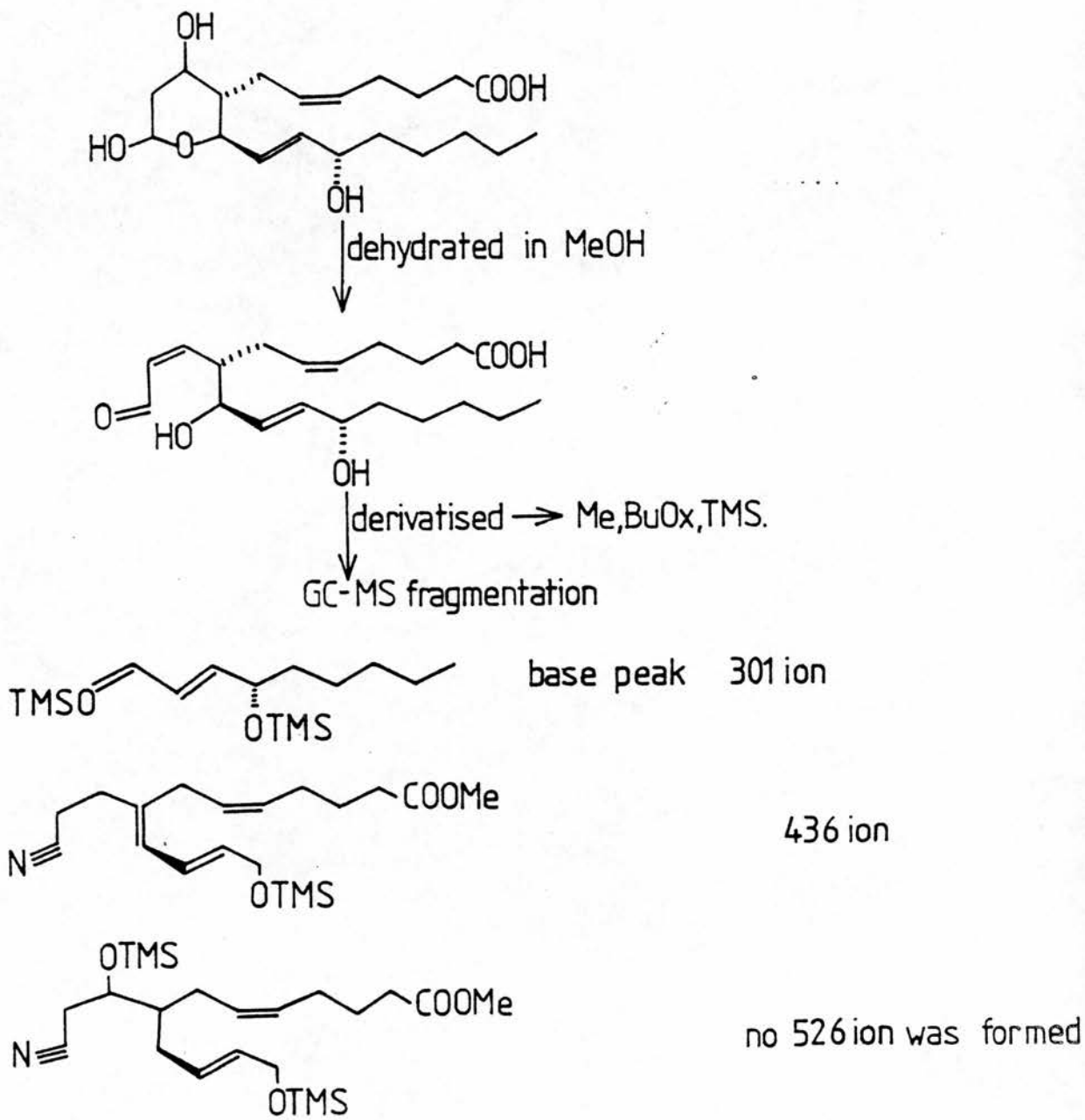


FIGURE 3.13: GC-MS traces of TXB_2 samples extracted from platelet rich plasma.

has a retention time of about 15 minutes) in the way that is possible with radioimmunoassay (RIA). However, the GLC-MS assay has the advantage that the material measured can be conclusively identified as TXB_2. Initial RIA procedures for TXB_2 suffered from fairly high cross reactivities with PGD_2, which is not only synthesised by platelets but can result from isomerisation of PGH_2 in the presence of serum albumin (261). In fact, background interference in this assay was reasonably low. Fig. 3.13 allows comparison of sample traces with standards (Fig. 3.8). TXB_2 standard was stored in ethyl acetate because when stored in methanol for long periods of time, some of the TXB_2 dehydrated (Fig. 3.14). This dehydrated TXB_2 produced an interfering 301 peak which ran with a slightly shorter retention time than that of TXB_2 itself. Dehydration did not occur when the TXB_2 was stored in ethyl acetate.

The analogues EP037 and EP045 did not inhibit the biosynthesis of TXA_2 by platelets. EP092 has a slight inhibitory action in concentrations above 2.5 µg/ml. This effect of EP092 is evident with much higher concentrations than required for this antagonist action: 0.2 µg/ml EP092 will shift the dose response curve for 11,9-epoxymethano PGH_2 10-fold. However, EP043 shows marked inhibition of thromboxane biosynthesis in concentrations in which this analogue is active as an inhibitor of AA-induced platelet aggregation. This may explain why EP043 is as effective as an inhibitor of AA as 11,9-epoxymethano PGH_2 induced aggregation (1 µg/ml EP043 shifts both AA and 11,9-em PGH_2 with a dose ratio of 3.23), whereas most of the analogues tested inhibit 11,9-epoxymethano PGH_2 to a greater extent than AA. (1.6 µg/ml EP045 shifts 11,9-em PGH_2 a dose ratio of 3 and AA with a dose ratio of 1.94.) It is likely that this is a direct action of EP043, although whether this is due to inhibition of cyclo-oxygenase, or thromboxane synthetase, or both, cannot be determined from this experiment.

FIGURE 3.14: Dehydration of TXB_2 when stored in methanol.



GC-MS fragmentation of TXB_2Me, BuOx, TMS yields both 301 and 436 ions in the ratio of 19 : 1. However, the ratio of 301 : 436 ions in the interfering peak running before TXB_2 is 8 : 1 showing a significant increase in the amount of 436 ion present. Furthermore there is no evidence of any ions of molecular weight greater than 436.

It is likely that some of the TXB_2 stock has dehydrated when stored in methanol. The loss of the hydroxyl would explain the absence of the 526 ion and the prodominance of the 436 ion. The lower molecular weight of the dehydrated TXB_2 would also explain the shorter retention time of the interfering peak.

EP035 also inhibits the synthesis of TXB_2 by platelets. It has been reported that agents which increase cAMP levels in platelets, either directly or by inhibiting phosphodiesterase, inhibit both platelet aggregation and internal contraction induced by AA released from platelet phospholipids (262,263,264,265). This effect of cAMP can be partly attributed to inhibition of phospholipase A_2 but an additional action of cAMP to inhibit the biosynthesis of TXA_2 from AA has been proposed (264,265), although it is still controversial (262,263). Since AA is added exogenously in this experiment, the inhibition of phospholipase A_2 by cAMP is not important. The finding that PGD_2 and ZK36374 both inhibit TXB_2 synthesis suggests that an increase in cAMP levels will inhibit metabolism of AA; this is proposed to result from inhibition of cyclo-oxygenase (265). The effect of EP035 is probably a result of its action to stimulate adenyl cyclase, rather than a direct effect.

It is interesting that no compound completely inhibited the metabolism of AA to TXB_2. In fact, in most instances the inhibitory effect plateaued at about 50-60% inhibition of synthesis. Experiments where high concentrations of thromboxane synthetase inhibitors have been used should perhaps be cautiously interpreted, as TXA_2 synthesis may not be abolished. It is thus advisable to measure the degree of inhibition of TXA_2 synthesis obtained with a given concentration of TXA_2 synthetase inhibitor.

CHAPTER IV

AA Induced Death in Rabbits

INTRODUCTION

Countries of the western world have an especially high incidence of thrombosis, thought to reflect the unwise dietary habits and stressful way of life. Originally, interest centred around the dietary intake of fats, in particular the ratio of saturated to polyunsaturated fats. This led to the finding that corn oil, which is the major ingredient of margarine, and is composed predominantly of unsaturated fatty acids, does not cause coronary thrombosis when fed to rats whereas butterfat and lard, composed of saturated long-chain fatty acids, do (266a). Furthermore, infusion of long-chain saturated fatty acids into dogs causes extensive thrombosis and death (266b,267) which does not occur when unsaturated fatty acids or short-chain saturated fatty acids are given.

Dietary studies in animals and man have shown that over a period of 3-6 weeks on a diet which is rich in unsaturated or saturated fatty acids, the dietary fatty acids are reflected in the platelet phospholipid fatty acids. Since the nature of the fatty acids incorporated into the platelet phospholipids will influence the relative amounts of the various fatty acids released when the platelets are stimulated, this in turn will determine the nature and amounts of the biologically active substances formed as metabolites of these acids (268). Of the most common long-chain saturated fatty acids, interest focussed on arachidonic acid as mediator of these noxious effects. AA is a potent aggregating agent, both *in vitro* and *in vivo*, and is rapidly incorporated into platelet phospholipids and metabolised into TXA_2 when released. The levels of AA in human platelets can be raised by the ingestion of ethylarachidonate, and these platelets are hypersensitive to aggregating agents (269).

Unlike arachidonic acid, dihomo-γ-linolenic (DLL) acid and eicosapentaenoic acid (EPA) are not aggregating agents but inhibitors of the second wave of aggregation (270). Although ordinarily DLL and EPA are present in small amounts compared with AA, dietary supplementation of these anti-thrombotic acids increases the relative amounts present in platelet phospholipids. Oral administration of DLL leads to its rapid incorporation into platelet phospholipids (271,272,273) which is reported to inhibit platelet aggregation in the rat (263)(but not the rabbit (264)) induced by ADP or collagen. Similarly human platelet aggregation is reduced by the ingestion of DLL (274,275).

This effect was originally attributed to the conversion of DLL to PGE_1 which is a potent inhibitor of platelet aggregation and itself protects against experimental thrombosis in the rabbit, when given intravenously (276). In addition, the intermediate PGH_1 has been shown to block platelet aggregation by raising cAMP levels (277,278) although it has been suggested that non-enzymatic formation of PGE_1 or PGD_1 (from PGH) may be responsible for this effect (279). The assumption that PGE_1 is responsible for the protective effect of DLL no longer seems justifiable since it has been demonstrated that ^{14}C DLL is metabolised by platelets to ^{14}C 12 hydroxyheptadecadienoic acid (HHD) (279), ^{14}C PGE_1 being undetectable. HHD results from the conversion of PGH_1 by thromboxane synthetase (280), and the formation of TXA_1 is so low (281) that it has not been detected by some workers (282). It appears that the protective effect of DLL results from its action as a competing substrate, DLL being released from the phospholipids in place of AA, and metabolised to HHD, rather than the potent aggregating agent TXA_2.

EPA cannot aggregate platelets. Although both PGH_3 and TXA_3 are formed, they are reported to inhibit platelet aggregation by stimulating

platelet adenyl cyclase (278). However, recent experiments suggest that the formation of PGD_3 and PGE_3 by platelets (283) masks the true aggregatory action of TXA_3, as this is weak compared with TXA_2 (284). The additional conversion of EPA to PGI_3 (278,283,285,286) which has similar properties and potency to prostacyclin itself (287) suggests that EPA could offer dietary protection against thrombosis (288). This has been linked to the low incidence of myocardial infarction and increased bleeding time in Greenland Eskimos whose diet is high in EPA and low in AA, resulting in equal levels of AA and EPA in the platelet phospholipids.

Myocardial ischemia is thought to be related to abnormalities of platelet function (289,290) where increased in-vivo aggregation (283) may partly occlude coronary arteries, severely reducing blood flow. This will result in decreased oxygen supply while the oxygen demand is unaltered. Both myocardial ischemia and vasospasm are implicated in cases of stroke, angina pectoris, myocardial infarction and sudden death. The potent vasospastic constriction of arteries and aggregation of platelets by TXA_2, along with the significance attributed to AA in the dietary studies, suggest a role for TXA_2 in the pathogenesis of these diseases.

Certainly a stable analogue of TXA_2, carbocyclic thromboxane A_2 (CTA_2) has been shown to induce severe coronary vasoconstriction and coronary vasospasm which leads to sudden death in rabbits within 10 minutes (292). Similarly, experimental stroke and heart attack induced by TXA_2 is prevented by the thromboxane antagonist EG626. Although experimental models where a bolus of TXA_2 is added exogenously are rather extreme, myocardial ischemia is associated with dramatically increased TXB_2 concentrations (293) and when induced by

ligation of the coronary artery, myocardial ischemia was prevented by the TXA_2 antagonist, pinane TXA_2 (294). Similarly, aspirin inhibits the early myocardial release of TXB_2 and the associated ventricular ectopic activity following acute coronary occlusion in dogs (295).

Patients with arterial thrombosis, deep vein thrombosis or recurrent venous thrombosis show an increased production of PG endoperoxides by their platelets *in vitro* (296). Such an increase is also reported in patients who have survived myocardial infarction (297) and this is associated with an increase in the sensitivity of the platelets to the thromboxane mimic 11,9-epoxymethano PGH_2. A similar increase in sensitivity to this mimic was found in platelets from patients with angina pectoris (298) and increased TXA_2 production in pacing-induced angina (299). Although these patient studies are still limited, they do suggest a correlation between platelet hyperactivity and thrombo-embolic episodes.

Several animal models, which produce an experimental condition similar to thrombosis, have been described. Basically, these involve the measurement of the formation of intravascular thrombi. This can be achieved indirectly by continuously measuring platelet count which will fall in response to intravascular aggregation and adhesion. A continuous recording of platelet count can be obtained by passing blood from the carotid artery directly through an autocounter (300). Direct measurement of platelet aggregates can be achieved by allowing a thrombus to develop, on a thread inserted into an extracorporeal shunt (301) or on a strip of collagen tissue superfused by the blood (302). Here the weight of the thrombus determines the degree and rate of thrombus formation. Alternatively, thrombus formation can be studied microscopically as in the microcirculation of the hamster cheek pouch (303) or by trapping the thrombi onto a porous disc through which single platelets

and other blood components can pass (296). In these experiments thrombus formation can be induced by aggregating agents (300), a mild electrical current (303,305,306) or by contact with a pro-aggregatory surface (302,304,307). However, since no drugs are available which reliably prevent either arterial or venous thrombosis, the relevance of these animal models in assessing potential anti-thrombotic agents cannot be established. The major difficulty is the choice of aggregatory stimulus because the actual stimulus for thrombus formation *in vivo* is, as yet, unknown.

AA given intravenously to rabbits produces acute pulmonary thrombosis leading to sudden death within 2-5 minutes (308). Since fatty acids closely related to AA do not produce this phenomenon (308, 309) and AA-induced death is inhibited by aspirin (308,310,311), it is unlikely that a non-specific detergent action of AA is responsible (312). Indeed it is probable that TXA_2 is responsible for the pathophysiologic events leading to sudden death, as is suggested for the acute thrombocytopenia and fall in blood pressure observed with lower doses of AA (313). In this section the effects on AA-induced death of intravenous infusions of EP035, EP037, EP045 and EP092 to rabbits have been studied. EP035, EP045 and EP092 protect against death when given 2 minutes before the dose of AA. This protective effect is lost in 15 minutes. EP037 affords minimal protection; indeed, a weak agonist action is evident with the doses given. The lethal effects of AA are suggested to be due to its conversion to TXA_2, as both the thromboxane synthetase inhibitor UK-37,248-01 and the thromboxane antagonists EP035, EP043 and EP092 afford protection.

MATERIALS AND METHODS

Preparation of drugs

Arachidonic acid was purified as mentioned in Chapter III. Initially a 0.9% saline solution of AA was made from a stock solution of 10 mg/ml AA in ethanol. The saline solution of 1 mg/ml AA contained 10% ethanol and required two drops of molar NaOH to form a true solution. Infusions of saline containing 10% ethanol and NaOH as well as oleic acid containing 10% ethanol and NaOH were performed as controls. In later experiments a titration procedure was used to allow AA to go into solution in saline without any ethanol. The sodium salt of AA was made by adding 10% more molar NaOH than is required for complete neutralisation of AA. The ethanol was removed by evaporation under a N_2 jet, then the residue dissolved in 0.9% saline and the solution warmed to 40°C for a few minutes.

The analogues were prepared as sodium salts and dissolved in 0.9% saline as above. Indomethacin (Merck, Sharp and Dohme) was dissolved by sonication at room temperature in 0.05M sodium hydroxide potassium phosphate buffer (pH 8). A buffer control was performed. UK-37,248-01 was dissolved in saline; although this compound is very water soluble it precipitates out of solution at neutral pH.

Experimental procedure

Adult male rabbits, weighing between 2-3 kg where possible, were anaesthetised with 6 ml/kg of a 25% urethane solution. The trachea was cannulated. The carotid artery was cannulated with a polythene catheter filled with 0.9% saline and 100 u/ml heparin. This was attached to a transducer for the recording of arterial blood pressure. Calibration of the blood pressure was achieved using a mercury manometer. The ear vein was used for the intravenous infusion of drugs; the butterfly

needle which was used for infusion of the anaesthetic was kept patent by flushing with a 10 u/ml solution of heparin.

In some experiments a small coil was attached to the skin of the animal's chest and connected to a force transducer. This gave a crude measure of respiration.

The animal was left for one hour after surgery. Drugs to be tested were infused intravenously (i.v.) 2 or 15 minutes before the infusion of AA. Indomethacin was the only drug to be given intra peritoneally (i.p.) and was allowed to act for 1 hour before the addition of AA.

RESULTS

0.3 mg/kg AA infused i.v. for 1 minute caused severe respiratory distress and a precipitous fall in blood pressure characterised by a reduction in pulse pressure; death occurred within 2 minutes in 40% of the animals. With 1 mg/kg AA similar effects were seen but the mortality rose to 75% (Table 4.1).

Typical traces are shown in Fig. 4.1. Oleic acid and ethanol controls are also shown. When respiration was measured, it was found to increase markedly after the infusion of AA, for example from 216-240 breaths/minute. These respiratory effects precede the fall in blood pressure very slightly (Fig. 4.2).

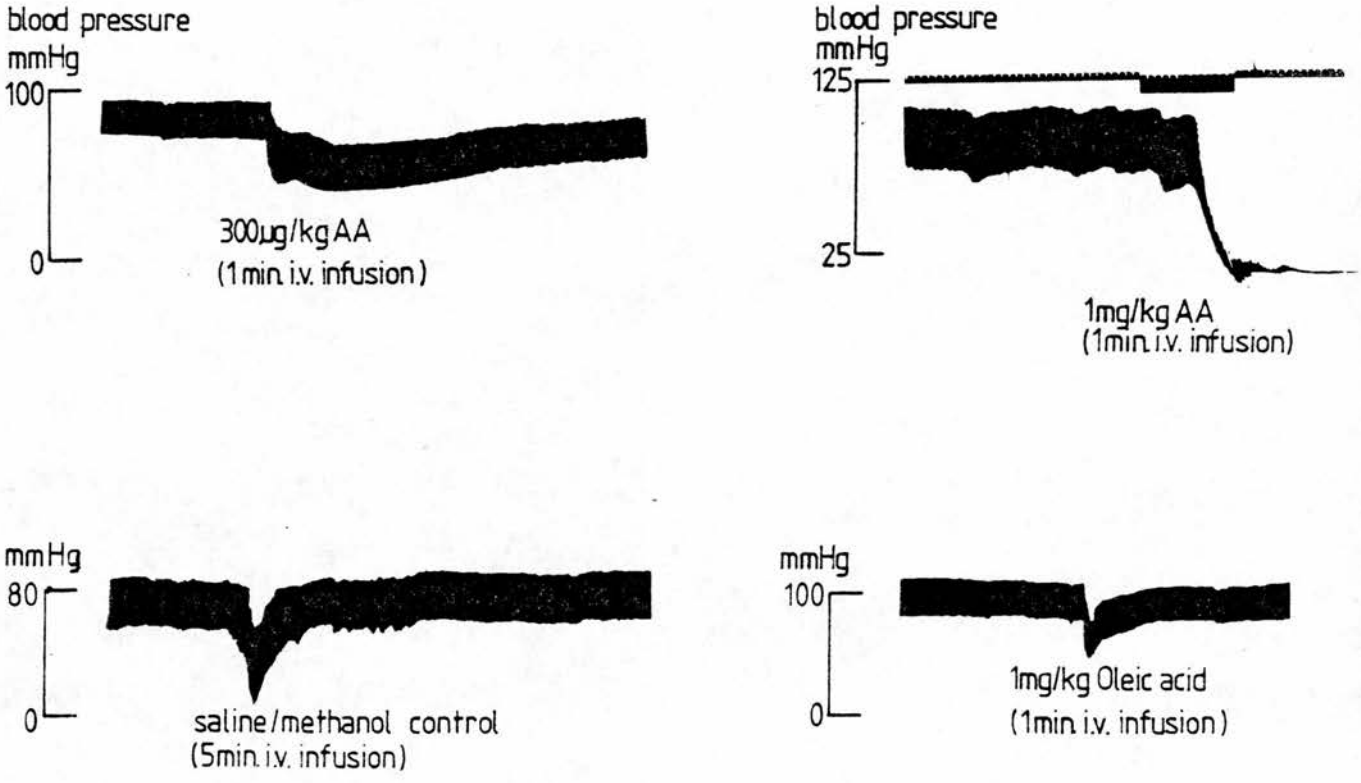


FIGURE 4.1: Control responses to AA, oleic acid and saline/methanol on rabbit blood pressure.

