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In vivo platelet aggregation in the rat: dependence on extracellular divalent cation and inhibition by non-steroidal anti-inflammatory drugs

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1 Using the Technicon Autocounter, the mechanisms involved in collagen-induced platelet aggregation *in vivo* have been studied without the interference of an anticoagulant.

2 Extracellular divalent cation was essential for *in vivo* platelet aggregation.

3 Non-steroidal anti-inflammatory drugs completely inhibited the aggregation induced by collagen in platelet-rich plasma in *in vitro* or *ex vivo* studies. *In vivo* only a maximum of 50% inhibition was achieved when release of thromboxane A_2 (TXA_2) was completely inhibited. Therefore *in vivo*, collagen causes aggregation through more than one pathway which operate independently of each other and which are all dependent on extracellular divalent cation.

4 *In vivo*, when different doses of collagen were compared, aggregation produced by low doses of collagen was more dependent upon prostaglandin endoperoxide/TXA_2 formation.

Introduction

Plasma calcium ion is required in one or more of the mechanisms by which platelets adhere to collagen and release their granule contents (Kinlough-Rathbone, Cazenave, Packham & Mustard, 1980). Heptinstall & Taylor (1979) also suggest that extracellular divalent cation is required for platelet aggregation by both ADP and collagen and that subsequent secretion is mediated by calcium ion in the cytoplasm. It is clear that *in vitro* studies on platelet aggregation, by necessity carried out in an anticoagulated medium, must be influenced by the presence of that anticoagulant (Kinlough-Rathbone & Mustard, 1971; Buchanan & Hirsh, 1975; Scrutton & Egan, 1979). A study of platelet aggregation in the absence of an anticoagulant would therefore be of interest. An *in vivo* technique using a modified Technicon Autocounter enables platelet aggregation to be studied *in vivo* without the interference of an anticoagulant (Smith & Freuler, 1973).

Injury to the blood vessel wall can reveal subendothelial collagen. Platelets adhere to the exposed collagen and are activated. Subsequent aggregation may be partly due to ADP released from erythrocytes (Born & Wehmeier, 1979) and thromboxane A_2 (TXA_2) released from platelets, which is a potent vasoconstrictor and aggregating agent (Samuelsson, 1976). Prostacyclin (PGI_2) has directly opposing actions to TXA_2 and is released from the vessel wall (Gryglewski, Bunting, Moncada, Flower & Vane, 1976). Both PGI_2 and TXA_2 are produced from the prostaglandin endoperoxide PGH_2. The hypothesis that blood vessels use platelet PGH_2 for PGI_2 synthesis (Bunting, Gryglewski, Moncada & Vane, 1976) is disputed by Needleman, Wyche & Raz (1979). Therefore unlike *in vitro* platelet aggregation studies using collagen, where the aggregation occurs as a direct result of platelet activiation, the *in vivo* effect of collagen-induced platelet aggregation may be due to several interactions occurring within the vascular system. It is likely that in haemostasis and thrombosis a similar interplay exists.

Compared with another *in vivo* model (Page, Paul & Morley, 1982) the Technicon Autocounter system uses low doses of aggregating agents which allow repeated and reproducible injections to be made in one anaesthetized animal and so enables an intra-assay design to be carried out. Also, the effect of aggregating agent and inhibitor can be studied in the natural environment of untreated platelets in the absence of any anticoagulant.

This paper describes the effect of trisodium citrate, Ca^{2+} and non-steroidal anti-inflammatory drugs (NSAIDs) on collagen-induced aggregation which shows the importance of extracellular divalent cation and the arachidonate pathway *in vivo* in the rat.

Methods

In vitro *study*

Male Sprague-Dawley rats (350 g–450 g) were anaesthetized with pentobarbitone (60 mg kg^{-1} i.p.) and blood collected from a cannulated carotid artery into polypropylene tubes containing sufficient 3.15% trisodium citrate to provide a 1 in 10 dilution by blood. The tube was centrifuged at 120 *g* for 15 min and the resultant platelet-rich-plasma (PRP) dispensed into 0.2 ml aliquots. Each sample was preheated at 37°C then stirred at 1000 rev min^{-1} before addition of drug. NSAID or vehicle was added and 3 min later collagen (2.5–5.0 $\mu g\,ml^{-1}$, final concentration) was added. Aggregation was monitored using a blood platelet aggregometer (ADG Instruments Ltd) and Servoscribe 1S potentiometric recorder. An incubation time of 10 min was used in some experiments. Inhibition of collagen-induced platelet aggregation was assessed by comparing the unit fall in transmission produced in NSAID-treated PRP with the fall in transmission observed in vehicle-treated PRP and expressing the difference as a percentage. All items in contact with blood were siliconized. The number of platelets in PRP was $800–1000 \times 10^3\,\mu l^{-1}$.