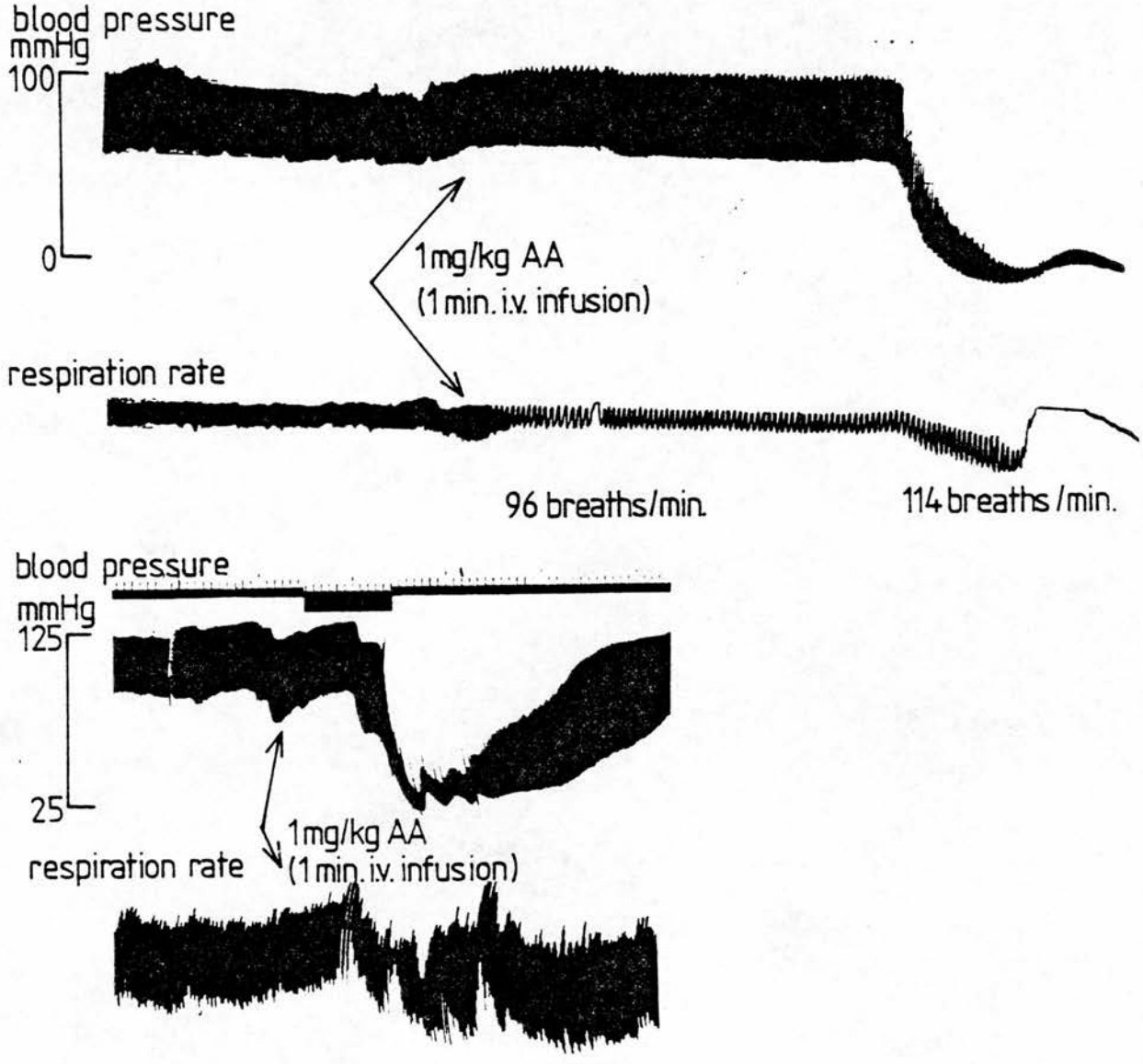


FIGURE 4.2: The effects of an intravenous infusion of AA on both rabbit blood pressure and respiration rate.

TABLE 4.1: Mortality in rabbits infused with two concentrations of AA

Concentration of AA (mg/kg)	Weight of rabbit (kg)	Death	Survival	Mortality
0.3	2.40		+	$^{3}/_{8}$
	2.36	+		
	2.30		+	
	3.00		+	
	2.04	+		
	4.00		+	
	2.48		+	
	2.68	+		
1.0	3.80	+		$^{13}/_{17}$
	2.98	+		
	2.30		+	
	2.40	+		
	2.30		+	
	3.00	+		
	2.48		+	
	4.00	+		
	2.80	+		
	2.15	+		
	3.27	+		
	2.92	+		
	3.00	+		
	2.60	+		
	2.40		+	
	2.20	+		
	2.40	+		

The results of infusion of the analogues 2 minutes before the addition of AA are given in Table 4.2. EP037 (5 mg/kg) did not protect against AA-induced death. Indeed, in this concentration it showed marked agonist activity causing a fall in blood pressure and considerable respiratory distress. An infusion of 10 mg/kg EP037 was lethal (Fig. 4.3). EP035, EP045 and EP092 protect against AA-induced death, in concentrations of 1-5 mg/kg. This protective effect is lost

TABLE 4.2: Survival of rabbits pretreated with EP035, EP037, EP045 and EP092 two minutes before an infusion of AA (1 mg/kg)

Weight of rabbit (kg)	Analogue	Concentration of analogue (mg/kg)	Concentration of AA (mg/kg)	Death	Survival	% survival
2.20	EP035	0.5	1.0	+		33.3
2.43		0.5	1.0	+		
2.15		0.5	1.0		+	
2.25	EP035	5.0	1.0		+	100
2.20		5.0	1.0		+	
2.10		5.0	1.0		+	
2.30	EP037	5.0	1.0		+	33.3
2.20		5.0	1.0	+		
2.04		5.0	1.0	+		
2.26	EP037	10.0	0	+		0
2.14	EP045	0.5	1.0	+		33.3
2.46		0.5	1.0	+		
2.70		0.5	1.0		+	
2.20	EP045	1.0	1.0		+	100
2.40		1.0	1.0		+	
2.30		1.0	1.0		+	
2.39	EP045	5.0	1.0		+	100
2.40		5.0	1.0		+	
2.54		5.0	1.0		+	
2.70	EP092	0.5	1.0		+	33.3
2.65		0.5	1.0	+		
2.44		0.5	1.0	+		
2.63	EP092	2.0	1.0		+	100
3.45		2.0	1.0		+	
2.54		2.0	1.0		+	

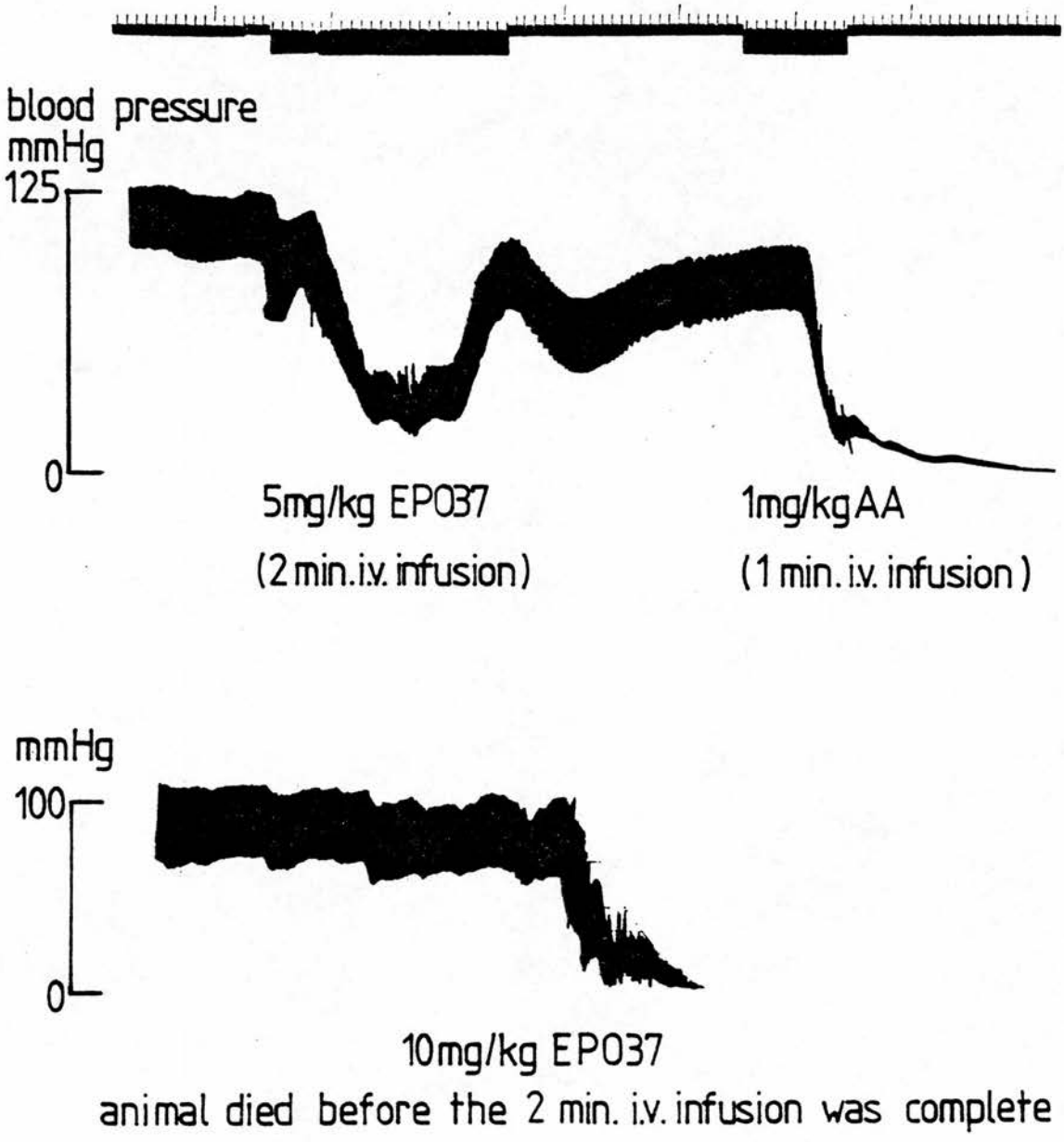


FIGURE 4.3: The agonist action of EP037 when infused intravenously into rabbits (5-10 mg/kg).

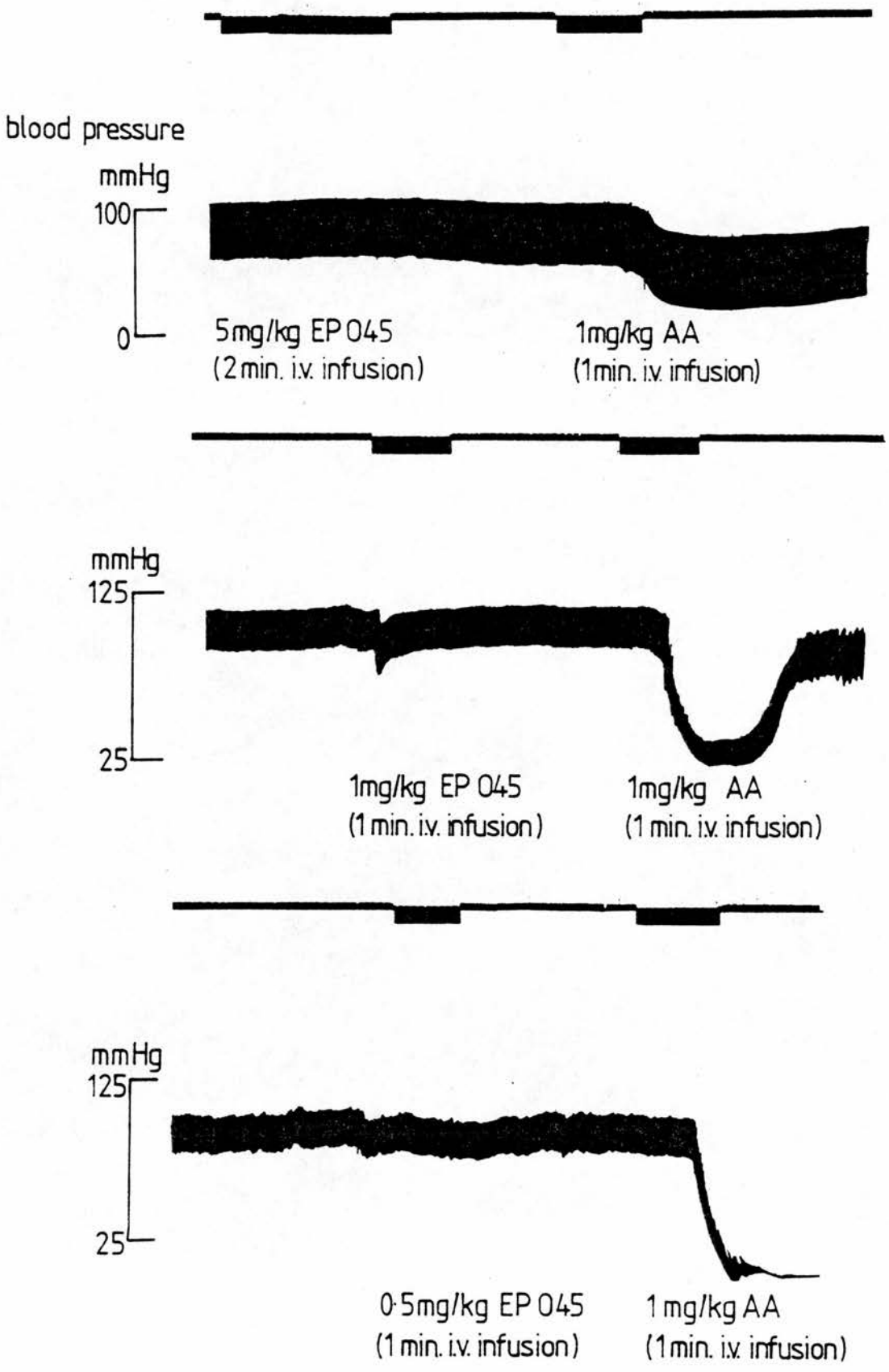


FIGURE 4.4: Protection against AA-induced death in rabbit with various concentrations of EP045.

with doses of less than 1 mg/kg and is also lost if AA is infused 15 minutes rather than 2 minutes after infusion of the analogue. Typical traces are shown in Fig. 4.4. Although both 1 mg/kg and 5 mg/kg EP045 afford 100% protection against AA-induced death, the traces show that distress to the animal is much more severe with the lower dose of EP045. The fall in blood pressure observed, when protected against AA-induced death, is similar to that obtained following intravenous injection of PGE_2 or I_2 (Fig. 4.5) as there is no diminution in pulse pressure.

Both the cyclo-oxygenase inhibitor indomethacin (0.042 - 0.125 mg/kg i.p.) and the thromboxane synthetase inhibitor UK-37,248-01 (2 mg/kg i.v.) protect against the lethal effects of AA. This effect of indomethacin is lost when the dose is lowered to 0.0125 mg/kg (Table 4.3).

TABLE 4.3: Protective effects of indomethacin and UK-37,248-10 against AA-induced death

Weight of rabbit (kg)	Inhibitor	Concentration of inhibitor (mg/kg)	Concentration of AA (mg/kg)	Death	Survival
2.30	indomethacin	0.125	1.0		+
2.08		0.125	1.0		+
2.30		0.125	2.0		+
2.18	indomethacin	0.042	1.0		+
2.00		0.042	1.0		+
2.09		0.042	2.0	+	
2.70	indomethacin	0.0125	1.0	+	
2.15	UK-37,248-01	2.0	1.0		+
2.20		2.0	1.0		+
2.35		2.0	1.0		+

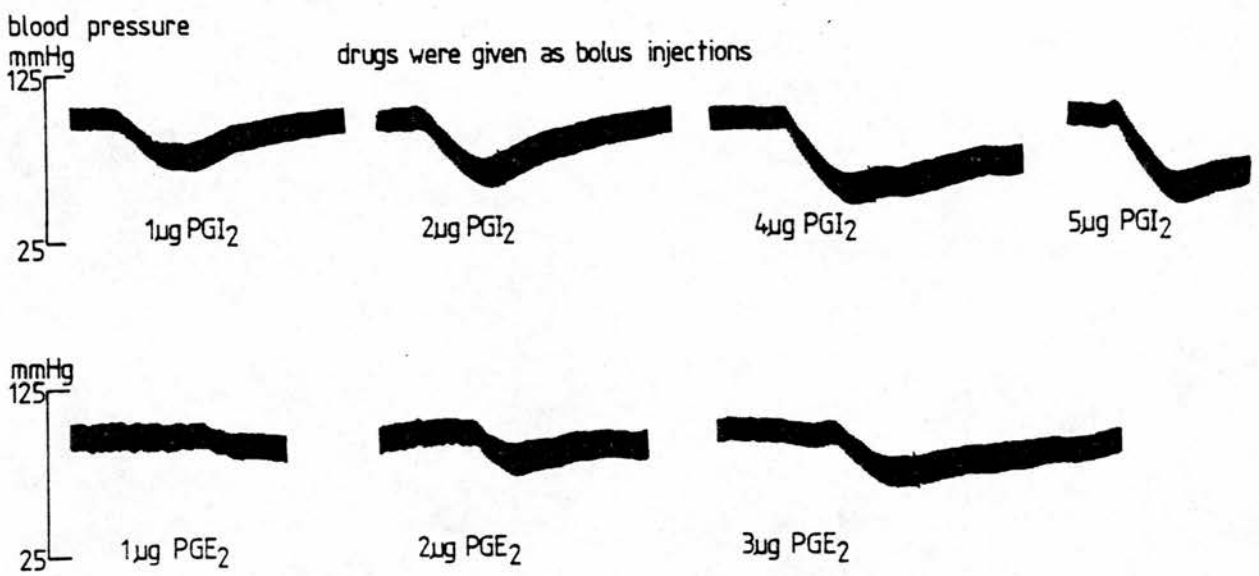


FIGURE 4.5: The effects of intravenous injections of PGE_2 and PGI_2 on rabbit blood pressure.

DISCUSSION

Although experimental evidence suggests that increased sensitivity to the AA metabolite TXA_2 may contribute to the platelet hyperactivity and vasospasm which are evident in pathological conditions such as thrombosis or sudden death, it does not indicate that AA is the causal factor responsible for intravascular platelet aggregation. The experimental model of AA-induced death in rabbits is not intended to mimic the pathological condition of sudden death. Since AA constitutes only 2.5% of the non-esterified fatty acid pool in the blood stream (314), if AA acts as a trigger *in vivo*, it is probable that another agent will stimulate its release from endogenous phospholipid stores where AA is abundant (315).

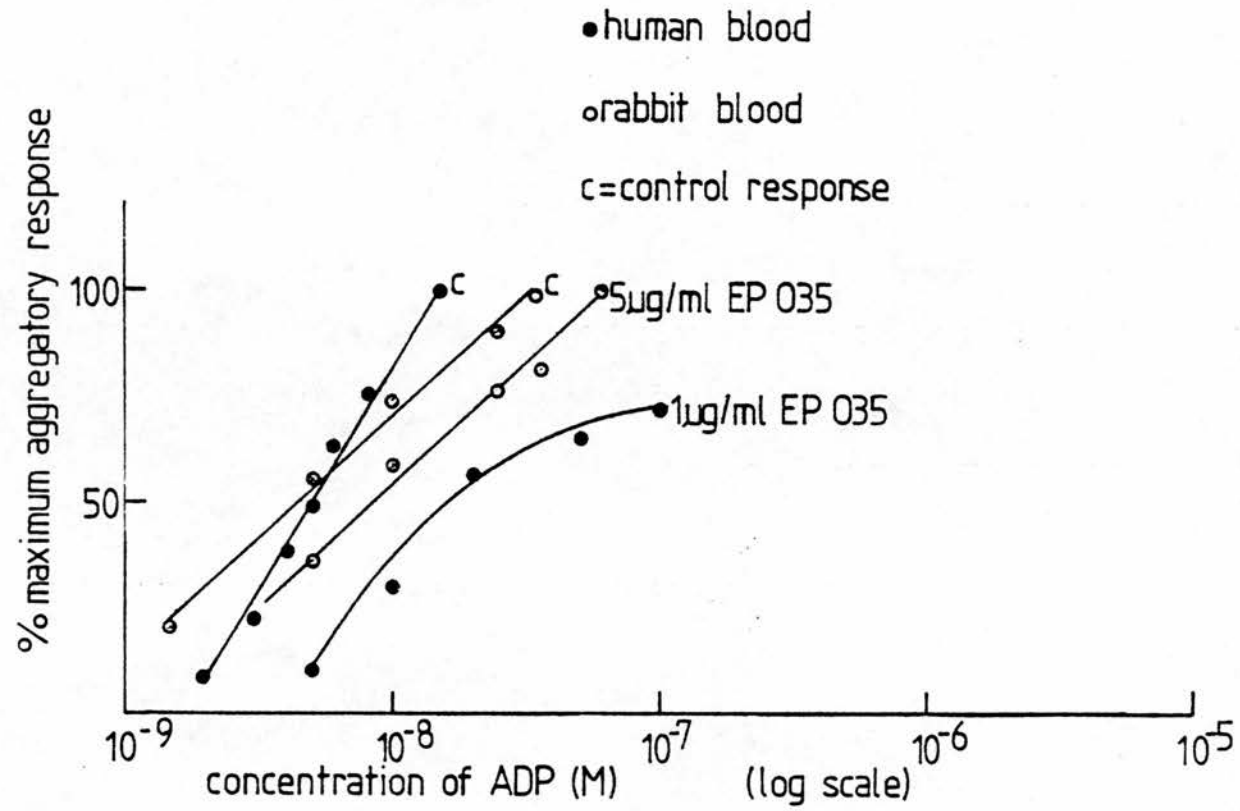
Since the analogues tested are thromboxane antagonists, they will be useful anti-thrombotic agents only if AA and thus TXA_2 play a prominant role. However, it is not known which experimental model, if any, most closely resembles the pathological condition of thrombosis, so it was thought that AA-induced death was a good test for in-vivo activity of these analogues. If the analogues protect against such an extreme situation where AA swamps the system, they will probably be useful agents if excessive TXA_2 production is causally related to thrombosis.

EP035, EP045 and EP092 (1-5 mg/kg) all exhibited in-vivo activity and protected against AA-induced death. This action was short lived as it was lost within 15 minutes of the infusion, suggesting rapid metabolism of these compounds in the body. Unfortunately, there was not sufficient EP043 to use in these experiments. Using this rabbit model, EP037 appears to be a weak partial agonist. In concentrations of 5-10 mg/kg EP037 exhibited an agonist action (a marked fall in blood pressure and respiratory distress), the higher proving lethal. Indeed,

Dr. R.L. Jones has found that on sensitive preparations of the rabbit aorta EP037 can elicit a very weak contractile effect. This is no more than 5% of the maximum response.

The discovery that both thromboxane antagonists and the thromboxane synthetase inhibitor UK-37,248-01 protect against the lethal effects of AA, support the suggestion that the metabolism of AA to TXA_2 is required for this activity. EP035, EP045 and EP092 will antagonise both the vasoconstrictor and aggregatory actions of TXA_2. However, the vasospastic action alone of TXA_2 may be sufficient to induce sudden death as the potent vasoconstrictor carbocyclic TXA_2 can induce sudden death in rabbits even although it inhibits rather than aggregates platelets (292). Indeed strips of the rabbit intrapulmonary artery have been found to rapidly metabolise exogenous AA to TXA_2, which contracts the tissue (316). Previously it was thought that vascular tissues metabolised AA almost exclusively to prostacyclin and that platelets were responsible for the metabolism of AA to TXA_2. The additional action of TXA_2 to aggregate platelets probably accounts for the rapidity of its effects; death being induced within 2 minutes, rather than 10 minutes when vasoconstriction alone is responsible.

The protective effects of EP035, EP045 and EP092 are particularly encouraging as rabbit platelets are less sensitive to those antagonists than human platelets (Fig. 4.6). Originally, rats were used in this model but they proved to be relatively insensitive to the lethal effects of AA, requiring at least 5 mg/kg AA compared to 1 mg/kg with the rabbit. The resistance of rats to AA induced mortality has recently been related to the failure of rats to produce large amounts of TXA_2 in response to AA infusion (316).



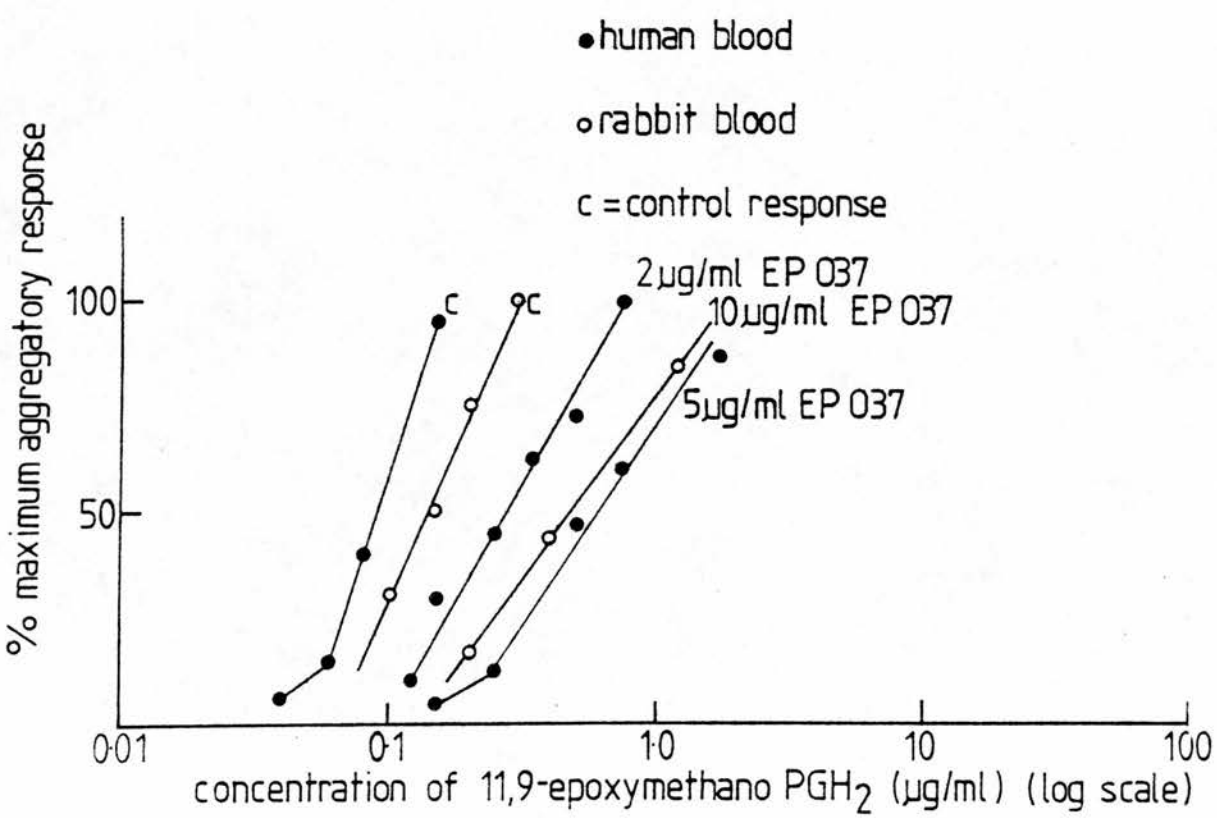


FIGURE 4.6: Comparison of the anti-aggregatory effects of EP035 and EP037 on rabbit and human platelets.

CHAPTER V

The Binding of ^{3}H 9,11-epoxymethano PGH_2 to Human Platelets

INTRODUCTION

The first successful proposal for a general theory of drug action was made by Clark, that stimulant drugs occupied specific receptors and that the response depended on the proportion of receptors occupied (317,318). Receptors were postulated to be specific molecular sites or structures in (or on) an effector cell, with which molecules of a specific agonist must react in order to elicit the characteristic response of the cell to the agonist. The Langmuir isotherm relationship developed for the adsorption of gases on metals was found to apply to many enzyme concentration velocity curves and Clark extended this mass law relationship to the action of drugs.

Subsequently, Gaddum extended this approach to the action of antagonists which he supposed to fill a receptor site without activating it (319). The parallel shift to the right in the log dose-response curve for an agonist, produced by an antagonist, was explained by competition between the antagonist and agonist, the agonist being able to physically displace the antagonist from the receptors. However, experimental evidence suggested that the antagonist was not being displaced and even when 90-99% of the receptors were occupied, a maximum response could be elicited if sufficient agonist was added. This led to the concept of spare receptors and the suggestion that an agonist need occupy a small fraction of the receptors to produce a maximum response (320). In competitive antagonism, agonist and antagonist presented simultaneously in solution are now thought to compete for receptors to the exclusion of the other; the response determined by the concentration of the two drugs and their relative affinity constants.

Since mass law equations applied to drug antagonism refer to events on receptors rather than observable responses, it is necessary

to postulate some relationship between receptor activation and response. Usually the limited assumption has been made that an agonist must occupy the same number of receptors in the presence or absence of an antagonist to produce a given response. This useful assumption has led to the use of the dose-ratio (the ratio of the concentrations of agonist in the presence and absence of antagonist to produce a given response) to estimate the affinity constant (K_B) of an antagonist for a particular receptor.

K_B values help the classification of drugs according to the receptors on which they act. If the use of different agonists give the same K_B value for a particular antagonist on a given tissue, it is strong provisional evidence that the agonists act on a common receptor. This is a consequence of the mass law and applies whatever the affinity or efficacy of the agonist. Also, comparison of receptors in different tissues which are activated by the same agonist can be made by comparing K_B values for a common antagonist.

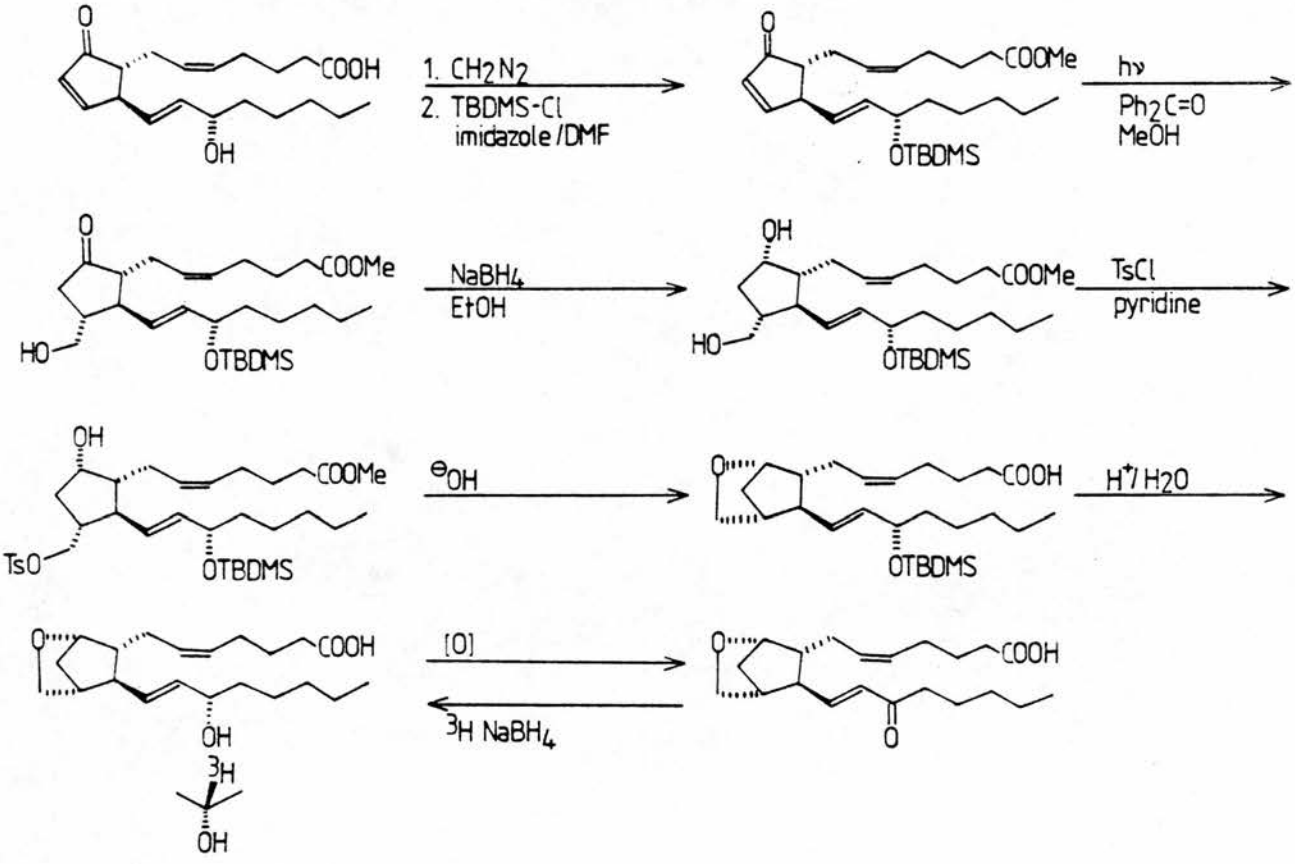
Although methods have been described to measure the affinity constant of an agonist (321), it is customary to use equipotent molar ratios as a measure of the effectiveness of different agonists at a given receptor. This is a much more useful concept as the response of an agonist is dependent on both the affinity and the efficacy of a drug, where efficacy is a measure of the effectiveness of the drug-receptor complex (322). (Antagonists are thought to have zero efficacy.) The equipotent molar ratio is used to compare the relative potencies of different agonists at a given receptor site. It is the ratio of the concentration of one agonist compared to another agonist, required to produce the same response. These equipotent molar ratios can be used alongside antagonist K_B values in the classification of receptors.

It is only relatively recently that the existence of drug receptors could be demonstrated directly, rather than deduced from pharmacological experiments. This advance was a result of the development of receptor-specific, radio-labelled ligands which have enabled binding to receptor sites to be studied directly. The general approach has been to study the physiochemical interaction between the radio-labelled ligand and the plasma membrane either in the intact cell or in an isolated membrane preparation. However, a number of basic criteria must be fulfilled before this binding can be said to represent selective binding to the receptor in question. These are:

1. *specificity:* concentrations of drugs which are pharmacologically effective at this particular receptor should displace the saturable component of binding, while pharmacologically effective concentrations of drugs acting at different receptors should be ineffective. Known steric and structure-activity relationships should be complied with, and the receptor should have a high affinity for the radio-labelled ligand consistent with the sensitivity of the tissue to the ligand.

2. *saturability:* a component of the binding should saturate with increasing concentration of the radio-labelled ligand, since the number of receptors is finite.

3. *distribution:* the saturable component of the specific receptor binding should be restricted to those tissues which are known to show the appropriate pharmacological response.



TBDMS = tertiary butyl dimethylsilyl chloride

hν = light

$Ph_2C=O$ = benzophenone

$NaBH_4$ = sodium borohydride

TsCl = tosyl chloride

FIGURE 5.1: Preparation of ^3H 9,11-epoxymethano PGH_2 from natural PGA_2.

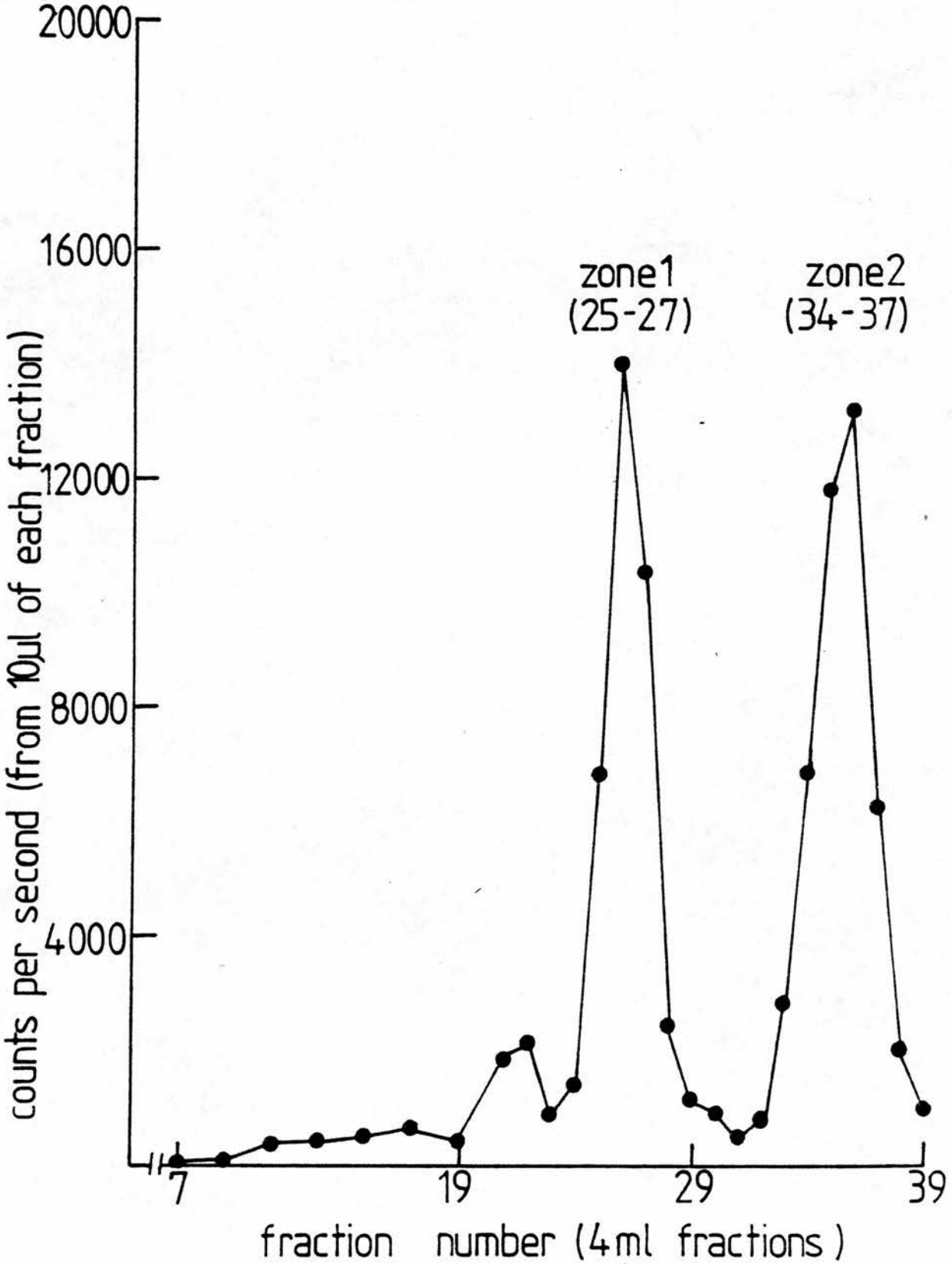
Although binding studies of ^3H PGI_2, ^3H PGE_1 and ^3H PGD_2 to both whole platelets and to platelet membrane preparations have been reported (207-211), there have been no reports of tritiated PGH_2 or TXA_2 binding studies. As TXA_2 is very acid labile, having a half-life of about 30 seconds, a number of stable compounds have been developed (120-130) which mimic the biological actions of TXA_2, perhaps by interaction with a common receptor.

To provide further information on this matter, a radio-labelled thromboxane mimic, ^3H 9,11-epoxymethano PGH_2 was prepared, and its binding to whole platelets studied (323). Although it would be more desirable to study the binding of ^3H TXA_2 itself, classical binding studies would be hampered by its instability. In this section the preparation of this radio-labelled ligand and the development of a binding assay for whole platelets is described. The difficulties in interpreting binding data, Scatchard analysis and displacement experiments are discussed. Possible ways of improving these binding studies to make the results more meaningful are proposed.

MATERIALS AND METHODS

Preparation of ^3H 9,11-epoxymethano PGH_2 (by Dr. R.L. Jones and N.H. Wilson)

15-oxo-9,11-epoxymethano PGH_2 was prepared from natural PGA_2 (324) by tosylation and base cyclisation (Fig. 5.1) and supplied to Amersham Radiochemical Centre for reduction with tritiated sodium borohydride ($NaBH_4$). This is achieved by first taking up the compound in 3-4 ml ethanol then cooling to -20°C. Excess solid ^3H $NaBH_4$ (1 Ci at $\geq$ 20 Ci/mmol) is added and the reaction mixture left for 30 minutes at -20°C followed by 30 minutes at room temperature. 50 ml of distilled water are added and the pH taken to 4 with dilute hydrochloric acid,



Zone I is 15(R) ^{3}H 9,11-epoxymethano PGH_2

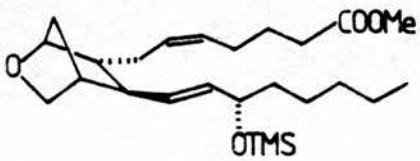
Zone II is 15(S) " " "

FIGURE 5.2: Radio TLC of ^{3}H 9,11-epoxymethano PGH_2

before partitioning twice with 50 ml of diethyl ether. This is evaporated to dryness and the residue vacuum dessicated. The residue was stored in methanol at -20°C by Amersham and 25 m Ci batches of the crude product sent when required.

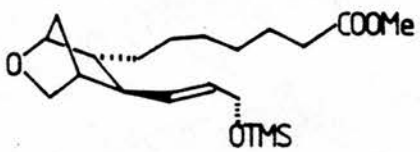
The crude ^{3}H 9,11-epoxymethano PGH_2 was separated into the 15(R) and 15(S) forms by liquid-gel partition chromatography. A N1114-20% LH20 Lipidex column (70 ml) was used and eluted with 100/100/5 hexane/dichloroethane/ethanol containing 0.1% acetic acid. Fractions 25, 26, 27 (4 ml) inclusive were pooled as zone I and fractions 34, 35, 36, 37 inclusive as zone II. The small peak in fractions 21/22 could be 13,14 dihydro material or some residual 15-oxo-9,11-epoxymethano PGH_2 (Fig. 5.2).

Analysis by radio TLC showed that zones I and II ran as two distinct peaks, zone II running coincident with 15(S) 9,11-epoxymethano PGH_2. Testing of zone II on the rabbit aorta showed contractile activity similar to 11,9-epoxymethano PGH_2. On the dog saphenous vein, zone II showed activity equivalent to 16 µg/ml 9,11-epoxymethano PGH_2. This value gave an estimate of 13.9 Ci/mmole for the specific activity of 15(S) ^{3}H 9,11-epoxymethano PGH_2 (zone II). The value agreed with that determined by GLC-MS analysis, where the methyl ester, TMS ether of zones I and II were prepared and 200 ng samples injected and full mass spectra obtained to allow estimations of the tritium/protium content (Table 5.1, Fig. 5.3).

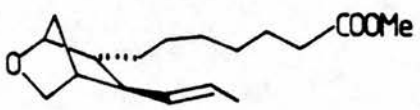


9,11-epoxymethanoPGH$_2$, Me, TMS. m+/e = 436

Major fragments

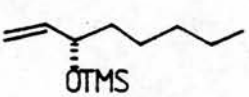


m/e = 365



m/e = 275

m/e = 199



^{3}H 9,11-epoxymethano PGH$_2$ fragments to form the same ions, although those containing ^{3}H will be 2 mass units heavier (367, 277, 201).

FIGURE 5.3: Fragmentation of 9,11-epoxymethano PGH$_2$, Me, TMS by GC-MS.