Ex vivo *study*

After anaesthesia as above the jugular vein was cannulated for administration of drug and the carotid artery cannulated for collection of a blood sample. A dose of NSAID or vehicle was given. The dose of drug chosen was one which was only partially effective *in vivo*. Five min later the blood sample was collected and processed as above to obtain PRP. Then 0.2 ml samples of PRP were challenged with collagen (10 $\mu g\,ml^{-1}$) 30, 40, 50 and 60 min after collection of blood and the response monitored as above.

In vivo *study*

After anaesthesia as above the trachea was cannulated to ensure a free airway and a jugular vein was cannulated for injection of drug. A carotid artery was cannulated using a double cannula (Smith & Freuler, 1973) to allow continuous withdrawal of a sample (0.1 ml min^{-1}) of circulating blood. Only at this point was blood anticoagulated with 3.15% trisodium citrate (0.015 ml min^{-1}). Using the Technicon Autocounter, the sample was then diluted with ammonium oxalate (1%) and saponin (0.002%) to lyse the erythrocytes. The platelets were counted optically and the number of platelets continuously recorded on pre-calibrated chart paper. NSAIDs and $CaCl_2$ were given 5 min before injection of collagen. Sodium citrate was infused for 15 min and collagen injected after 10 min.

Blood sampling from anaesthetized animals

To construct a graph of % fall in platelet count against thromboxane B_2 (TXB_2) formation, samples of blood were collected by cardiac puncture 1 min after bolus injection of collagen (20, 40, 80 and 160 $\mu g\,kg^{-1}$). Then 2.2 ml of blood was withdrawn into a syringe containing 300 μl of the EDTA and indomethacin solution. This was centrifuged at 1500 *g* for 15 min and the supernatant collected and frozen at −20°C. In all other sampling procedures a carotid artery was cannulated 10 min prior to injection of collagen and a total volume of 1 ml was collected in a 1 ml syringe containing 120 μl of the EDTA and indomethacin solution. The syringe was centrifuged at 1500 *g* for 6 min and the supernatant collected and frozen at −20°C.

Radioimmunoassay of TXB_2

The frozen plasma samples were thawed and TXB_2 content was determined without extraction using appropriate plasma controls. Blank plasma was prepared by injection of indomethacin (10 mg kg^{-1}), into a rat 24 h and 1 h before anaesthesia when blood was collected from the carotid artery, processed as above and stored at −20°C until required. The [^{3}H]-TXB_2 (specific activity 139 Ci $mmol^{-1}$ was stored in ethanol at −20°C until required. An aliquot was taken, the ethanol removed and the residue dissolved in Tris gel buffer such that 0.1 ml gave about 3000 c.p.m. The final dilution of antiserum was 1:2000, the sensitivity of the assay was 30 pg 100 μl^{-1} (defined as the amount of TXB_2 added which displaces 10% of bound labelled ligand) and cross-reactivity was TXB_2, 100%; prostaglandin D_2 (PGD_2), 0.9%; $PGF_{2\alpha}$, 0.1%. PGE_2, 0.02%; PGE_1, 0.1%. Dextran-coated charcoal was used to adsorb the free TXB_2. Bound TXB_2 in the supernatant was poured into 8 ml scintillation cocktail and counted using a Packard Tri-Carb 2425 scintillation counter. A computer program was devised to compose a logit log plot of concentration of TXB_2 against % binding (Rodbard, Bridson & Rayford, 1969) which produced a linear relationship between x and y coordinates. From this the levels of TXB_2 in plasma samples were calculated. The correlation coefficient was estimated (r). An r value significantly greater than zero at 0.001% (d.f. = 7, $n = 9$) signified that the assay was precise. The inter-assay coefficient of variation was 16.4%.