TABLE 5.1: Estimation of the specific activities of both 15(S) and 15(R) ^{3}H 9,11-epoxymethano PGH_2 by GLC-MS

	Fragment ion	Assignment	Protium/tritium intensities	tritium / protium + tritium ratio
15(R) zone I	365/367	M-71	10.8/14.2	
	275/277	M-71-90	11.3/15.0	
	199/201	ω chain	19.6/12.3	
		sum=	41.7/41.5	0.445
15(S) zone II	365/367	M-71	13.2/18.8	
	275/277	M-71-90	16.4/21.6	
	199/201	ω chain	32.9/22.2	
		sum=	62.5/62.6	0.500
15(S) again	365/367	M-71	17.6/23.5	
	275/277	M-71-90	19.0/24.0	
	199/201	ω chain	42.6/29.5	
		sum=	79.2/77.0	0.493
				mean = 0.496

Amersham reduction allows a maximum specific activity of 28.9 Ci/m atom.

∴ specific activity of 15(R) = 28.9 x 0.445 = 12.9 Ci/mmole
specific activity of 15(S) = 28.9 x 0.496 = 14.3 Ci/mmole.

Binding studies

Reagents used:

1. CPD - for 100 ml - 0.33 g citric acid
   2.63 g sodium citrate
   0.22 g monosodium phosphate
   2.55 g dextrose

2. platelet medium - for 10 litres - 81.82 g sodium chloride
   3.73 g potassium chloride
   9.01 g glucose
   12.08 g citric acid
   5.88 g sodium citrate

   adjusted to pH 7.5 with 2 M Tris (121.14 g Tris/500 ml).

Method:

100 ml of blood was withdrawn from the antecubital vein of healthy volunteers into 14 ml CPD and centrifuged at 160 g for 20 minutes. The platelet-rich plasma was collected and treated with indomethacin (10^{-5}M) to inhibit TXA_2 production, and PGE_1 (1.7 x 10^{-8}M) to prevent aggregation by the thromboxane mimics. The PRP was further centrifuged at 1600 g for 10 minutes and the resulting pellet suspended in platelet medium to give a final platelet count in the region of 5 x 10^8 platelets/ml.

A stock solution of 500 ng/ml : 4 μCi/ml ^{3}H 9,11-epoxymethano PGH_2 15(S) was prepared in methanol and appropriate amounts dispensed into Eppendorf tubes and blown dry. 1 ml of platelet suspension was added to each tube, six tubes at a time, the tubes whirlimixed then incubated at room temperature for a given period of time. Incubation was terminated by centrifugation in an eppendorf centrifuge at 15000 g for two minutes. The supernatant was removed rapidly and added to scintillant (10.5 g PPO to 1.5 l toluene and 900 ml 2-ethoxy ethanol) to be counted later. Any remaining supernatant was cleaned from the pellet with a cotton bud. The pellet was digested with hyamine hydroxide (1M) (Fisons) by incubation at 50°C for 5 minutes. A few drops of 2M hydrochloric acid were added to each tube and the pellet was transferred to scintillant. The acid serves to neutralise the strong alkali (hyamine hydroxide) and prevents chemiluminescence interfering with the liquid scintillation counting.

Both supernatant and pellet samples were counted for 10 minutes. In displacement experiments prostaglandins and analogues were added in saline simultaneously with the platelet suspension at the start of the incubation.

Determination of platelet count

Reagents used:

Solution A - for 100 ml - 1 g sodium citrate
0.002 g mercuric chloride
0.2 g brilliant cresyl blue
warmed to 45°C.

Solution B - for 100 ml - 20 g urea

Method:

Platelet count was determined by the cresyl blue dye method. Equal volumes of solutions A and B were mixed and filtered. 50 µl platelet-rich plasma was added to 1 ml of the dye mixture, and samples were taken up by a glass capillary and used to fill both counting chambers of a haemocytometer. This was left to sediment for 15 minutes inside a petri dish containing moist filter paper. Each counting chamber was marked with squares and the number of platelets in 5 squares, usually four corner and one middle, were counted. The mean value of the number of platelets estimated from the 2 counting chambers was multiplied by 10^4 and taken as the number of platelets per µl of platelet rich plasma.

Liquid scintillation counting

Both supernatant and pellet samples were added to 10 ml PPO scintillant and counted for 10 minutes using a Philips PW4540 liquid scintillation analyser. This gave a value of counts per minute (cpm) for each sample. Sample cpm were corrected to disintegrations per minute (dpm) by correcting for the amount of quenching in the sample. Biological samples frequently exhibit chemical and colour quenching where the maximum photon yield for a given radioactive source is not achieved due to sample inhomogenity, adverse energy transfer and

non-transparency of the liquid scintillator to the photon emitted. Correction for quenching is achieved by determination of the efficiency of counting for each sample. Efficiency is defined as the ratio of the observed cpm to the true dpm.

To determine the efficiency of counting the scintillation counter machine constants were first determined by constructing a quench curve where a calibrated tritium standard, ^3H hexadecane, was used to provide a known number of dpm, and the cpm were determined at different levels of quenching, where chloroform ($CHCl_3$) was used as the quenching agent (Table 5.2).

TABLE 5.2: Quench curve protocol

Vial No.	µl Chloroform added	DPM added
1	-	-
2/3/4	-	85300
5/6/7	20	"
8/9/10	50	"
11/12/13	100	"
14/15/16	200	"
17/18/19	500	"
20/21/22	1000	"

The quench curve is plotted by measuring efficiency of counting, $^{cpm}/_{dpm}$ (y axis) against ratio (x axis). Either sample channel's ratio or external standard ratio was used. The quench curve is described by the equation $y = k_0 + k_1 x + K_2 x^2$ where K_0, K_1 and K_2 are constants. Typical quench curves and the values of K_0, K_1 and K_2 for these are given in Fig. 5.4. These constants are used to determine the efficiency of counting in each sample so that the dpm could be calculated.

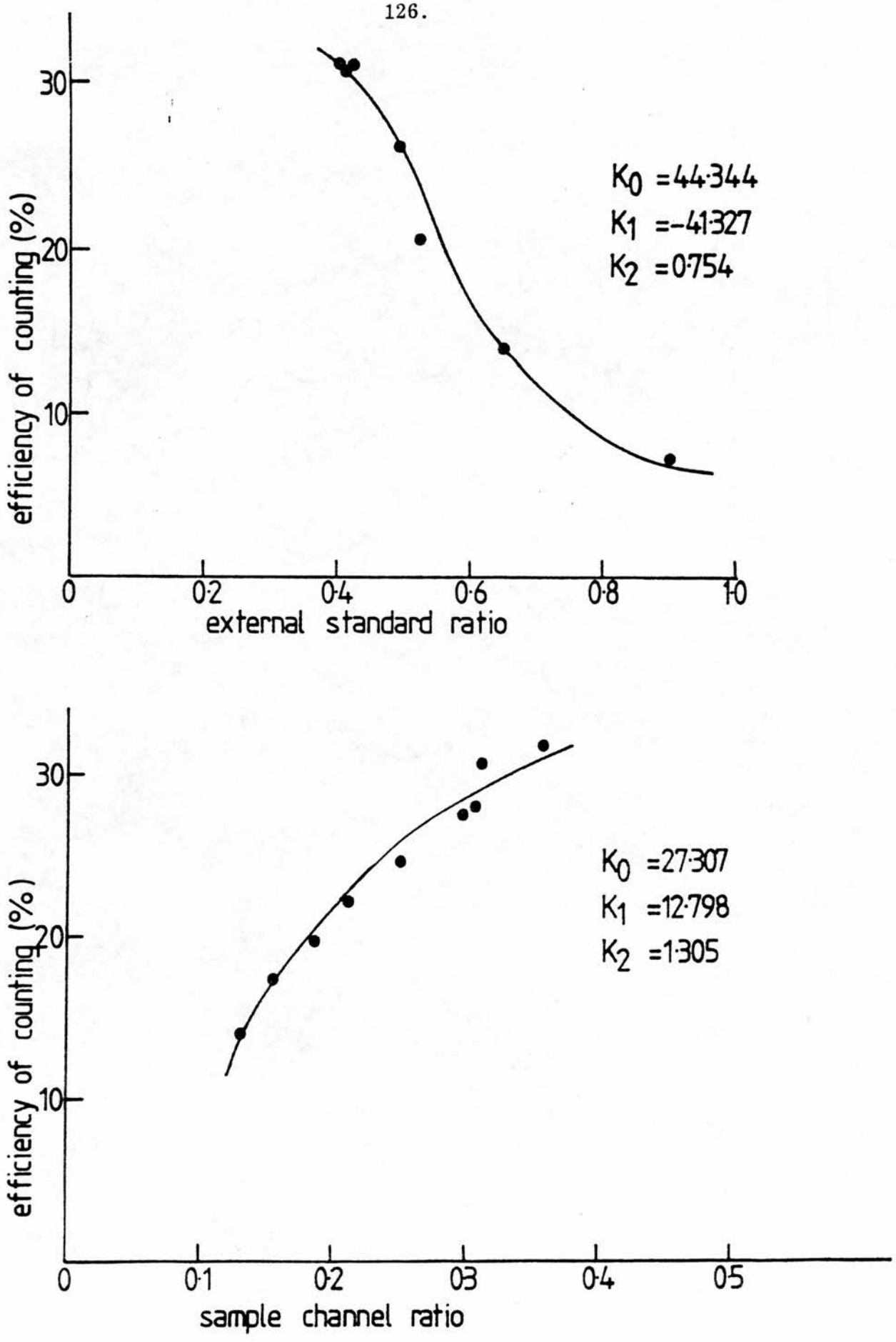


FIGURE 5.4: Typical quench curves for tritium where chloroform is used as the quenching agent.

RESULTS

The tritiated thromboxane mimic was found to have a specific activity of 13.9 Ci/mmol. Due to the limited number of specific binding sites usually present it is desirable to work with a ligand of as high specific activity as possible. The method used here to prepare ^{3}H 9,11-epoxymethano PGH_2 from natural PGA_2 has the disadvantage that only one tritium can be incorporated into each molecule of 9,11-epoxymethano PGH_2: the more tritium atoms present in each molecule, the greater the specific activity of the ligand. Although tritium labelling always affords ligands of low specific activity compared to those containing radioactive iodine (^{125}I), the labelled product is usually indistinguishable biologically from the unlabelled, whereas biological activity is often reduced with mono-iodinated, and lost altogether with di-iodinated compounds.

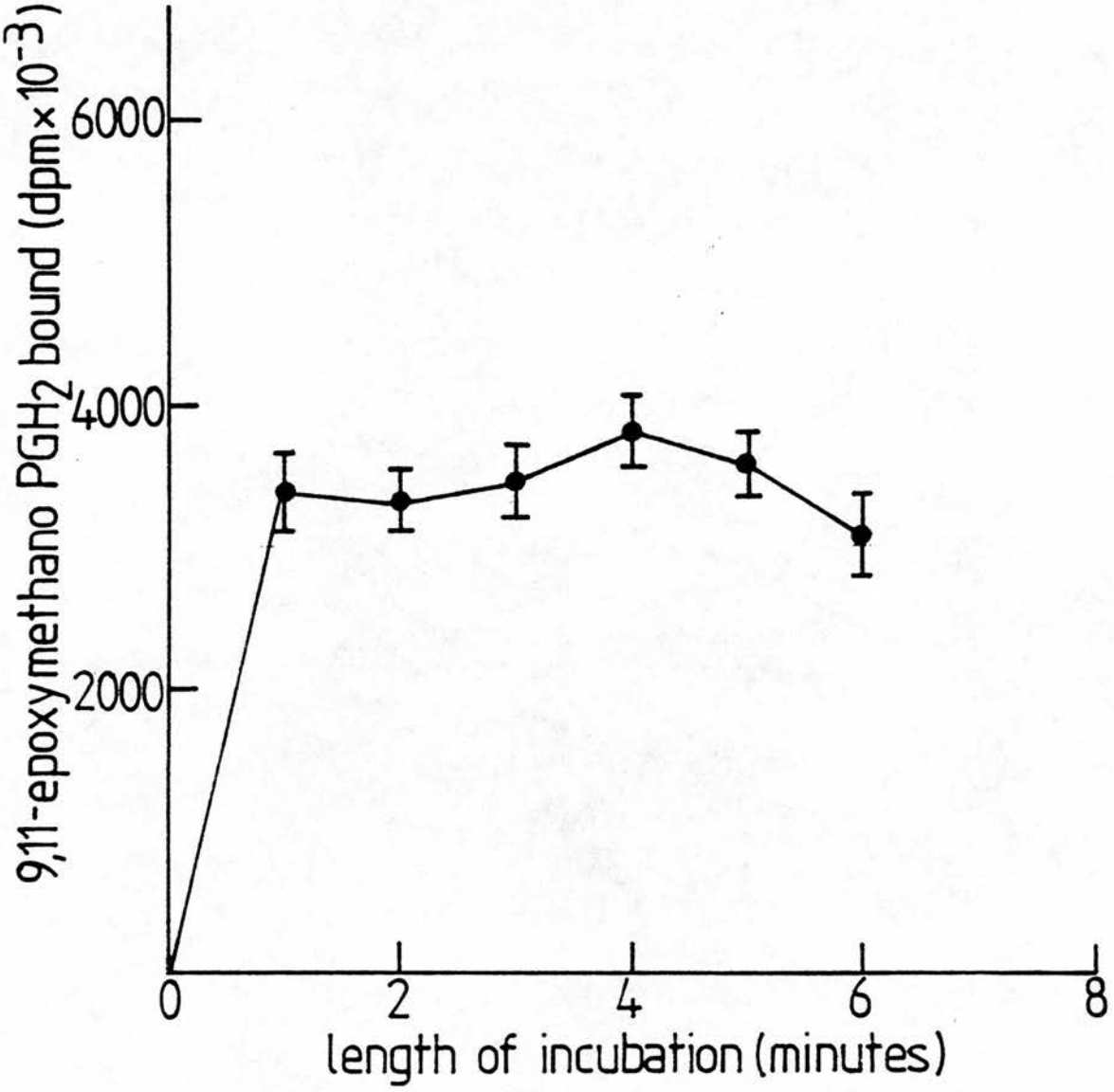
To test for binding of ^{3}H 9,11-epoxymethano PGH_2 to platelets, it was decided to look initially for binding to whole platelets. Particulate preparations have the advantage that they enable bound and free ligand to be separated, both quickly and easily, by centrifugation. Separation is begun while the binding reaction is at equilibrium and it is essential to use a separation technique which minimises the dissociation of the receptor-ligand complexes, particularly if this dissociation is rapid. Here separation was achieved by centrifugation in an eppendorf centrifuge at 15000 g for 2 minutes. The supernatant was removed rapidly with a pasteur pipette and any remaining supernatant was cleared from the pellet with a cotton bud. This allowed the bound and free ligand to be completely separated within 3-4 minutes, as a maximum of 6 samples were processed at any one time. Although centrifugation procedures have the disadvantage of less efficient washing of the

particulate pellet, as compared to filtration techniques, they have the advantage that a much more rapid separation can be achieved.

Incubation of the washed platelet suspension with a fixed concentration of ^{3}H 9,11-epoxymethano PGH_2 (0.07 μM) for increasing lengths of time indicated considerable binding of 9,11-epoxymethano PGH_2 by platelets. The amount of ^{3}H 9,11-epoxymethano PGH_2 bound plateaued within one minute and remained approximately at this level for 6-8 minutes after the start of the incubation, when the level began to fall slightly (Fig. 5.5). This binding could be rapidly displaced by an excess of non-radioactive 9,11-epoxymethano PGH_2 (11.42 μM).

The number of specific binding sites and the affinity of the ligand for these binding sites can be determined by incubating various concentrations of the ligand with a fixed number of platelets. The concentration of the ligand can be increased either by increasing the amount of radioligand added and so increasing the number of cpm added or by adding a fixed amount of radioactivity and increasing amounts of unlabelled ligand, which effectively increases the ligand concentration by diluting the specific activity of the radioligand (325). In the preliminary Scatchard analysis, experiments were performed using the first of these two methods.

The simplest condition occurs when one molecule of the ligand binds to one receptor site, the binding sites being identical and non-interacting, and the ligand is present in excess so that its free concentration does not change significantly during the course of the binding reaction.



Each result is the mean and standard error of 10 observations.

FIGURE 5.5: Time course of binding of ^{3}H 9,11-epoxymethano PGH_2 to whole platelets.

$C_B + C_L \rightleftharpoons C_{BL}$

where C_{BL} = molar concentration of ligand occupied sites

C_B = concentration of free sites

C_L = concentration of free ligand

The association constant $K = \dfrac{C_{BL}}{C_B \times C_L}$

Let $C°_B$ = total concentration of binding sites

then $K = \dfrac{C_{BL}}{(C°_B - C_{BL})C_L}$ $\left[C_{BL} = \dfrac{C°_B K C_L}{1 + KC_L} \right]$

$$K\,(C°_B - C_{BL}) = \frac{C_{BL}}{C_L}$$

The Scatchard plot of C_{BL}/C_L (y axis) against C_{BL} (x axis) is a straight line if K is a constant (326). The equilibrium dissociation constant (K_D) is estimated as the negative reciprocal of the slope (K) of the line of best fit. The intercept on the x axis (where C_{BL}/C_L is zero) gives $C°_B$ the number of binding sites. The Scatchard plot gives a value for both the equilibrium dissociation constant of the ligand and the number of ligand binding sites. The same binding curve would be obtained if there were more than one binding site per receptor provided that the sites are non-interacting.

The Scatchard plot for a system with two classes of distinct binding sites is described by the equation:

$$(C_{BL})_t = (C°_B)_1 \frac{K_1 C_L}{1+K_1 C_L} + (C°_B)_2 \frac{K_2 C_L}{1+K_2 C_L}$$

The plot is now curvilinear rather than linear, and the two asymptotes can be used to derive the dissociation constants for both binding sites as well as the number of both classes of binding sites.

Since the amount of fresh human blood available was restricted due to the limited number of donors, binding of ^{3}H 9,11-epoxymethano PGH_2 to fresh platelets was compared to binding to platelets obtained from the Blood Transfusion Unit, Royal Infirmary of Edinburgh, which were a maximum of 24 hours old. ^{3}H 9,11-epoxymethano PGH_2 in concentrations of 0.86 - 7.14 nM, was incubated with 1 ml of the platelet suspension for 4 minutes at room temperature. Table 5.3 gives the dpm bound, for both types of platelets, over this concentration range.

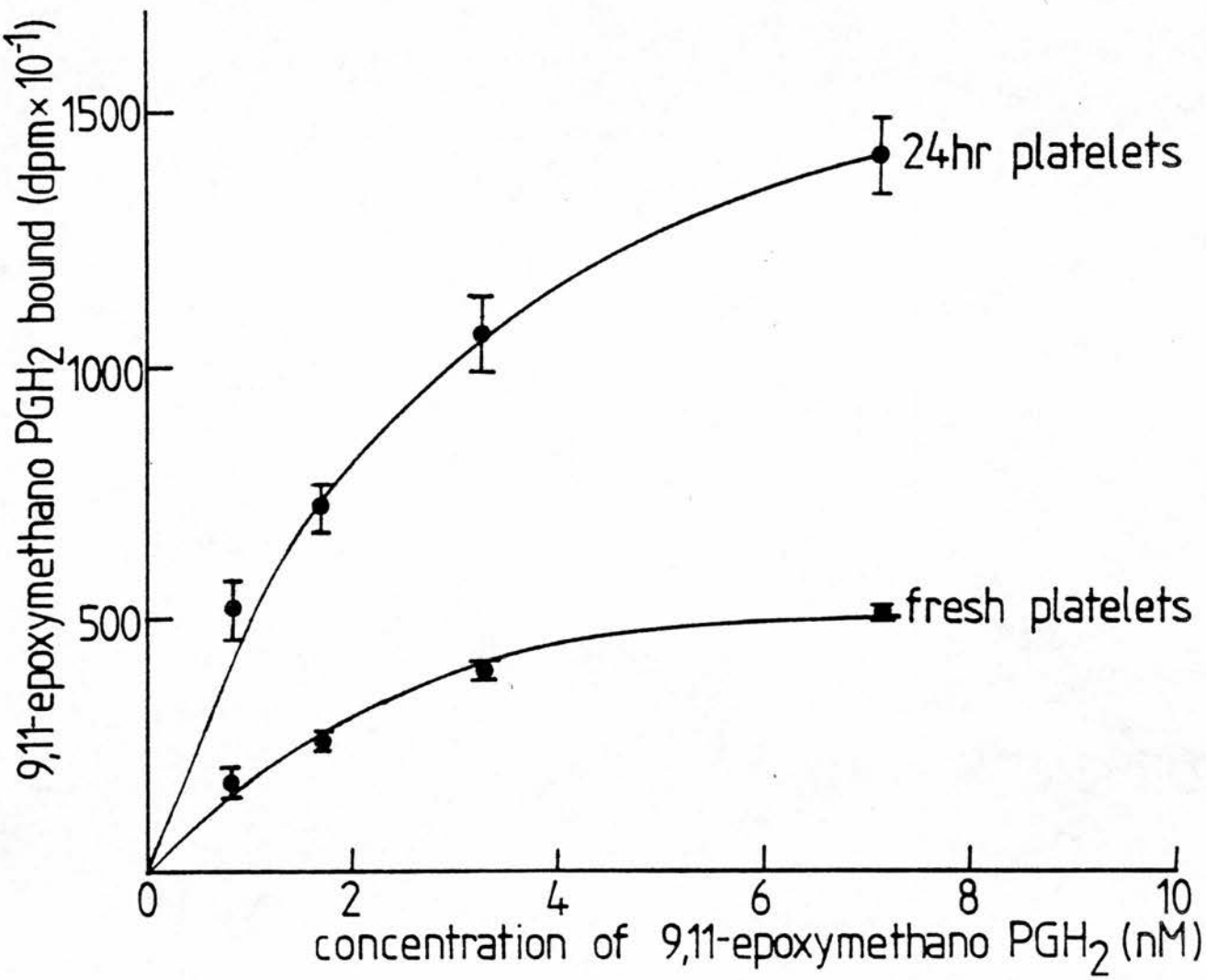
TABLE 5.3: The binding of ^{3}H 9,11-epoxymethano PGH_2 to both fresh and 24-hour platelets

	Concentration of 9,11-epoxymethano PGH_2 (nM)			
	0.86	1.72	3.57	7.14
DPM bound to 1 ml of fresh platelets	1824 ± 247	2631 ± 213	3998 ± 169	6173 ± 100
DPM bound to 1 ml of 24-hr platelets	5235 ± 616	7259 ± 536	10669 ± 885	14106 ± 812

These values represent the mean and standard error of 18 values, 3 donors.

Although considerably more ^{3}H 9,11-epoxymethano PGH_2 was bound by the 24-hour platelets this will reflect the higher platelet count (mean of 5.4×10^{8} platelets/ml compared to 2.62×10^{8} platelets/ml) rather than a greater capacity to bind 9,11-epoxymethano PGH_2. Since the binding of 9,11-epoxymethano PGH_2 followed a similar pattern with both types of platelets (Fig. 5.6), 24-hour platelets have been used throughout this study so that sufficient platelets were available to study binding over a much wider range of concentrations.

For Scatchard analysis of this binding, increasing concentrations of ^{3}H 9,11-epoxymethano PGH_2, ranging from 0.86 to 23.14 nM, were



Each result is the mean and standard error of 18 observations, 2 donors.

FIGURE 5.6: Comparison of binding of ^{3}H 9,11-epoxymethano PGH_2 to fresh and 24-hr platelets.

incubated for 4 minutes at room temperature with 1 ml of the platelet suspension. The dpm bound were converted into moles of ^{3}H 9,11-epoxymethano PGH_2 bound (specific activity of 13.9 Ci/mmole is equivalent to 13.9 x 2.2 x 10^6 dpm/n mole, i.e. 30,600,000 dpm/n mole) and divided by the platelet count to obtain the number of f moles bound/ 10^8 platelets. The values of 9,11-epoxymethano PGH_2 bound over this concentration range are given in Table 5.4.

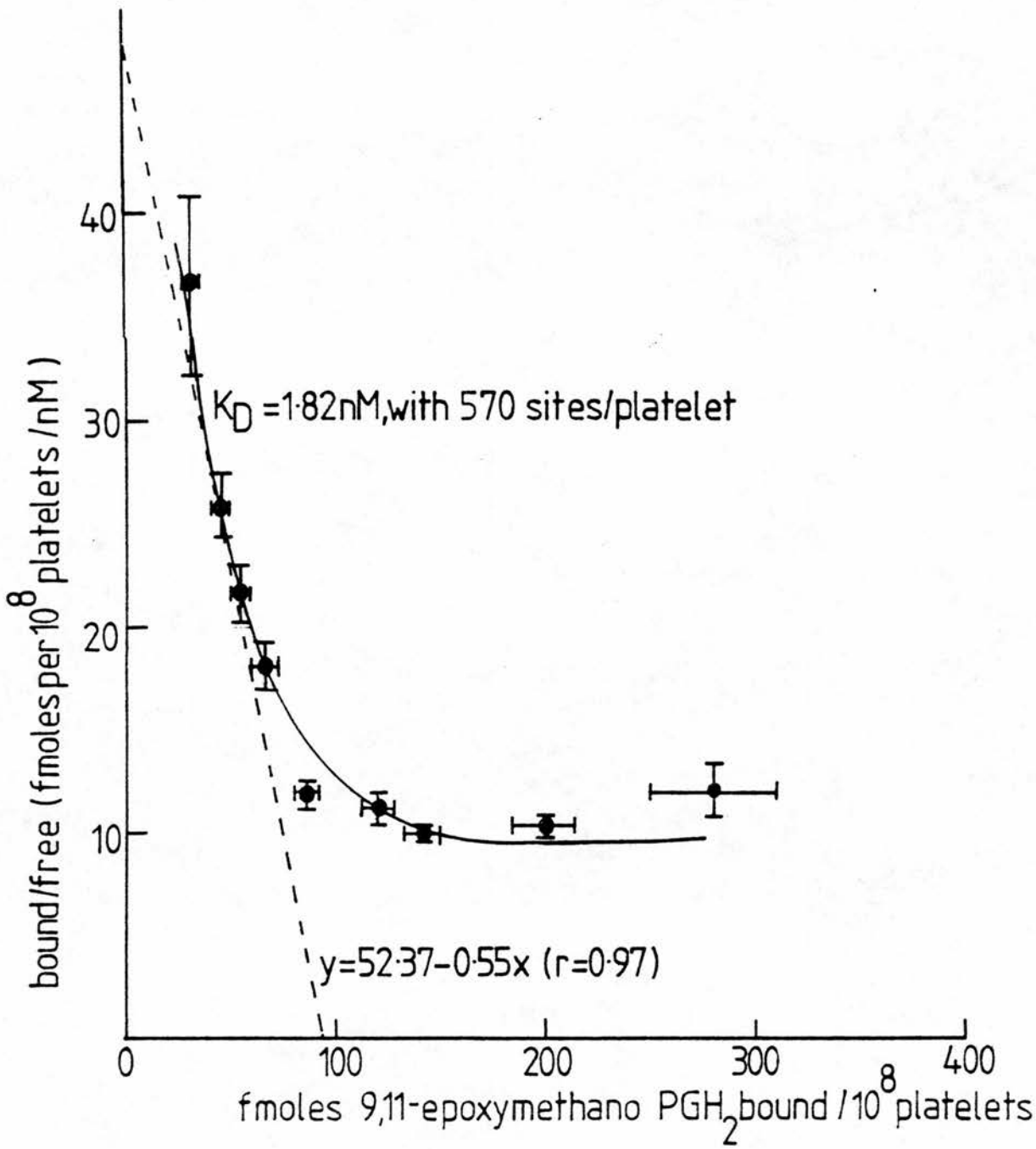
Ideally, radioligand binding studies should be performed so that at equilibrium less than 10% of the added radioligand is bound to the tissue (327). A loss of up to 10% of the added radioligand concentration will not significantly affect the estimation of binding constants if it is assumed that the added radioligand concentration is equal to the actual free concentration at equilibrium. The free concentration of 9,11-epoxymethano PGH_2 was in fact measured and was very close to that added. Indeed, in some cases (over) correction for quenching gave a final value slightly greater than the concentration added. Comparison of the dpm bound to the dpm added, over this concentration range, showed that 5.8 - 10.6% of the total counts were bound. A Scatchard plot of bound/free versus bound is shown in Fig. 5.7. Free is taken as the concentration of 9,11-epoxymethano PGH_2 added. Scatchard analysis yielded a hyperbolic plot indicating two types of binding. The higher affinity site has an equilibrium dissociation constant of 1.82 nM and a capacity of 570 sites per platelet. The lower affinity binding was non-saturable and was thought to reflect linear concentration of the lipophilic ligand in the platelet.

These results were expressed in the form of a Michaelis-Menten plot (bound against free) to check if the binding curve could be explained as the sum of a hyperbola (binding to a limited number of

TABLE 5.4: Scatchard analysis of the concentration-dependent binding of ^{3}H 15(S) 9,11-epoxymethano PGH_2 binding to whole human platelets

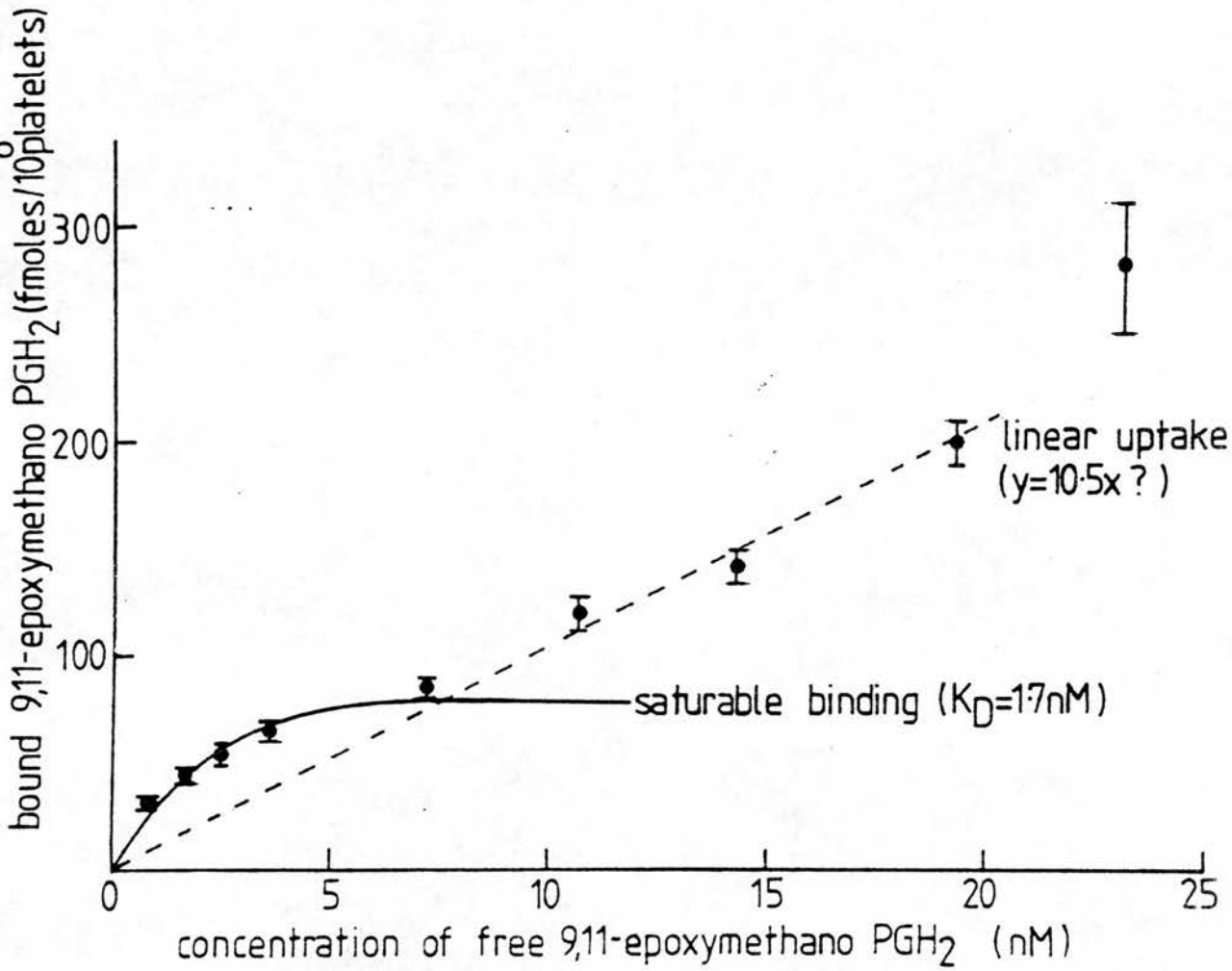
	Concentrations of ^{3}H 15(S) 9,11-epoxymethano PGH_2 added (nM)								
	0.86	1.70	2.50	3.57	7.14	10.71	14.29	19.28	23.14
Calculated bound 9,11-epoxymethano PGH_2 (f moles/10^8 platelets)	31.48 ±3.70	43.93 ±3.24	54.20 ±4.22	64.57 ±5.35	85.37 ±4.91	120.20 ±8.00	142.40 ±7.80	199.40 ±10.60	280.80 ±30.00
Bound / free (added)	36.64 ±4.30	25.84 ±1.90	21.68 ±1.70	18.09 ±1.50	11.90 ±0.70	11.20 ±0.74	9.96 ±0.03	10.33 ±0.55	12.10 ±1.30

These values represent the mean and standard errors of 24 results, 6 donors.



Each result is the mean and standard error of 24 observations, 6 donors. The free concentration has been taken as the concentration of ^3H 9,11-epoxymethano PGH_2 added to the platelets.

FIGURE 5.7: Scatchard analysis of the concentration-dependent binding of ^3H 15(S)9,11-epoxymethano PGH_2 to whole platelets.



A possible explanation of the observed plot is that it represents the sum of binding to a limited number of sites (hyperbola) and linear uptake of the lipophilic ligand.

FIGURE 5.8: Michaelis-Menten plot of the concentration-dependent binding of ^{3}H 15(S) 9,11-epoxymethano PGH_2 to whole platelets.

sites) and a linear component (Fig. 5.8). If the point (23.14, 280.8) is omitted, the other points fit this explanation. The plot of C_{BL} (y axis) against C_L (x axis) is described by the Michaelis Menten equation:

$$C_{BL} = \frac{C°_B C_L}{K_D + C_L}$$

where C_{BL} is the molar concentration of ligand occupied sites, $C°_B$ the total number of binding sites, C_L the concentration of free ligand and K_D the equilibrium dissociation constant. This equation can be rearranged to give:

$$K_D = \frac{C°_B C_L}{C_{BL}} - C_L$$

when $C°_B/C_{BL}$ is two, i.e. when half the total number of binding sites are occupied, K_D is equal to C_L, the concentration of ligand required to occupy 50% of the binding sites. The equilibrium dissociation constant (K_D) estimated from this hyperbola is 1.7 nM.

However, it is not necessarily correct to ignore the last point (23.14, 280.8). When one or more points appear atypical, perhaps due to error in experimental technique or measurement, methods have been described to give these points little credance when analysing Scatchard data so that the values estimated for the number of binding sites and the dissociation constant will not be greatly affected by these points (328, 329). This appears to be a slightly misleading practice in cases where these atypical values represent a 'real effect', as will be shown later for 9,11-epoxymethano PGH_2. Certainly, it did not seem justifiable to ignore this point as this concentration of 9,11-

experimental points	(0·86, 31·48) (1·7, 43·93) (2·5, 54·2) (3·57, 64·57)
points expected for linear uptake only	(0·86, 9·03) (1·7, 17·85) (2·5, 26·25) (3·57, 37·48)
points resulting when linear uptake is subtracted	(0·86, 22·45) (1·7, 26·08) (2·5, 27·95) (3·57, 27·08)

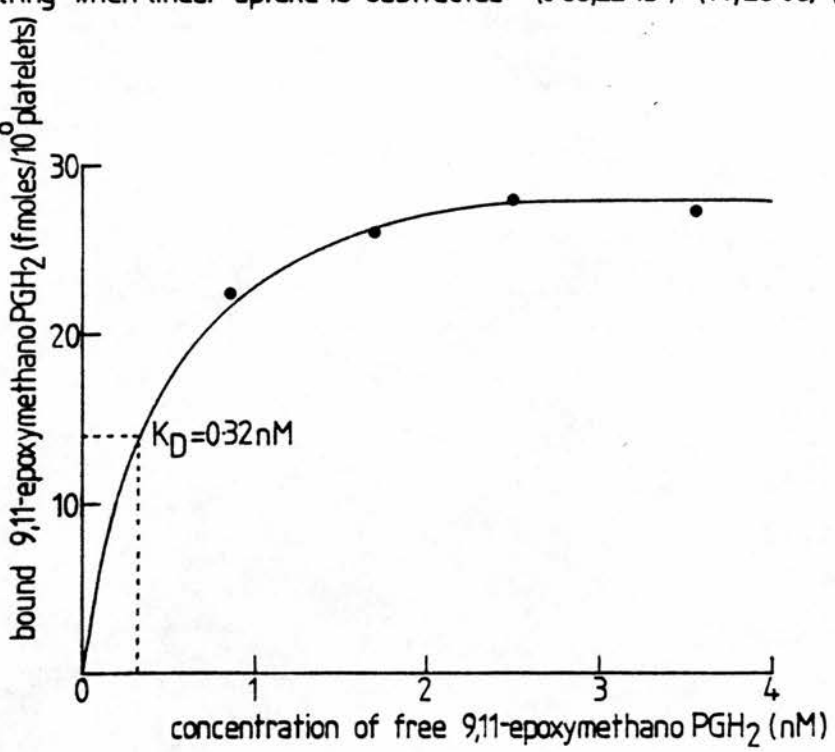


FIGURE 5.9: Michaelis-Menten plot of binding of ^{3}H 15(S) 9,11-epoxymethano PGH_2 to whole platelets, when the effect of linear uptake of the ligand is subtracted.

epoxymethano PGH_2 produced a marked increase in binding in each of the six individuals tested. Furthermore, the dissociation constant of 1.7 nM measured from the Michaelis-Menten plot is fairly close to that estimated from Scatchard analysis, 1.82 nM. However, if the effect of linear uptake is subtracted from the points of the hyperbola, the equilibrium dissociation constant is estimated as 0.32 nM (Fig. 5.9).

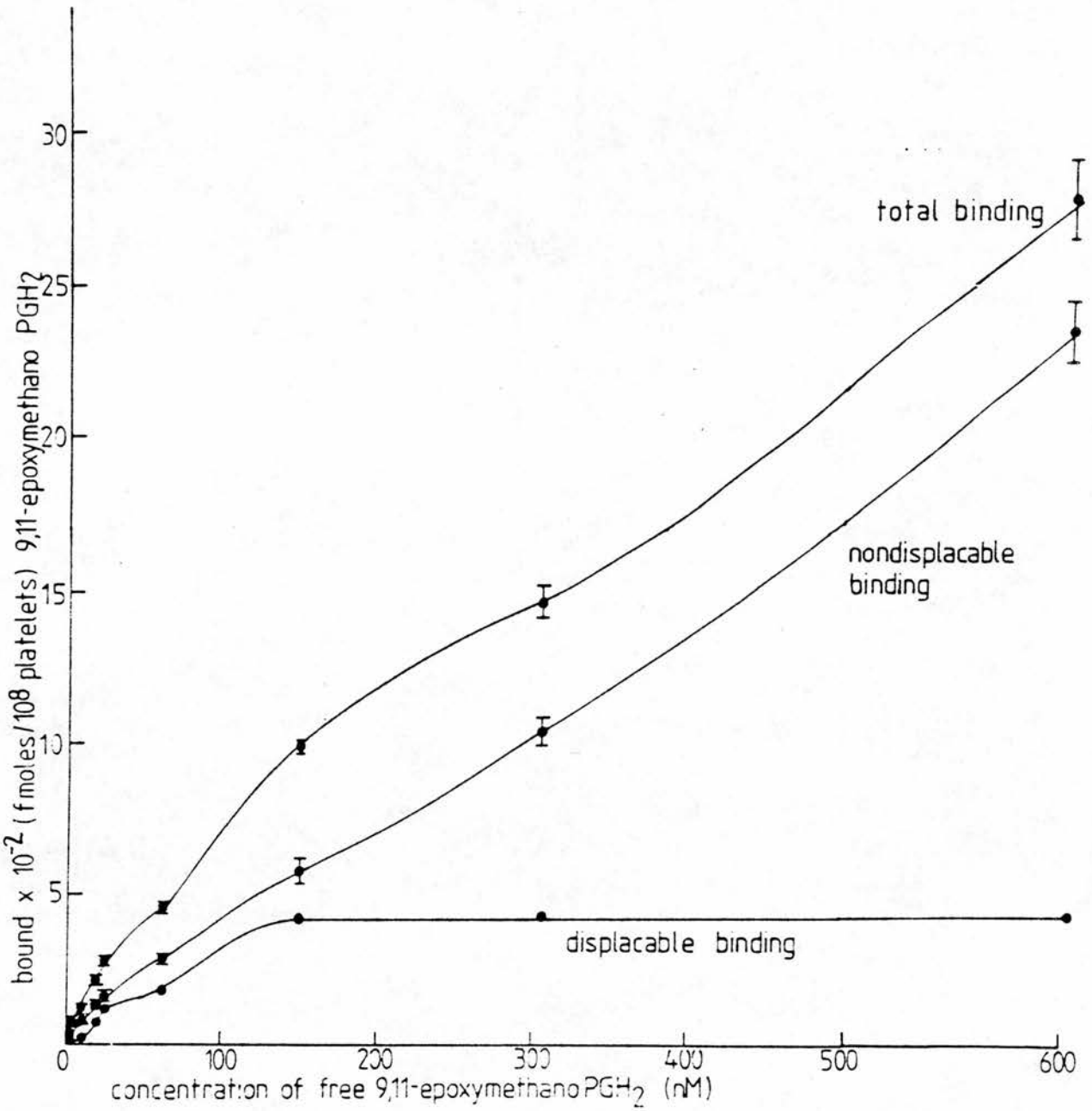
Further investigations were then performed in which the binding of ^3H (15S) 9,11-epoxymethano PGH_2 was studied over a much greater concentration range (0.86-607 nM). The higher concentrations were achieved by diluting the specific activity of the radioligand with cold 9,11-epoxymethano PGH_2. Each concentration was tested in the presence and absence of excess cold 9,11-epoxymethano PGH_2 (11.4 μM). Since only specific binding should be displacable, it was hoped that subtraction of the values obtained for binding in presence of excess 'cold' from total binding would give a value for specific binding (Table 5.5). Michaelis-Menten plots of this data are shown in Fig. 5.10. The specific binding curve is complex revealing a small saturable component at low concentrations and an S-shaped saturable component at higher concentrations which represented by far the major component of binding. This finding emphasises the importance of analysing data over a wide concentration range. With Scatchard analysis it is advisable to obtain plots with points which came very close to crossing both the y and the x axes, so that the graph can easily be extrapolated to cross both axes.