Drugs and solutions

Drugs used were: collagen, bovine achilles tendon, type 1 (Diamed Diagnostics), calcium chloride (Fisons), sodium citrate (Fisons). Indomethacin (gift from MSD) and sulphinpyrazone (gift from Geigy Pharmaceuticals) were dissolved in Na_2CO_3 solution. Piroxicam (gift from Pfizer) was dissolved by dropwise addition of 1M NaOH onto the powder and made up to volume with H_2O. The thioether metabolite of sulphinpyrazone (G-25671) was a gift from Dr R. Wallis and was dissolved by sonication in NaOH and saline with subsequent heating. The EDTA and indomethacin solution was prepared by adding 1 ml indomethacin (1 mg ml^{-1}) to 4 ml EDTA (1%) in normal saline. For radioimmunoassay Tris gel buffer was used (6.35 g Tris HCl and 1.18 g Tris base dissolved in 1 g l^{-1} gelatin solution). The [^{3}H]-TXB_2 was supplied by Amersham. TXB_2 and antiserum were kindly provided by Dr J. Salmon.

Scintillation mixture contained 5 g diphenyloxazole dissolved in 750 ml toluene made up to 1 l with Triton-X. PGD_2, E_1, E_2, and $F_{2\alpha}$ were gifts from Upjohn Co.

Statistical analyses

Difference between two means was calculated using Student's *t* test. The F-distribution was used to compare two variances.

Results

In vitro *study*

Complete inhibition of collagen-induced aggregation was produced by indomethacin (5 μg ml^{-1}), piroxicam (0.5 μg ml^{-1}) and G-25671 (5 ug ml^{-1}) (Table 1). Sulphinpyrazone (500 μg ml^{-1}) did not inhibit aggregation even after a 10 min pre-incubation period.

Ex vivo *study*

Indomethacin (8 mg kg^{-1}), piroxicam (800 μg kg^{-1}), G-25671 (8 mg kg^{-1}) and sulphinpyrazone (80 mg kg^{-1}) all produced complete inhibition of platelet aggregation ($n = 4$ in each case) when the respective PRP was challenged, at 30, 40, 50 and 60 min after collection of blood, with collagen.

Blood sampling from anaesthetized animals

The TXB_2 levels obtained by removing blood samples by cardiac puncture were very variable with standard errors of about 28% of the mean. In later experiments therefore, samples were taken from a carotid artery and a procedure devised to minimise thromboxane formation *in vitro* (see Methods). Figure 1 shows that plasma levels of TXB_2 were less using this new method (significant with low dose collagen, $P < 0.01$) and that standard errors were improved (variance (F) significant ($P < 0.01$) at high dose collagen.) The cannulations required to facilitate continuous blood sampling did not elevate TXB_2 levels.

In vivo *study*

Figure 2a shows a typical trace where collagen, injected at 15 min intervals, produced reproducible falls in platelet count. The response to collagen was measured by calculating the maximum % fall in platelet count that occurred after the injection of collagen. Indomethacin inhibited the fall in platelet count (Figure 2b) and this inhibition was expressed as a percentage of the mean of the two preceding collagen injections.

In the rat there was a significant correlation between the % fall in platelet count and the log dose of collagen ($r = 0.96$, $P < 0.001$). However, when a graph of % fall in platelet count was plotted against TXB_2 levels in plasma, a curve was formed (Figure

Table 1 *In vitro* inhibition of collagen-induced platelet aggregation (2.5–5.0 μg ml^{-1})

Drug	*Final cuvette concentration* (μg ml^{-1})	*% Inhibition*	n
Indomethacin	5	100	6
	0.5	47 ± 20.3	4
Piroxicam	0.5	100	6
	0.05	40 ± 20.0	2
G-25671	5	100	5
	0.5	21.9 ± 14.3	4
Sulphinpyrazone	1000	0	2
	500	0	4
	500*	0	3

Values are presented as mean ± s.e.mean; drugs were incubated for 3 min except * which was 10 min.

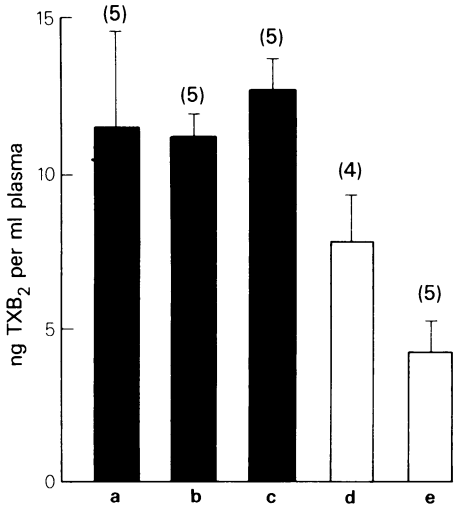

Figure 1 Levels of thromboxane B_2 (TXB_2) in plasma 1 min after injection of high dose, 80 $\mu g\,kg^{-1}$, (solid columns) or low dose, 40 $\mu g\,kg^{-1}$, (open columns) collagen. Samples were obtained by cardiac puncture (a and d) or from a carotid artery (b, c and e). In (c) the remaining carotid artery was cannulated to facilitate continuous platelet counting. When the TXB_2 levels, measured by radioimmunoassay, of samples obtained either by cardiac puncture or from the carotid artery were compared the standard error was significantly lower ($P < 0.01$) using the carotid artery technique (b compared with a). Using low dose collagen the levels of TXB_2 were significantly greater ($P < 0.01$) in plasma obtained via cardiac puncture (d) than in plasma obtained by cannulation (e). The extra cannulation required for continuous blood sampling did not significantly raise TXB_2 levels (c compared with b). The number of observations is indicated in parentheses.

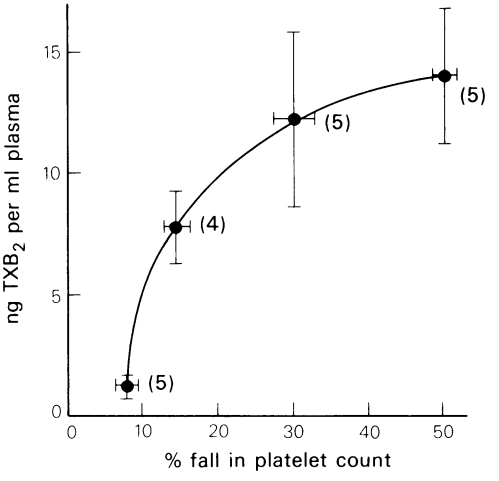

Figure 3 Rats received injections of collagen 20, 40, 80 or 160 $\mu g\,kg^{-1}$ and the % fall in platelet count recorded. One min after injection of collagen, a sample of blood was obtained by cardiac puncture and the thromboxane B_2 (TXB_2) measured by radioimmunoassay. The number of observations is indicated in parentheses.

3). This shows that the aggregation produced by low dose collagen depends more on arachidonate metabolism, as reflected by elevation in plasma TXB_2 levels, than do high doses of collagen.

Calcium chloride (0.1 mg kg^{-1} min^{-1}) potentiated ($43.0 \pm 7.1\%$, $n = 7$) and 3.15% tri-sodium citrate (0.5 ml kg^{-1} min^{-1}) completely inhibited low dose collagen-induced intravascular aggregation (Figure 4). The aggregation produced by high doses of collagen was only partially inhibited ($67.0 \pm 2.1\%$, $n = 4$) by tri-sodium citrate. Indomethacin produced

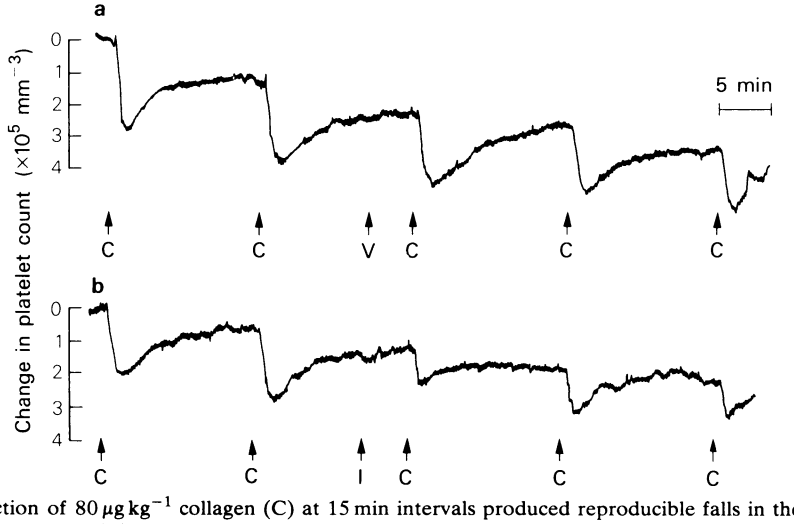

Figure 2 Injection of 80 $\mu g\,kg^{-1}$ collagen (C) at 15 min intervals produced reproducible falls in the circulating count. In (a) when vehicle (V) was injected 5 min prior to an injection of C, no effect on platelet count was observed. In (b) 8 mg kg^{-1} indomethacin (I) was injected 5 min prior to an injection of C when inhibition of platelet aggregation was observed.