There is considerable non-displacable binding: this ranges from 51.5% at low concentrations to 84.6% at high concentrations, and will considerably reduce the accuracy with which displacable binding can be determined. Non-specific binding has proved to be high in opiate binding studies (330). This is partly due to physical solution of the

TABLE 5.5: A Binding of ^{3}H 9,11-epoxymethano PGH_2 to whole human platelets

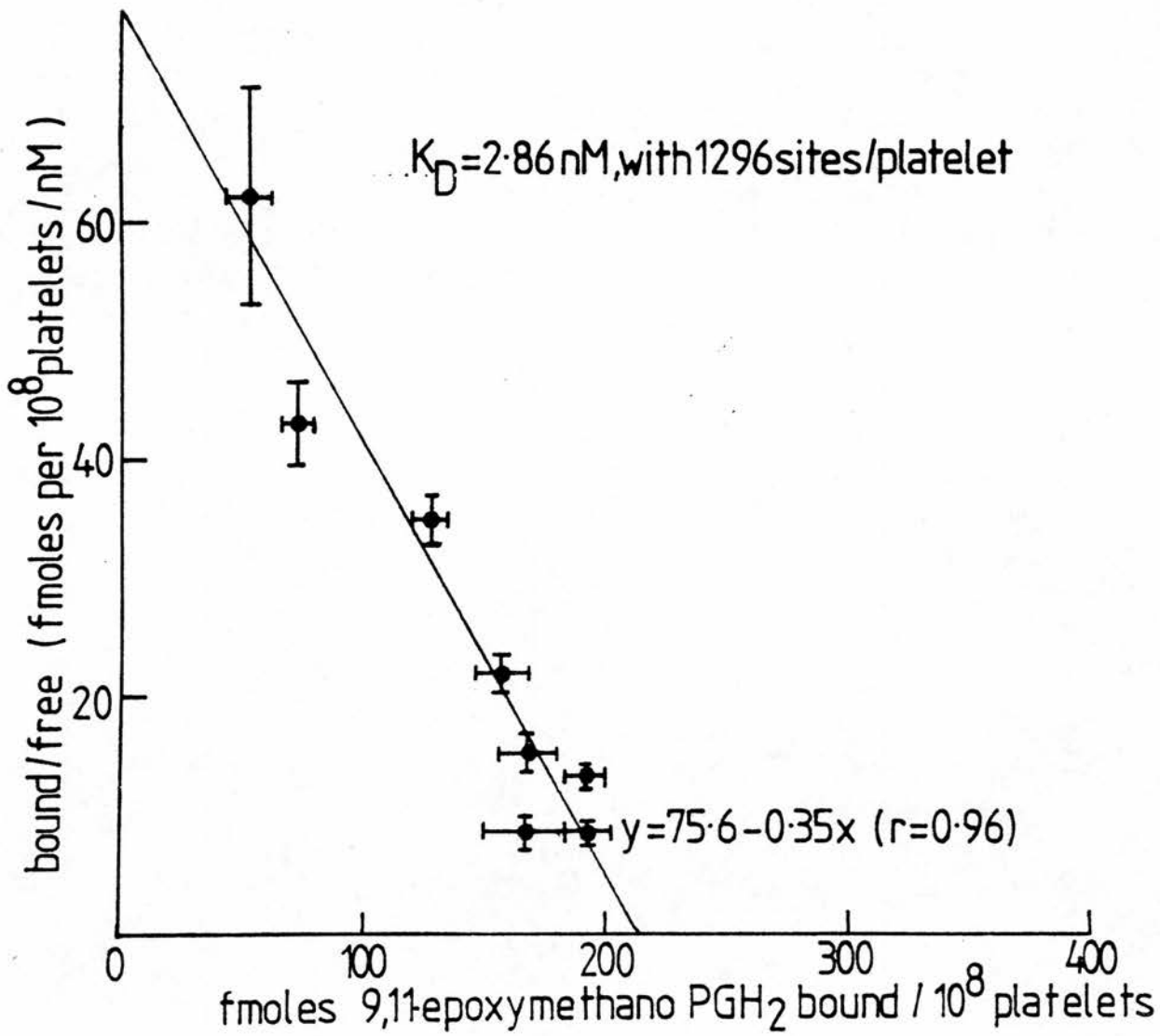
	Concentration of ^{3}H 9,11-epoxymethano PGH_2 added (nM)												
	0.86	1.7	2.5	3.57	7.14	10.71	14.29	19.28	23.14	60.7	151.75	303.5	607.0
Bound (f moles/ 10^8 platelets)	22.8 ±1.5	31.1 ±1.8	39.4 ±2.0	60.9 ±4.3	90.5 ±5.7	113.4 ±4.6	156.1 ±5.4	207.8 ±11.7	272.5 ±16.8	455.3 ±16.8	985.7 ±15.1	1460.9 ±41.9	2777.1 ±125.0
B Binding in the presence of 11.4 μM 9,11-epoxymethano PGH_2													
Bound (f moles/ 10^8 platelets)	11.7 ±1.8	16.3 ±2.7	23.2 ±3.0	35.3 ±3.1	52.6 ±6.2	73.3 ±5.2	92.4 ±3.8	128.8 ±9.9	153.6 ±5.0	280.6 ±10.3	571.6 ±48.5	1Q36.5 ±41.9	2350.8 ±103.7
C Estimated non-displacable binding													
Bound (f moles/ 10^8 platelets)	11.0	14.8	26.2	25.6	37.9	40.2	63.7	79.1	118.9	174.7	414.1	424.5	426.3

These results are the mean and standard errors of 12-18 results, 3-4 donors



Each result is the mean and standard error of 12-18 observations, 3-4 donors.

FIGURE 5.10: Michaelis-menten plot of binding of ^3H 15(S) 9,11-epoxymethano PGH_2 to whole platelets over a greater concentration range. Displacable binding has been estimated as the portion of total binding displacable by excess cold 15(S) 9,11-epoxymethano PGH_2 (11.4 μM).

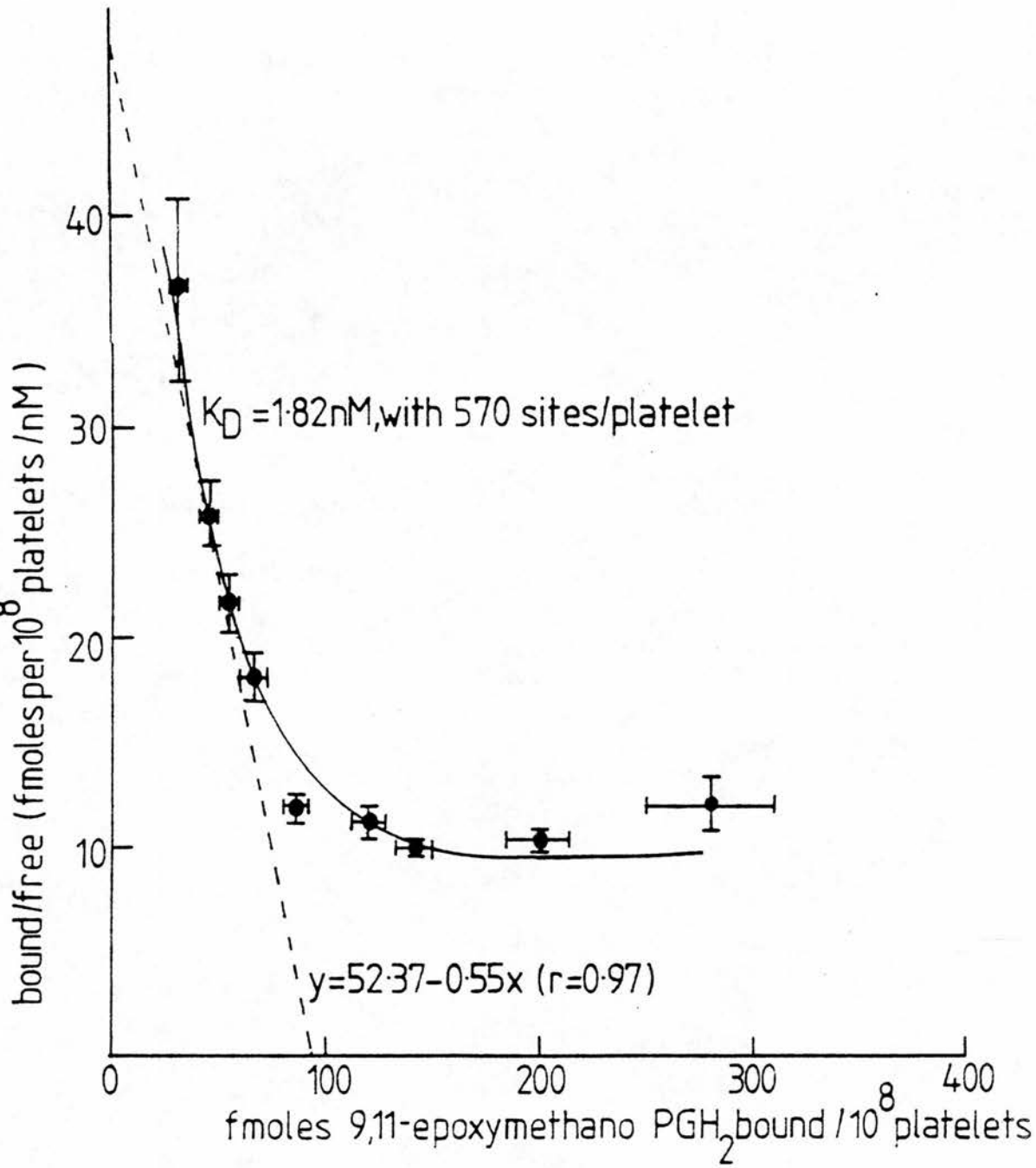


Each result is the mean and standard error of 18 observations, 3 donors.

(A)

FIGURE 5.11: Comparison of Scatchard analysis of the binding of 15(R) with 15(S) ^3H 9,11-epoxymethano PGH_2 to whole platelets.

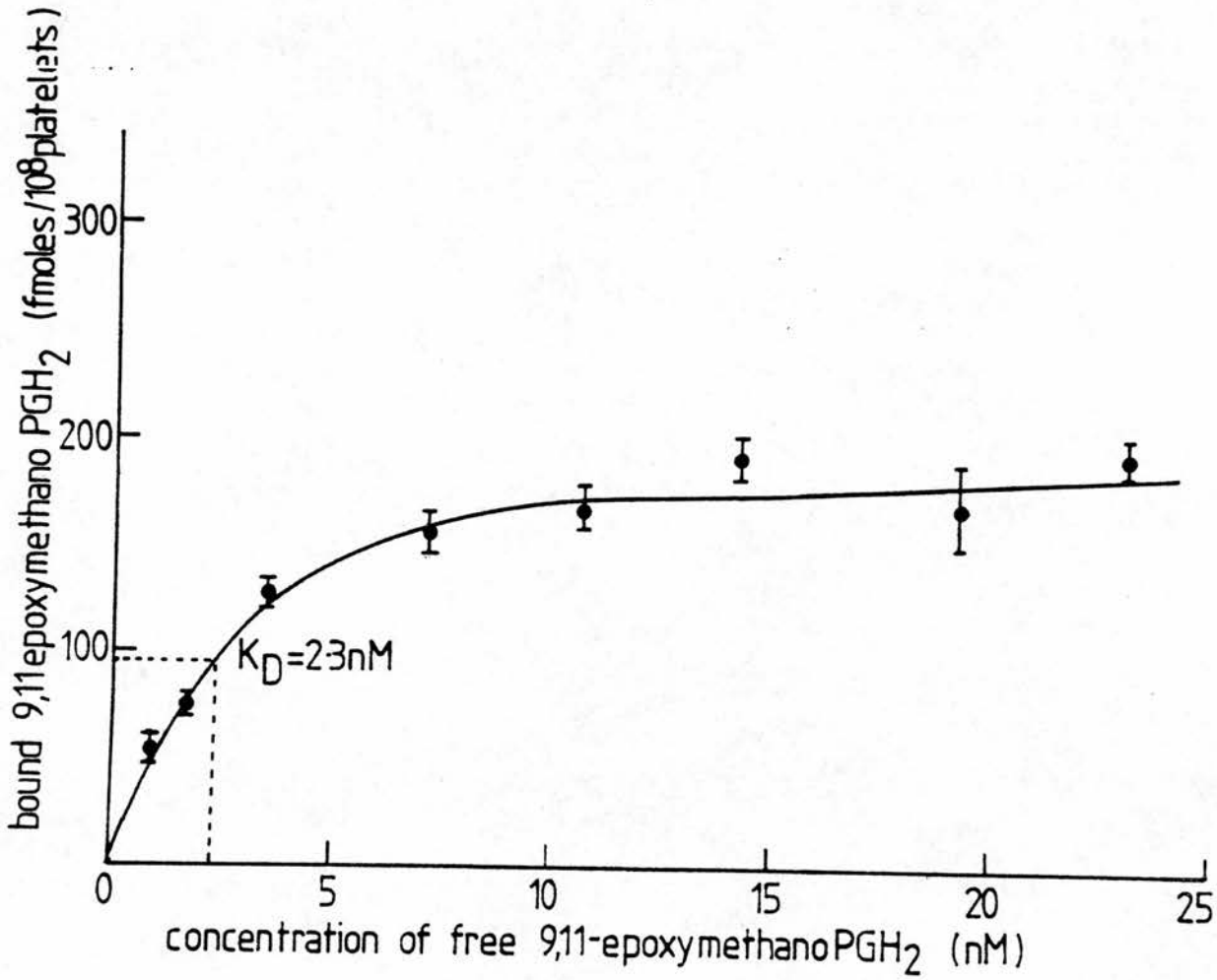
(A) concentration-dependent binding of ^3H 15(R)-9,11-epoxymethano PGH_2 to whole platelets.



Each result is the mean and standard of 24 observations, 6 donors.

(B)

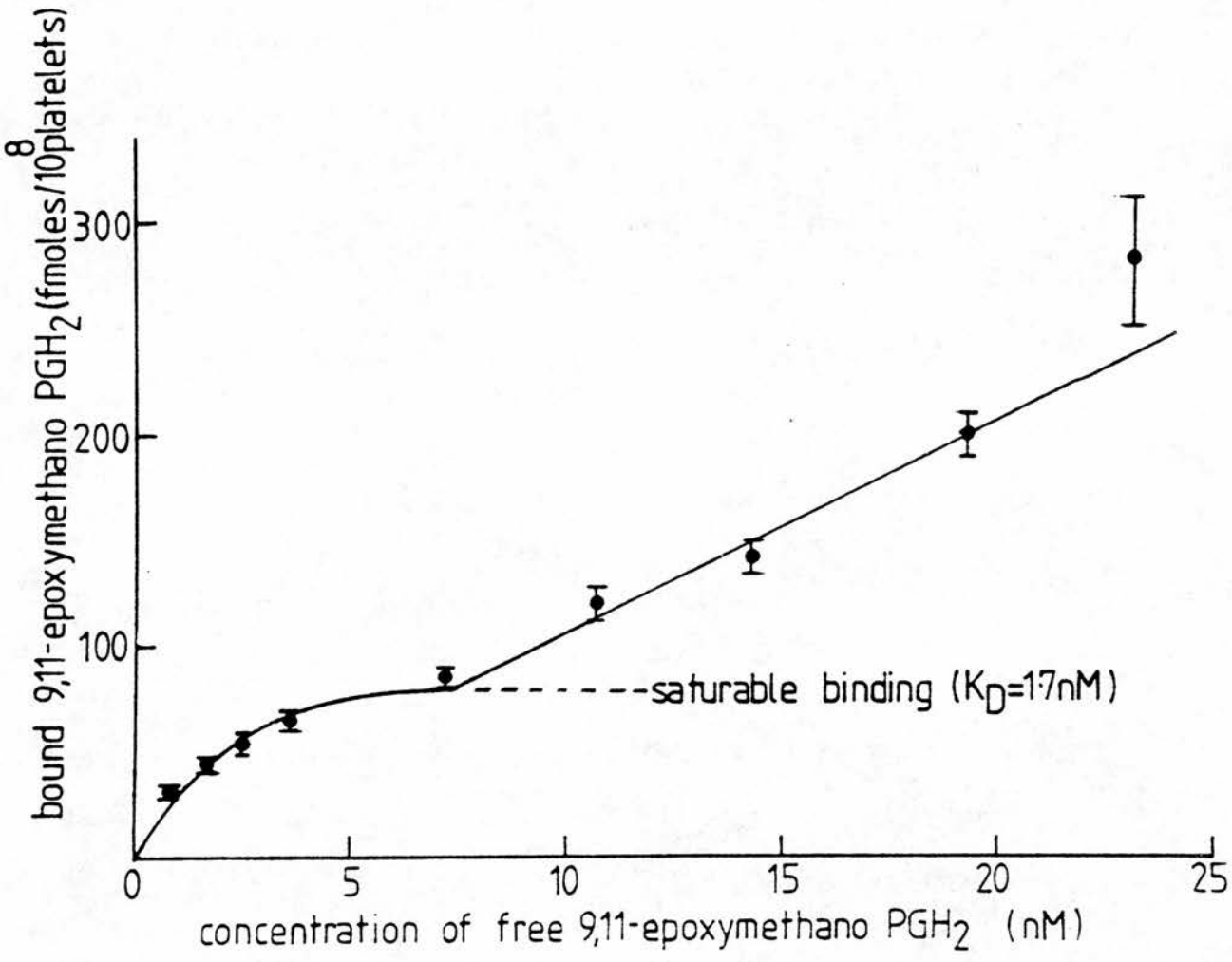
FIGURE 5.11: (B) concentration-dependent binding of ^3H 15(S)-9,11-epoxymethano PGH_2 binding to whole platelets.



(A)

FIGURE 5.12: Comparison of Michaelis-menten plots of the binding of 15(R) with 15(S) ^{3}H 9,11-epoxymethano PGH_2 to whole platelets.

(A) - concentration-dependent binding of ^{3}H 15(R) 9,11-epoxymethano PGH_2 to whole platelets.



(B)

FIGURE 5.12: (B) - concentration-dependent binding of ^{3}H 15(S) 9,11-epoxymethano PGH_2 to whole platelets.

lipophilic opiate molecules in the lipid membranes but is also due to a non-specific saturable binding associated with the ionic interaction between the protonated nitrogen of the opiate and ionic groups on the membrane. This latter type of binding is exhibited by both (+) and (-) configurations of the opiate and is unrelated to the strongly stereospecific binding of the (-) opiate to the receptor.

The binding of the 15(R) configuration of ^{3}H 9,11-epoxymethano PGH_2 to platelets was studied. Concentrations of 15(R) ^{3}H 9,11-expoxymethano PGH_2 ranging from 0.86 - 23.14 nM were incubated for 4 minutes at room temperature with 1 ml of the platelet suspension (Table 5.6). Fig. 5.11 shows a comparison of the Scatchard plots obtained for the 15(R) and 15(S) forms of ^{3}H 9,11-epoxymethano PGH_2. Fig. 5.12 shows a comparison of the Michaelis-Menten plots.

The data clearly suggests that in low concentrations (0.86 - 20 nM) both 15(R) and 15(S) bind non-specifically to a limited number of binding sites, presumably on the surface of the platelet membrane. The specific receptor-binding of 15(S) does not become apparent until slightly higher concentrations are used ($\geqslant$20 nM). Indeed, it is at these concentrations that 9,11-epoxymethano PGH_2 is active as an aggregating agent. If both the non-specific saturable binding and non-saturable lipophilic binding are subtracted from the total binding, an estimate of the stereospecific receptor binding should be achieved. The Michaelis-Menten plot of this binding is shown in Fig. 5.13 from this plot the dissociation constant can be estimated at 65 nM and the number of binding sites as 2400 per platelet. Both of these estimates are very approximate.

Displacement studies were carried out using a fixed concentration of ^{3}H 15(S) 9,11-epoxymethano PGH_2 (71 nM) which was incubated

TABLE 5.6: Scatchard analysis of the concentration-dependent binding of ^{3}H 15(R) 9,11-epoxymethano PGH_2 binding to whole human platelets

	Concentration of ^{3}H 15(R) 9,11-epoxymethano PGH_2 added (nM)							
	0.86	1.70	3.57	7.14	10.71	14.29	19.28	23.14
Calculated bound 9,11-epoxymethano PGH_2 (f moles/10^8 platelets)	53.61 ±7.76	73.15 ±5.81	127.90 ±7.26	157.76 ±10.94	166.83 ±11.05	193.72 ±9.94	167.15 ±18.80	193.28 ±7.96
Bound / free (added)	62.34 ±9.02	43.03 ±3.40	35.80 ±2.03	22.09 ±1.53	15.57 ±1.03	13.55 ±0.69	8.67 ±0.97	8.35 ±0.34

These values represent the mean and standard errors of 24 results, 6 donors.