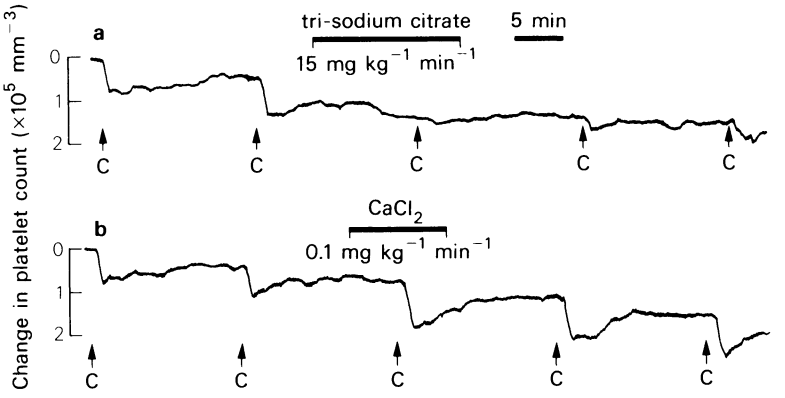

Figure 4 Tri-sodium citrate infused during injection of collagen 40 μg kg^{-1} (C) prevented completely the fall in platelet count observed previously (a). Infusion of $CaCl_2$ (b) potentiated the effect of C.

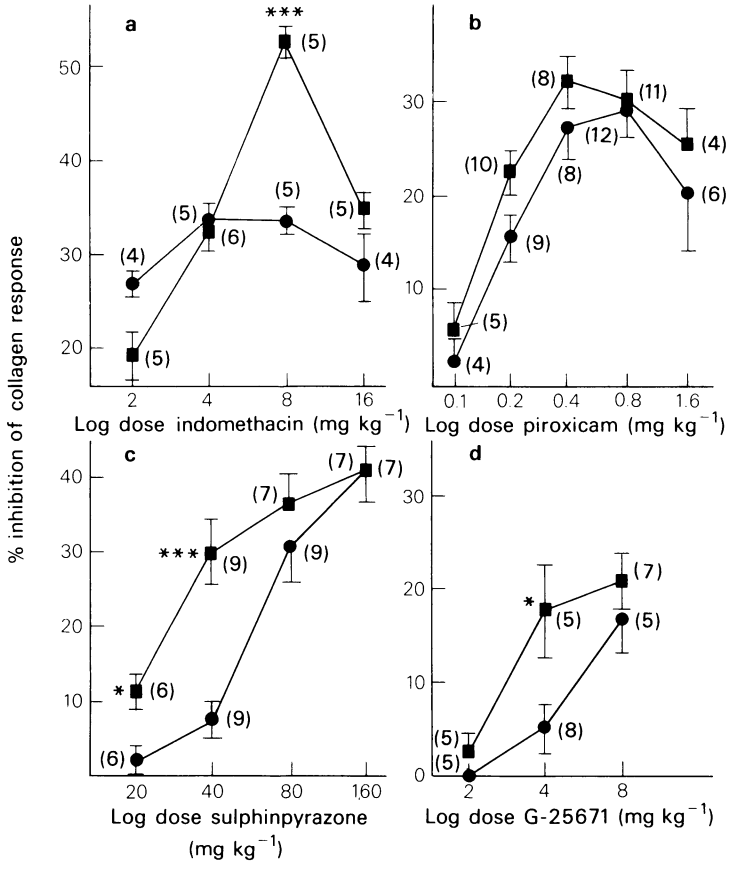

Figure 5 Inhibition of intravascular platelet aggregation. All drugs were more effective at inhibiting low dose (■) than high dose (●) collagen-induced platelet aggregation which was significant at 8 mg kg^{-1} indomethacin ($P < 0.001$), 20 mg kg^{-1} and 40 mg kg^{-1} sulphinpyrazone ($P < 0.05$ and $P < 0.001$ respectively) and 4 mg kg^{-1} G-25671 ($P < 0.05$). The number of experiments is indicated in parentheses.

Table 2 Reduction of thromboxane B_2 (TXB_2) levels in plasma, after injection of collagen, by non-steroidal anti-inflammatory drugs (NSAIDs)

	TXB_2 levels in plasma (ng ml^{-1})	
NSAID (mg kg^{-1})	*Collagen* (40 μg kg^{-1})	*Collagen* (80 μg kg^{-1})
—	4.2 ± 1.0	11.2 ± 0.7
Indomethacin (8)	ND	ND
Piroxicam (0.8)	ND	ND
G-25671 (8)	ND	ND
Sulphinpyrazone (40)	ND	1.5 ± 0.6
Sulphinpyrazone (80)	ND	ND

Values are presented as mean ± s.e.mean; ND = not detected. Sensitivity of assay = 0.3 ng ml^{-1}; $n = 5$ in each case. Prior to collagen injection, TXB_2 was not detected in the plasma.

52.5% inhibition of low dose (40 μg kg^{-1}) collagen-induced aggregation which was significantly different from the inhibition observed using high dose (80 μg kg^{-1}) collagen (Figure 5a). Piroxicam showed similar results to indomethacin (Figure 5b) but only up to 32.2% inhibition was observed. Higher doses of indomethacin (16 mg kg^{-1}) and piroxicam (1600 μg kg^{-1}) produced an unexpected reduction in inhibition. Samples taken for radioimmunoassay showed that these drugs reduced the amount of TXB_2 to below detectable levels (Table 2).

Sulphinpyrazone (40 mg kg^{-1}) partially inhibited collagen-induced aggregation, being more effective at inhibiting low dose collagen than high dose collagen (Figure 5c). At 40 mg kg^{-1} sulphinpyrazone, TXB_2 was not detected in plasma from rats injected with low dose collagen. TXB_2 levels were not detected at high dose collagen when 80 mg kg^{-1} sulphinpyrazone was used. Basal levels of TXB_2 were also below detectable levels and it was assumed that undetectable levels of TXB_2 indicated complete inhibition of release of TXA_2. Sulphinpyrazone, however, produced even more inhibition of aggregation at 160 mg kg^{-1}. G-25671 (8 mg kg^{-1}) produced only 21.2% inhibition of collagen-induced platelet aggretation (Figure 5d). It was also found to be more effective at inhibiting aggregation due to low dose collagen than high dose collagen-induced aggregation. Since a dose of 16 mg kg^{-1} G-25671 produced toxic reactions in rats, reliable results could not be obtained at this dose level.

Discussion

One of the first events in haemostasis and thrombosis is adhesion of platelets to exposed sub-endothelial elements such as collagen. We therefore decided to study the mechanisms involved in collagen-induced intravascular platelet aggregation. Cazenave, Packham & Mustard (1973) have shown that plasma calcium may have a role in adhesion and Lages & Weiss (1981) suggest aggregation responses in platelets exhibit an absolute dependence on the presence of extracellular divalent cation. The effects of NSAIDs on collagen-induced aggregation were therefore compared in *in vivo*, *ex vivo* and *in vitro* situations.

It was possible to produce complete inhibition of collagen-induced aggregation *in vitro* and *ex vivo* using indomethacin, piroxicam and G-25671. The doses used *ex vivo* were the same as those that caused only partial inhibition *in vivo*. Sulphinpyrazone was only effective *ex vivo* but not *in vitro*. Since sulphinpyrazone was effective *ex vivo* this may show that during the processing of blood to obtain PRP the more active metabolite (G-25671) was formed. Ali & McDonald (1977) state that the inhibitory effect of sulphinpyrazone *in vitro* is largely determined by the strength of collagen stimulus, and that the competitive inhibition of cyclo-oxygenase exhibited by sulphinpyrazone may not be observed if the collagen stimulus is too strong. For this reason, sub-maximal doses of collagen (2.5–5.0 μg ml^{-1}) were used *in vitro* whilst challenge with a higher dose (10 μg ml^{-1}) of collagen was used to test the *ex vivo* effectiveness of the NSAIDs.