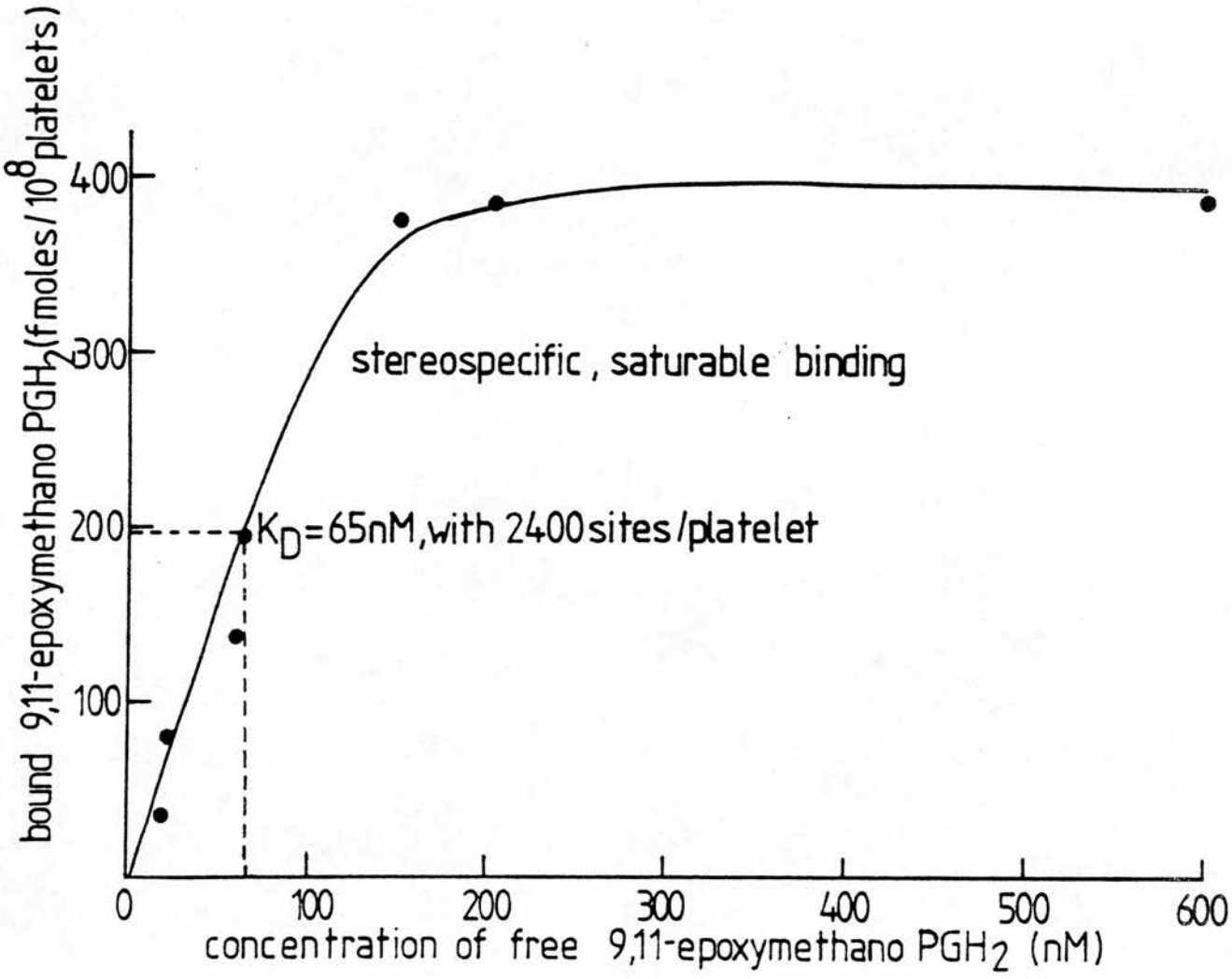


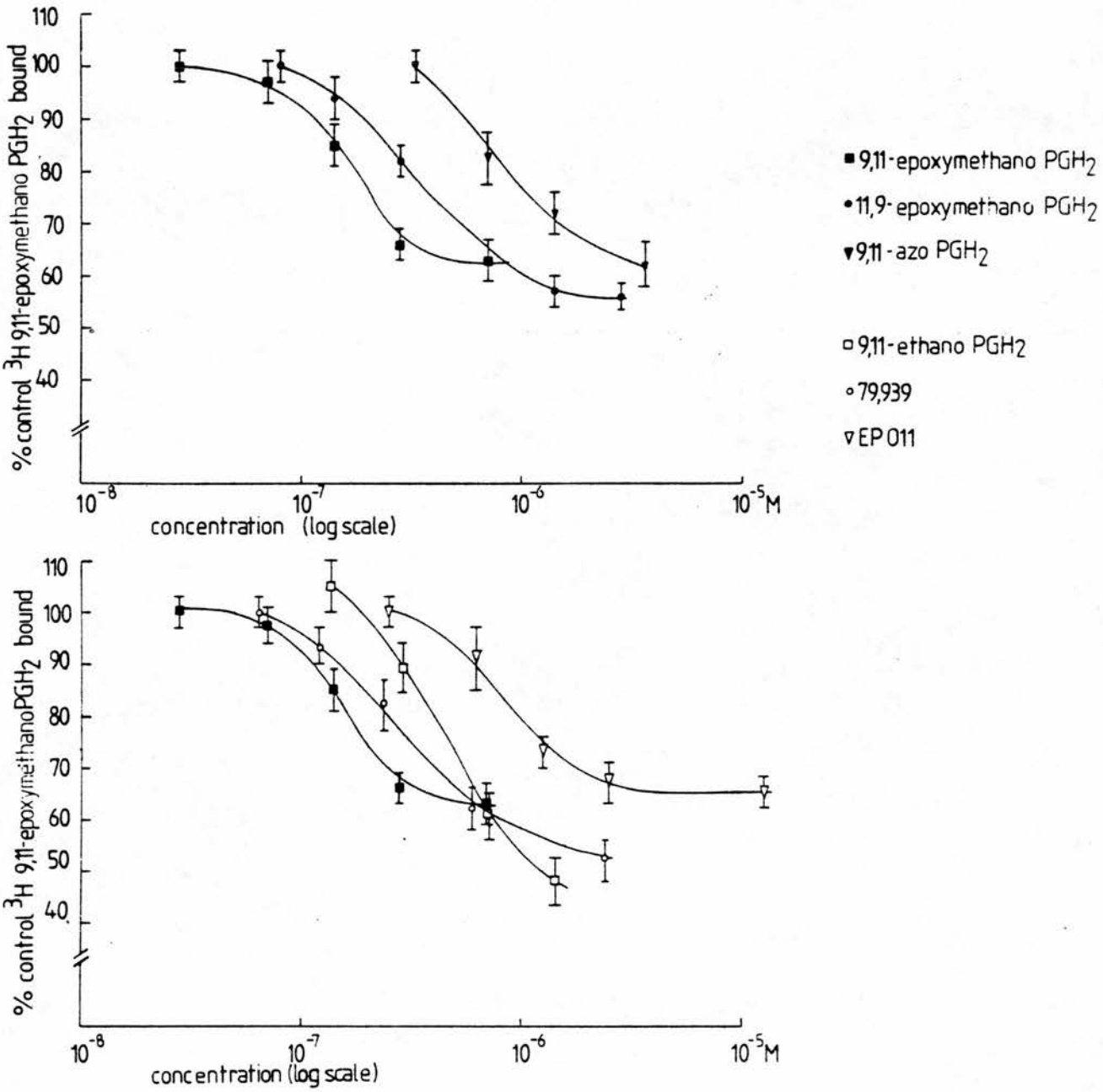
FIGURE 5.13: Michaelis-Menten plot of the stereospecific, saturable binding of ^{3}H 15(S) 9,11-epoxymethano PGH_2 to whole platelets.

Stereospecific, saturable binding has been estimated by subtracting the saturable component of binding evident with both 15(R) and 15(S) ^{3}H 9,11-epoxymethano PGH_2 from total displacable binding observed for 15(S) 9,11-epoxymethano PGH_2.

TABLE 5.7: Displacement of ^{3}H 15(S) 9,11-epoxymethano PGH_2 binding to whole platelets, by analogues with thromboxane-like activity

Analogue	Concentration (μM)	% Bound
9,11-epoxymethano PGH_2	0.071	97.2 ± 3.7
	0.142	84.9 ± 4.3
	0.284	66.6 ± 3.5
	0.710	62.5 ± 4.2
11,9-epoxymethano PGH_2	0.142	93.9 ± 4.0
	0.284	81.8 ± 4.6
	1.42	57.1 ± 4.4
	2.84	57.3 ± 2.3
9,11-azo PGH_2	0.287	100.0 ± 5.9
	0.718	84.4 ± 5.6
	1.436	72.5 ± 4.2
	3.59	62.0 ± 3.8
	7.18	46.2 ± 6.2
9,11-ethano PGH_2	0.143	105.7 ± 9.6
	0.286	89.2 ± 9.4
	0.714	61.0 ± 5.7
	1.43	56.3 ± 5.0
	2.86	48.0 ± 4.6
EP011	0.249	101.5 ± 6.6
	0.623	91.3 ± 6.3
	1.247	73.6 ± 4.8
	2.494	67.3 ± 4.2
	12.469	65.7 ± 3.0
ICI 79,939	0.119	93.0 ± 4.6
	0.237	82.6 ± 4.9
	0.594	61.8 ± 3.9
	2.375	52.5 ± 4.2

Results are given as the mean and standard error of 12-18 observations, 3 donors.



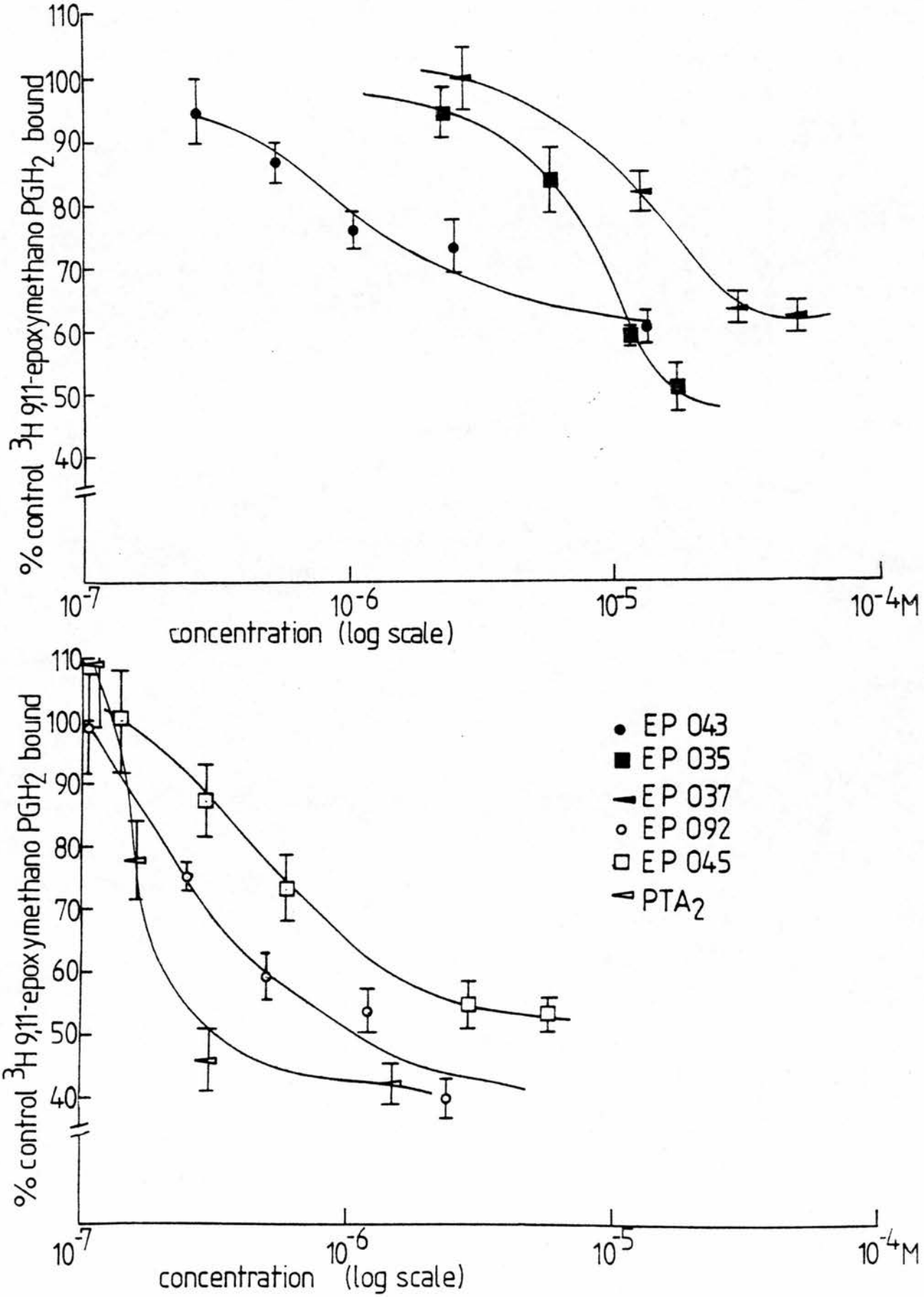
Each result is the mean and standard error of 12-18 observations, 3 donors.

FIGURE 5.14: Displacement of ^{3}H 15(S) 9,11-epoxymethano PGH$_2$ (71 nM) bound to whole platelets by analogues with thromboxane-like activity.

TABLE 5.8: Displacement of ^{3}H 15(S) 9,11-epoxymethano PGH_2 binding to whole platelets, by analogues with thromboxane-antagonist activity

Analogue	Concentration (μM)	% Bond
EP035	2.31	94.7 ± 4.1
	5.775	83.6 ± 6.2
	11.55	58.2 ± 3.4
	17.325	50.8 ± 3.9
EP037	2.674	100.0 ± 5.0
	13.369	81.8 ± 3.3
	20.053	63.0 ± 3.2
	40.107	62.1 ± 2.6
EP043	0.267	94.7 ± 5.7
	0.534	86.9 ± 3.2
	1.068	75.9 ± 3.3
	2.67	73.1 ± 4.8
	14.35	60.0 ± 3.2
EP045	0.133	100.0 ± 8.3
	0.267	87.1 ± 6.1
	0.534	73.1 ± 5.5
	1.335	54.4 ± 4.2
	2.670	53.1 ± 1.5
EP092	0.062	100.0 ± 8.7
	0.248	74.9 ± 3.7
	0.496	59.2 ± 4.1
	1.240	53.5 ± 3.1
	2.480	39.2 ± 3.6
pinane TXA_2	0.61	107.5 ± 9.1
	1.575	77.6 ± 6.7
	3.05	46.4 ± 4.9
	15.25	42.2 ± 3.4

Results are given as the mean and standard error of 12-18 observations, 3 donors.



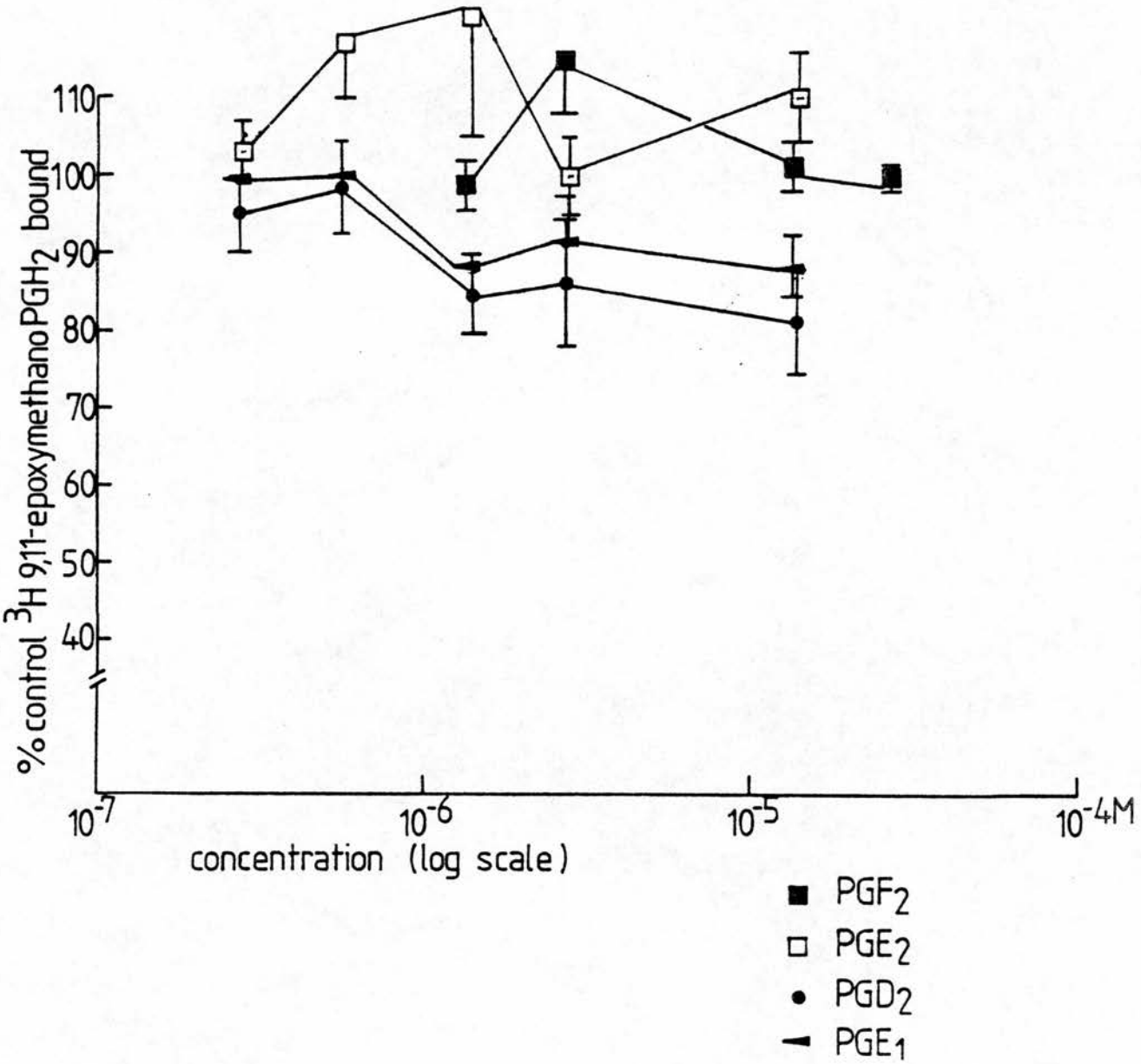
Each result is the mean and standard error of 12-18 observations, 3 donors.

FIGURE 5.15: Displacement of ^{3}H 15(S) 9,11-epoxymethano PGH_2 (71 nM) bound to whole platelets by analogues with thromboxane-antagonist activity

TABLE 5.9: Displacement of ^{3}H 15(S) 9,11-epoxymethano PGH_2 binding to whole platelets, by natural prostaglandins thought to act on receptors distinct from the TXA_2 receptor

Prostaglandin	Concentration (μM)	% Bound
$PGF_{2\alpha}$	1.374	99.0 ± 3.3
	2.747	114.6 ± 5.9
	13.736	101.7 ± 2.7
	27.472	99.2 ± 2.4
PGE_2	0.276	103.2 ± 4.2
	0.552	116.1 ± 6.0
	1.381	119.0 ± 14.0
	2.762	99.5 ± 5.5
	13.812	110.3 ± 6.6
PGD_2	0.276	95.2 ± 5.1
	0.552	98.4 ± 5.9
	1.381	84.2 ± 6.0
	2.762	86.6 ± 8.1
	13.812	81.1 ± 6.8
PGE_1	0.275	98.3 ± 4.4
	0.549	107.7 ± 5.8
	1.374	87.9 ± 6.0
	2.747	91.5 ± 5.8
	13.746	86.7 ± 6.1

Results are given as the mean and standard error of 12-18 observations, 3 donors.



Each result is the mean and standard error of 12-18 observations, 3 donors.

FIGURE 5.16: Displacement of ^{3}H 15(S) 9,11-epoxymethano PGH$_2$ (71 nM) bound to whole platelets by other prostaglandins.

simultaneously with the displacing agent and 1 ml of platelet suspension at room temperature for 6 minutes. Unfortunately due to the high non-specific binding only about 50% of the total binding is actually displacable. The results of these displacement studies form three groups: thromboxane mimics (Table 5.7, Fig. 5.14), thromboxane antagonists (Table 5.8, Fig. 5.15) and other prostaglandins (Table 5.9, Fig. 5.16).

DISCUSSION

When platelets are incubated with ^{3}H 15(S) 9,11-epoxymethano PGH_2, the bound radioactivity appears to be a measure of three types of binding:

1. the non-saturable uptake of the lipophilic ligand into the platelet membrane;
2. the non-specific saturable binding, possibly to ionic groups on the membrane;
3. the stereospecific, saturable binding to the receptor.

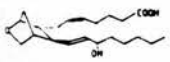
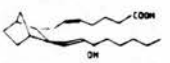
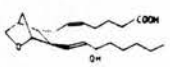
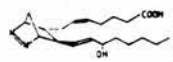
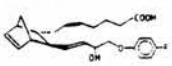
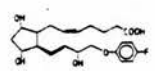
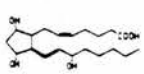
Both the 15(R) and 15(S) conformations of ^{3}H 9,11-epoxymethano PGH_2 bind in very low concentrations (0.86-23.14 nM) to a saturable binding site on the platelet membrane. The 15(S) form shows a dissociation constant of 1.82 nM and the 15(R) 2.86 nM for this site and give estimates of 570 and 1296 sites per platelet respectively. The nature of this interaction is not known but may involve electrostatic attraction between the carboxylate ion of 9,11-epoxymethano PGH_2 and a charged nitrogen species. Both 15(R) and 15(S) forms of the ligand will be taken up into the lipophilic membranes of the platelet. This effect is non-saturable and will not be displaced by excess 'cold' 9,11-epoxymethano PGH_2. Uptake of the lipophilic ligand can be estimated by measuring the

binding of ^3H 15(S) 9,11-epoxymethano PGH_2 in the presence of excess cold. If this is subtracted from the total binding it gives a measure of the displacable component of binding and if the non-specific saturable component is also subtracted, the resultant binding gives a measure of the stereospecific receptor binding. Analysis of the data suggests that ^3H 15(S) 9,11-epoxymethano PGH_2 binds with a dissociation constant of 65 nM to a limited number of sites, estimated at 2400 per platelet.

The binding of ^3H 15(S) 9,11-epoxymethano PGH_2 to whole platelets is both stereospecific and saturable, and the receptor has a high affinity for the radioligand. Furthermore, the saturable component of binding can be displaced by thromboxane mimics and antagonists but not by $PGF_{2\alpha}$, PGD_2, PGE_2 or PGE_1 (in concentrations up to 14 μM) which are thought to act on $PGF_{2\alpha}$, PGD_2, PGE_2, PGE_2/I_2 receptors respectively. Indeed, the $PGF_{2\alpha}$ analogue ICI 79,939, which has been shown to have thromboxane-like activity, can displace the saturable component of binding whereas $PGF_{2\alpha}$ itself cannot. Those compounds capable of producing irreversible platelet aggregation show high affinity for the saturable binding site and 9,11-ethano PGH_2, which produces only reversible aggregation and antagonises the action of 11,9-epoxymethano PGH_2 consistent with its proposed partial agonist action, shows a particularly high affinity for the binding site. A comparison of displacement of ^3H 9,11-epoxymethano PGH_2 binding by these agonists and their aggregatory actions on human platelets are shown in Fig. 5.17.

The affinity constant of an antagonist can be estimated from the concentration of the antagonist required to displace 50% of the saturable binding;

$$K_D = \frac{x_{B50}}{1 + C_L}$$

ompound	Structure	Concentration required for 50% displacement of saturable binding (μM)	Aggregatory action on platelets	Concentration required for aggregation (μM) *
-epoxy nano PGH_2		0.2	Irreversible	0.54
-ethano +		0.89	Reversible only	10-50
-epoxy nano PGH_2		2.1	Irreversible	0.3
-azo PGH_2		1.2	Irreversible	0.073
+ 011		1.9	Irreversible	0.34
+ 79,939		0.27	Irreversible	0.72
α		>30	No effect	>100
037		16	Blocks aggregation	-

The concentration of labelled ligand was 0.071 μM. Labelled and unlabelled compounds were added simultaneously and incubated for 6 minutes at room temperature.

+ racemic compounds

* reversible aggregation - wave of 20 units magnitude on chart recorder - mean of at least 3 determinations.

FIGURE 5.17: A comparison of displacement of ^3H 9,11-epoxymethano PGH_2 binding and aggregatory actions on human platelets.

where K_D is the equilibrium dissociation constant of the antagonist;

x_{B50} is the concentration required to displace 50% of the binding;

C_L is the concentration of the radioligand.

Table 5.10 shows a comparison of the affinity constant ($^1/K_D$) of the antagonists estimated from the displacement studies compared to those estimated by Dr. R.L. Jones from the shift of the Log dose-response curve to the aggregating agent 11,9-epoxymethano PGH_2. The same order of potency is seen for the affinity constants measured by these two methods.

The results of these displacement studies are consistent with the stereospecific saturable component of binding reflecting binding to the thromboxane receptor since only the drugs which are pharmacologically effective at this receptor are capable of displacing this component of binding. Furthermore, both agonists and antagonists show displacement of binding in the concentration range in which they are effective as inducers or inhibitors of platelet aggregation.

The variation in the maximum amount of displacable binding reflects the degree of non-saturable binding. This varies from donor to donor and as these results are the mean of only three donors, the variation is considerable. In retrospect it may have been better to standardise each displacement experiment by including a control concentration of cold 9,11-epoxymethano PGH_2 which would displace all the saturable binding. This could have been taken as 100% displacement for the particular platelets tested, and the displacement observed for the analogues measured with respect to this control.

The non-saturable binding is particularly high owing to the lipophilic nature of 9,11-epoxymethano PGH_2, which has a partition coefficient of 19 between $CHCl_3$ and H_2O (pH 7.4) compared to 0.0091 for a water soluble compound, $PGF_{2\alpha}$. To minimise the non-saturable uptake

TABLE 5.10: Estimates of the affinity constants (x 10^{-6} M) of the analogues from both displacement and aggregation experiments

Analogue	Concentration required for 50% displacement (μM)	Equilibrium dissociation constant (10^6 M)	Affinity constant (displacement experiments)	Affinity constant (aggregation experiments)*
EP037	16.0	15.0	0.067	0.46
EP043	1.0	0.99	1.01	1.2
EP045	0.51	0.53	1.88	0.87
EP092	0.25	0.24	4.16	20.0
Pinane TXA_2	2.0	1.9	0.526	0.42

*The agonist used in these determinations is 11,9- not 9,11-epoxymethano PGH_2.

of the ligand it may have been better to use a less lipophilic radioligand. ICI 79,939 (Fig. 1.4) is very much more water soluble but will also bind to $PGF_{2\alpha}$ receptors with a high affinity. Although it is not likely that platelets contain $PGF_{2\alpha}$ receptors, as $PGF_{2\alpha}$ has little effect on platelet function, if the binding assay is extended to study the thromboxane receptor of vascular tissue, then the $PGF_{2\alpha}$ analogue would no longer be useful as $PGF_{2\alpha}$ is a potent vasoconstrictor. A binding assay using platelet homogenates may serve to limit some of the complications introduced by uptake but may provide an additional problem as the enzyme thromboxane synthetase is present in the microsomal fraction. Since 9,11-epoxymethano PGH_2 is reported to inhibit thromboxane synthetase (250), this radioligand would no longer be useful as the enzyme would represent another binding site.

Further information could be gained about this binding site if radioligands with structures more akin to thromboxane A_2 rather than the endoperoxides could be prepared. This is a difficult area of chemistry particularly since the radioligand would have to be enantiomerically pure and most synthetic routes yield racemic mixtures. However, if additional radioligands, preferably more water soluble, can be prepared they would aid classification of this binding site. If the binding assay can be improved by using membrane fragments from osmotically-lysed human platelets this would have the additional benefit that PGE_1 would no longer be required to inhibit aggregation. (Although the concentration of PGE_1 added to inhibit aggregation is very low and unlikely to have any effect on 9,11-epoxymethano PGH_2 binding.) Also, a filtration method would be required to separate bound and free which would minimise the contamination of the pellet with the supernatant. This is always higher when centrifugation methods

are used as higher levels of unbound radioligand are trapped in the pellet water space.

Although these binding studies require considerable improvement, as a preliminary investigation they are quite promising. Hopefully, the development of more radioligands and an improved binding assay will allow the thromboxane receptors of both human platelets and blood vessels to be characterised.

CHAPTER VI

DISCUSSION

TXA_2 has potent biological actions including the stimulation of platelet aggregation, bronchoconstriction and contraction of pulmonary, coronary and systemic vascular smooth muscle. A number of stable compounds have been developed to inhibit the actions of TXA_2, either by inhibition of its synthesis (112,135,139,242-252) or by antagonism of its action at the receptor level (131-135). Analogues which mimic the actions of TXA_2 have also been developed (120-130), as TXA_2 itself is very unstable, having a half life of 30 seconds at physiologic pH.

However, very few of these analogues have been found to have a specific action at only one of these sites. Of the more commonly used analogues,

1. 9,11-epoxymethano PGH_2 is a full agonist on human platelets, a partial agonist on smooth muscle preparations and an inhibitor of thromboxane synthetase (120,250); (its isomer 11,9-epoxymethano PGH_2 appears to be a specific thromboxane agonist on all preparations tested (121,331));

2. pinane TXA_2 is an antagonist on platelets, a partial agonist on smooth muscle and also inhibits thromboxane synthetase (135);

3. carbocyclic TXA_2 is an antagonist on platelets, an agonist on smooth muscle and an inhibitor of thromboxane synthetase (130, 292).

In the search for a specific TXA_2 receptor antagonist, Dr. R.L. Jones and Dr. N.H. Wilson, of the Department of Pharmacology, University of Edinburgh, have prepared a number of analogues of 9,11-ethano PGH_2 (itself a partial agonist) with modified ω side chains.

Many of these analogues were found to have TXA_2 antagonist activity on both platelets and isolated smooth muscle preparations. The affinity constants of these antagonists were calculated according to the Schild equation:

$\log (DR - 1) = \log [B] + \log (K_B)$ where DR = dose ratio;

[B] = molar concentration of antagonist;

K_B = affinity constant of antagonist.

In each case a linear plot was obtained with a slope not significantly different from unity. This suggests a competitive type of antagonism. The molar concentration of antagonist giving a (DR - 1) value of 20 was used to calculate the affinity constant. Using the dog saphenous vein and guinea pig trachea as thromboxane sensitive tissues, similar affinity constants were found for the five analogues, EP035, EP037, EP043, EP045 and EP092 studied throughout this thesis. However, the affinity constants estimated from experiments using the rabbit aorta of human platelets were considerably lower. This is not true for EP037 which shows similar potency on all three vascular preparations, but only a tenth of this activity on human platelets (see Chapter I, Table 1.1).

The response of the vascular tissue to the standard agonist used, 11,9-epoxymethano PGH_2, is a sustained contraction. This is likely to be the direct result of receptor occupation. However, platelet aggregation is a much more complex process. With primary aggregation, the extent of aggregation may depend directly on the degree of activation of the external platelet receptors, but with secondary aggregation the response is amplified by the effects of ADP liberated during the release reaction and the AA metabolites PGG_2/H_2 and TXA_2 synthesised during

the second wave of aggregation. The autocatalytic nature of the platelet response creates an additional problem in that it is difficult to determine when equilibrium receptor occupancy has been reached. Indeed it is possible that complete aggregation occurs well before equilibrium occupancy is attained. The response is usually chosen rather arbitrarily as the degree of aggregation achieved 100 seconds after addition of the aggregating agent. With the smooth muscle preparations cumulative dose response curves to 11,9-epoxymethano PGH_2 were obtained, and equilibrium was assumed to be reached once the contraction observed with a given concentration of agonist had plateaued.

It seemed possible that the differences observed in the affinity constants of the antagonists determined from platelet aggregation might reflect the difficulty in establishing equilibrium and determining the extent of aggregation attributable to receptor occupancy by the agonist, rather than suggesting the existence of two different classes of thromboxane receptor. Furthermore, an additional effect of the antagonist on platelet function could alter the estimate for the affinity constant. With EP043, the affinity constant determined may be affected by its inhibitory action on the synthesis of TXA_2 from AA, which by decreasing the potentiating effect of TXA_2 on the second wave of aggregation, will increase the degree of inhibition compared with that attributable to receptor antagonism alone. Indeed, an affinity constant for thromboxane receptor blockade by EP035 cannot be determined because the dose-response curve for 11,9-epoxymethano PGH_2 is not parallel to the control, owing to a considerable inhibitory contribution from the increase in cAMP levels produced by EP035.

However, the differential activities of some analogues on smooth muscle and human platelets also suggested that the thromboxane receptor of platelets could indeed be different from that of vascular tissue:

1. EP011 is a full agonist on both smooth muscle and human platelets but 15-oxo-EP011 has no effect on human platelets although it is a full agonist on smooth muscle (332).

2. CTA_2 is a full agonist on smooth muscle yet antagonises platelet aggregation induced by AA or 11,9-epoxymethano PGH_2 (292).

3. PTA_2 is a partial agonist on smooth muscle preparations but an antagonist on human platelets (333).

4. The sodium salt of rac-9,11 : 11,12-dideoxa-9,11 : 11,12-diepithio-thromboxane A_2 will contract the rat aortic strip but has no effect on human platelet aggregation (334).

An alternative explanation for these differential activities is that they result from a difference in the number of spare receptors; if the number of spare receptors was restricted in platelets compared with smooth muscle, a weak agonist/partial agonist action on smooth muscle could conceivably be changed into an antagonist action on human platelets. Although such an argument could hold for a compound-like PTA_2, it is difficult to believe that the potent contractile action of CTA_2 could be transformed into an antagonist action simply through a reduction in the number of receptors available.

It seemed that radioligand binding studies might enable the affinity constants of these antagonists to be determined directly. A binding assay for the tritiated thromboxane mimic, ^3H 9,11-epoxymethano

PGH_2, using whole platelets was developed and the affinity constants determined from displacement experiments (see Chapter V, Table 5.10). Although the affinity constant estimates are still considerably less potent than those determined from smooth muscle preparations, the order of potency is much more similar to that of the antagonists on smooth muscle, than the order of potency determined from platelet aggregation affinity constants (Table 6.1). These results are not sufficient to determine whether there are 2 distinct classes of thromboxane receptors, but they indicate that this approach is worth pursuing.

TABLE 6.1: Comparison of the order of potency of the affinity constants for the antagonists, with respect to EP092, on the various preparations tested

Ratio of affinity constants	RA	DSV	GPT	HP (disp)	HP (agg)
EP092 : EP045	5.05	4.22	2.54	2.21	22.90
EP092 : EP043	6.18	4.43	4.20	4.12	16.67
EP092 : EP037	3.77	24.48	33.20	62.10	43.40
EP092 : EP035	45.65	7.15	22.70	31.00	-

RA - rabbit aorta; DSV - dog saphenous vein; GPT - guinea pig trachea
HP (disp) - human platelets - estimates determined from displacement experiments with 9,11-epoxymethano PGH_2 as agonist.
HP (agg) - human platelets - estimates determined from aggregation experiments with 11,9-epoxymethano PGH_2 as agonist.

Binding studies enable the affinity constant to be determined directly without complication from the unknown relationship between receptor occupancy and response. This may be particularly important where the compared responses are not only very different in character but one system is proteinacious (PRP) and the other an isotonic, protein-free bathing solution. The additional actions of EP043 and EP035 may not interfere with the determination of the affinity constant from binding experiments to as great an extent since the platelets are

treated with indomethacin (10^{-5}M), to inhibit TXA_2 production and PGE_1 (1.7×10^{-8}M) to prevent aggregation by the thromboxane mimics. The increase in cAMP levels induced by PGE_1 did not appear to affect binding of the tritiated thromboxane mimic.

Binding of ^{3}H 15(S) 9,11-epoxymethano PGH_2 to whole platelets appeared to be the result of three types of binding:

1. the non-saturable uptake of the lipophilic ligand into the platelet membranes;
2. the non-specific saturable binding, possibly to ionic groups on the membrane;
3. the stereospecific saturable binding of the ligand to the receptor.

It has been suggested that prostaglandins are taken up into cells only when specific uptake mechanisms exist (335), so that uptake of the lipophilic ligand will not be into the platelet cytoplasm but a result of physical solution of the lipophilic molecules in the lipid areas of platelet membranes. If this is the case, it suggests that the binding sites measured in this assay represent receptor sites on the platelet surface. Since only one class of stereospecific binding site was evident it would seem that only one type of receptor exists, rather than distinct receptors for the PG endoperoxides and for TXA_2. It has previously been suggested that all bisenoic prostaglandins act on a common receptor, present on the platelet surface membrane, and that TXA_2 acts on a distinct intracellular receptor (336). Unfortunately, there was no TXA_2 uncontaminated with PGH_2 to test for displacement of ^{3}H 9,11-epoxymethano PGH_2 to this site. However, since the thromboxane mimics and both the endoperoxide-like antagonists as well as PTA_2 (which is thromboxane-like

in structure), all displace binding, it is probable that they act on a common site, possibly on the surface of the platelet membrane.

Exogenously added AA is rapidly taken up by platelets where it is metabolised into PGH_2/TXA_2. It has been presumed that internal receptors exist where TXA_2 will bind to initiate platelet aggregation, contraction and release (336, 21), the PGH_2 and TXA_2 liberated from the platelets during this process can then promote further aggregation by acting on the surface receptors of neighbouring platelets. From these binding experiments no information can be gained about the distribution of thromboxane receptors in human platelets. However, binding studies to platelet homogenates should reveal the existence, either of an additional class of binding site (with a different affinity for the radioligand), or of considerably more receptor sites, if indeed there are receptors inaccessible to the radioligand during binding studies to whole platelets. ^3H 9,11-epoxymethano PGH_2 would not be useful for this purpose as it will also bind to the enzyme thromboxane synthetase, which it inhibits. The development of a binding assay to platelet homogenates and the preparation of additional radioligands, preferably more akin to TXA_2 in structure, could be of considerable assistance in the further characterisation of the thromboxane receptor(s) of platelets. Similar binding studies to smooth muscle preparations should help to clarify the position as to whether or not the receptor is distinct from that of platelets.

The suggestion that specific uptake mechanisms are required to allow prostaglandins to enter cells, raises the question of how these antagonists inhibit AA-induced aggregation? If TXA_2 produced within the platelet acts on intracellular receptors it seems probable that these receptors are accessible to the exogenously applied antagonist. Although

cyclo-oxygenase and thromboxane synthetase have been termed microsomal enzymes, this name may be slightly misleading as the microsomal fraction of platelets contains two-thirds of all the platelet particulate protein and most of the plasma membrane (337). The location of the platelet enzymes which metabolise AA still remains to be elucidated, and although the dense tubular system has been postulated (111-113) it has also been suggested that the enzyme is on the cell surface (338). It is conceivable that metabolism of AA occurs at the inner surface of the platelet membrane where internal receptors for TXA_2 exist. Such sites may be accessible to the antagonist through the lipidic platelet membrane. However, since these endoperoxide-like antagonists are exceptionally lipophilic, it is possible that they are able to enter cells more easily than the classical, more water-soluble prostaglandins.

Another possibility is that TXA_2 mediates aggregation by acting on the platelet surface receptor but acts intracellularly, perhaps by its proposed ionophoretic action, to mediate platelet contraction and release. Indeed EDTA (0.01M) can prevent aggregation induced by both PGG_2 and A23187 but not the internal contraction or secretion induced by these agents (108). It has also been reported that aggregation induced by both TXA_2 and A23187 is mediated by membrane proteins (339). The movement of anionic PG endoperoxides and TXA_2 from their membrane site of synthesis into the platelet cytosol will be accompanied by suitable cations to maintain electroneutrality (114). It is therefore plausible that TXA_2 will mediate aggregation through a membrane receptor site but will transport calcium from the lipid membrane site of synthesis into the aqueous cytosol to stimulate platelet contraction and the induction of the release reaction. Although thromboxane antagonists have been shown to inhibit aggregation induced by AA and thromboxane

mimics, their effect on platelet contraction and release has not as yet been studied, but is clearly a crucial experiment. If thromboxane antagonists only block the direct aggregatory action of TXA_2, it may explain why agents which raise cAMP levels are more effective inhibitors of aggregation, as these agents also suppress contraction and release by their Ca^{2+}-chelating action.

The possible dissociation of the aggregatory effects of TXA_2 from the contractile and release-inducing effects may be an important consideration in experiments designed to determine the relative importance of PGH_2 and TXA_2 in mediating AA-induced aggregation. It was previously suggested (Chapter I) that a possible explanation for the fact that PGH_2 itself only induces primary aggregation is that its rapid conversion into PGD_2 may result in the inhibition of its own aggregatory effect. Another explanation is that although PGH_2 has a direct aggregatory action its metabolism into TXA_2 is required for platelet contraction and release. In fact PGG_2, 11,9-epoxymethano PGH_2 and 9,11-epoxymethano PGH_2 have all been shown to have ionophoretic properties, with a 3-4 fold preference for Ca^{2+} over Mg^{2+} (117) and all three compounds are capable of inducing rapid and irreversible aggregation even in the presence of high concentrations of a thromboxane synthetase inhibitor. It is possible that the effectiveness of novel prostaglandin analogues will depend both on their affinity and efficacy for the thromboxane receptor, and their ionophoretic properties.

Increased platelet sensitivity to TXA_2 has been demonstrated both in survivors of myocardial infarction (297) and in patients with angina pectoris (298). Sensitivity to thromboxane action may be altered in two ways:

1. a change in receptor affinity and/or density;
2. a change in the stimulus-response coupling mechanism.

Binding studies may help to distinguish which of these is responsible for the increased sensitivity. If indeed increased sensitivity to TXA_2 is responsible for, or contributes to, thrombotic episodes, thromboxane antagonists may be of use in the inhibition of the action of TXA_2 *in vivo*. EP035, EP045 and EP092, but not EP037, were found to protect against AA-induced death in rabbits, thought to result from acute pulmonary thrombosis. This is particularly encouraging as the infusion of AA and resulting surge of TXA_2 produced may represent a more severe stimulus than would occur in the pathological situation, and rabbit platelets are not as sensitive as human platelets to the thromboxane antagonists tested.

Additional actions of these analogues, other than TXA_2 antagonism, may increase their usefulness as thrombotic agents:

1. an action to increase cAMP levels within the platelet, as was found for EP035, will maintain the platelets refractory to aggregation regardless of the particular agent responsible for the inhibition of platelet aggregation *in vivo*;

2. an action to inhibit thromboxane synthetase, as may be the case for EP043 (an action on cyclo-oxygenase cannot be excluded), may enable platelet endoperoxides to act as substrate for the prostacyclin synthetase enzyme of the vessel wall, resulting in a localised increase in prostacyclin levels which would serve to decrease the reactivity of the platelets (as in action 1) and reverse any platelet aggregates formed.

At the same time, the TXA_2 antagonist action would protect against the potent vasospastic and aggregatory actions of TXA_2

However, as pharmacological tools, specific receptor antagonists are more useful. Of the five analogues tested, only EP035 shows a marked effect on adenyl cyclase which it stimulates perhaps by activation of the PGI_2 receptor of platelets. EP043 has an ω side chain most similar to that of EP035 (diphenyl substitution) yet does not appear to increase cAMP levels. This suggests that EP043 does not activate prostacyclin receptors but it has not been tested for antagonist activity. EP043 does, however, show an additional action to inhibit the biosynthesis of TXA_2 from AA. EP092 at high concentrations (5-10 µg/ml) showed a slight effect to raise platelet cAMP levels. Although this effect was weak it was thought to be sufficient to affect platelet function. Also, in these concentrations EP092 inhibited the biosynthesis of TXB_2 from AA. These concentrations are about 10 times that required for antagonist activity.

EP037 and EP045 do not appear to have either of these additional actions on platelet function. However, since EP037 proved lethal to rabbits when infused intravenously in high concentrations (5-10 mg/kg), it seems that EP037 is in fact a weak partial agonist rather than a full antagonist. In conclusion, of the five analogues tested only EP045 appeared to specifically antagonise the effects of TXA_2 on human platelets, over a wide range of concentrations. However, in the concentrations in which they are effective as antagonists of 11,9-epoxymethano PGH_2 induced aggregation both EP045 and EP092 appear to be relatively specific.

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