Whereas complete inhibition of platelet aggregation was possible both *in vitro* and *ex vivo*, only partial inhibition was observed *in vivo*. An explanation is that citrate, in the commonly used concentration for anticoagulation, inhibits platelet function (Born & Cross, 1963; Mustard, Perry, Kinlough-Rathbone & Packham, 1975; Aurnses, Gjesdal & Abildgaard, 1981). When sodium citrate was infused *in vivo* low dose collagen-induced platelet aggregation was completely inhibited. Also, calcium chloride potentiated collagen-induced intravascular aggregation. This shows there is an absolute requirement for extracellular divalent cation in platelet aggregation *in vivo*. Indeed Scrutton & Egan (1979) have suggested that there are two distinct receptor sites on the platelet where extracellular divalent cation can attach and cause aggregation. Trisodium citrate was less effec-

tive at inhibiting high dose collagen-induced aggregation. In such an experiment it cannot be assumed that trisodium citrate will chelate all of the extracellular divalent cations and as such the remaining free cations may have been sufficient to permit some activation of the platelets by high dose collagen. Both *in vitro* and *ex vivo* studies therefore represent a false system where aggregation is dependent on the method of preparation of PRP, the concentration of agonist used and incubation time of the NSAID (Seuter, 1976).

In vitro studies have shown that several aggregatory pathways exist in the platelet. Some authors suggest two pathways exist in collagen-activated aggregation (Zucker & Peterson, 1970; Huzoor-Akbar & Ardlie, 1978; Best, Holland, Jones & Russell, 1980). One pathway involves the conversion of arachidonic acid in the platelet to prostaglandin endoperoxides by cyclo-oxygenase then to TXA_2 by thromboxane-synthetase. The other pathway is the platelet release reaction where the pro-aggregatory agents ADP and 5-HT are secreted. Botney, Swanson & Shuman (1979) suggest that collagen and thrombin induce aggregation through separate and specific mechanisms and several workers (including Packham, Guccione, Greenberg, Kinlough-Rathbone & Mustard, 1977) have shown evidence that indicates that a third pathway operates in thrombin-induced aggregation. A third pathway (Vargaftig, Chignard & Benveniste, 1981) may be that of release of platelet activating factor (Paf). *In vitro* work has provided conflicting evidence. Malmsten, Hamberg, Svensson & Samuelsson (1975) stated that ADP release is necessary for aggregation whereas several other authors have found that products of the thromboxane pathway can cause aggregation without secretion (Kinlough-Rathbone, Reimers, Mustard & Packham, 1976; Charo, Feinman, Detwiler, Smith, Ingerman & Silver, 1977; Parise, Venton & Le Breton, 1982). *In vivo*, 100% inhibition by NSAIDs cannot be expected as the thromboxane pathway has only a minor role in the adhesion of platelets to collagen (Kinlough-Rathbone *et al.*, 1980). Also *in vivo*, synergism between agents such as collagen, thrombin and ADP will exist (Kinlough-Rathbone, Packham & Mustard, 1977). The *in vivo* system presented here, therefore, allows the study of intravascular platelet aggregation where thrombin generation, release of ADP from erythrocytes (Born & Wehmeier, 1979) and the interaction of platelet-vessel wall may contribute to the aggregation produced as a result of collagen injection.

The aggregation produced by high doses of collagen depended less on arachidonic acid mobilization (measured as TXB_2) compared with low doses of collagen (Figure 3) and agrees with several *in vitro* studies (Ali & McDonald, 1978; Huzoor-Akbar & Ardlie, 1978; Best *et al.*, 1980). In this experiment blood was sampled by cardiac puncture and the resulting mean levels of TXB_2 in plasma were associated with a high degree of error. In the experiments using NSAIDs a 1 ml sampling technique was employed. This latter method minimized the manipulations associated with the collection and processing of blood and Figure 1 shows that the amount of TXB_2 formed after collection of the sample was significantly reduced (d compared with e, $P < 0.1$). This method also increased the reproducibility (a compared with b, $P < 0.05$). The level of TXB_2 in animals connected to the Autocounter was the same as TXB_2 levels in animals where only one carotid artery was cannulated. This suggests that the cannulations required to facilitate continuous platelet counting, although invasive, do not cause trauma which would elevate TXB_2 levels.

Indomethacin produced a maximal inhibition of 50% at low dose collagen which was significantly different from the inhibition at high dose collagen. Although not significant, piroxicam also showed this difference. This supports the findings in Figure 3 that low dose collagen-induced aggregation operates more through the prostaglandin endoperoxide/TXA_2 pathway than does high dose collagen-induced aggregation. An implication of this observation is that the efficacy of NSAIDs in the pathological conditions of thrombosis and atherosclerosis may depend on the amount of sub-endothelial collagen which is exposed to the blood. Piroxicam produced less inhibition than indomethacin (Figure 5). Indomethacin also inhibits platelet lipoxygenase (Siegel, McConnell & Cuatrecasas, 1979) the products of which may have a role in irreversible platelet aggregation (Dutilh, Haddeman, Don & Ten Hoor, 1981). In addition, indomethacin has a calcium antagonistic action (Northover, 1977). Therefore, some of the inhibitory effect of indomethacin may be due to actions other than cyclo-oxygenase inhibition. Piroxicam is a specific cyclo-oxygenase inhibitor (Carty, Stevens, Lombardino, Parry & Randall, 1980) and was ten times more active than indomethacin. At the maximum tolerated doses piroxicam and indomethacin became less effective. This may have been due to cyclo-oxygenase inhibition in the vessel wall whereby synthesis of PGI_2, a potent anti-aggregatory prostaglandin, would be reduced. Or, it may have been that high doses of the drugs caused lysis of erythrocytes thereby releasing ADP and synergizing the aggregation produced by collagen. Examination of plasma samples from animals dosed with these high doses showed some evidence of lysis. This effect remains to be investigated.

Both indomethacin and piroxicam reduced completely the levels of TXB_2 in plasma after injection of

collagen. Basal levels of TXB_2 were not detectable (results not shown). As inhibition of aggregation by these drugs was only partial then *in vivo* collagen-induced platelet aggregation occurs by more than one pathway. One pathway is the conversion of arachidonic acid into endoperoxides and TXA_2. The remaining pathway(s) must operate independently of the thromboxane pathway.

Sulphinpyrazone (40 mg kg^{-1} and 80 mg kg^{-1}) and G-25671 (4 mg kg^{-1} and 8 mg kg^{-1}) were more effective at inhibiting aggregation due to low dose collagen than that due to high dose collagen, thus confirming the above results that low dose collagen operates more through the thromboxane pathway than does high dose collagen. At 80 mg kg^{-1} sulphinpyrazone, TXB_2 production by both doses of collagen was completely inhibited yet even more inhibition was observed at 160 mg kg^{-1}. This suggests that sulphinpyrazone (160 mg kg^{-1}) inhibits another pathway and from the results it appears that this pathway is more important in aggregation due to high doses of collagen. Wiley, Chesterman, Morgan & Castaldi (1979) have shown that sulphinpyrazone can inhibit aggregation through a 'membrane effect', in addition to inhibiting prostaglandin synthesis. Vargaftig, Chignard, Benveniste, Lefort & Wal, (1981) have shown inhibition of Paf release by sulphinpyrazone *in vitro* in guinea-pigs. A possible explanation therefore is that sulphinpyrazone inhibits Paf release from platelets though preliminary studies (unpublished) have shown that injection of Paf in rats has no effect on aggregation.

Buchanan, Rosenfeld & Hirsh (1978) state that the prolonged inhibitory effect of sulphinpyrazone on *in vivo* collagen-induced aggregation is due to a potent metabolite and Pay, Wallis & Zelaschi (1981) have shown that, *in vitro*, a metabolite of sulphinpyrazone is largely responsible for the effect of the drug on the prostaglandin pathway. This metabolite, G-25671, produced partial inhibition of *in vivo* aggregation at lower doses than those of sulphinpyrazone (Figure 5d). A dose of 8 mg kg^{-1} completely inhibited production of TXB_2 generated by collagen (Table 2).

Figure 5 shows that inhibition of intravascular platelet aggregation by sulphinpyrazone and its metabolite produce similar dose-response curves though 16 mg kg^{-1} G-25671 was not tested. The increased inhibitory effect achieved by 160 mg kg^{-1} sulphinpyrazone, after TXB_2 production had been completely reduced, is entirely contrary to the decreased inhibitory effect of high doses of indomethacin (16 mg kg^{-1}) and piroxicam (1600 μg kg^{-1}). This suggests that NSAIDs act primarily through inhibition of TXA_2 synthesis but higher doses of drug act on other pathways to increase (sulphinpyrazone and G-25671) or decrease (indomethacin and piroxicam) their inhibitory effect.

This study has shown, therefore, that extracellular divalent cation is essential in collagen-induced intravascular platelet aggregation. The *in vivo* method presented here enables a study of the mechanisms of platelet aggregation without interference by an anticoagulant. Results show that arachidonic acid metabolism, as measured by plasma TXB_2 levels, is only partly responsible for the aggregation resulting from injection of collagen in rats, and, that its importance is relative to the amount of collagen in contact with the platelet.

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