

ASSESSMENT OF ANTITHROMBOTIC DRUGS IN VIVO AND EX VIVO

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ABSTRACT

THE ASSESSMENT OF ANTITHROMBOTIC DRUGS IN VIVO AND EX VIVO

The usefulness of drugs which inhibit platelet function and may be effective antithrombotic agents can only be demonstrated in clinical studies. Unfortunately, since the stimuli which cause thrombosis in patients are likely to vary extensively and since the mode of action of these drugs on such stimuli, although not completely understood, are likely to be different, the results from clinical studies cannot be generalized either for drug or thrombotic condition. Furthermore, the initial investigations with promising drugs cannot be made clinically for economic and ethical reasons. Therefore, there exists the need for some screening test(s) with which these drugs can be evaluated in vitro. In addition, there exists a need for an experimental in vivo test in order to study in greater detail the drug effects, their modes of action and possible synergism. Three drugs have been identified as possible anti-thrombotic drugs, these are aspirin, sulfinpyrazone and dipyridamole. Both dipyridamole and sulfinpyrazone appear to correct shortened platelet survival seen in patients with prosthetic heart valves whereas aspirin does not. However, aspirin in combination with a low dose of dipyridamole, which itself has no effect, does appear useful in correcting reduced platelet survival seen in similar patients. Sulfinpyrazone also has been shown to reduce the incidence of thrombosis of A/V shunts in patients undergoing renal dialysis. Nonetheless, when these drugs

are tested ex vivo, different results are observed. Dipyridamole and sulfinpyrazone appear to have little or no effect on platelet adherence, aggregation and release whereas aspirin has been shown to inhibit platelet function for the lifespan of the aspirin-exposed platelet. In spite of these in vivo-ex vivo inconsistencies, all three drugs have been shown to effectively inhibit thrombosis in various animal models.

In addition, little data is available on the relation between drug effect, dosage and plasma level. Aspirin, which appears to have a permanent effect on the platelet, has a circulating life span of less than 60 minutes. No studies have previously examined the effects of dipyridamole and sulfinpyrazone in vivo and their relation to their circulating plasma concentrations determined ex vivo.

The general objective of this study was to investigate the effects of these three drugs in vivo in an attempt to answer some questions concerning the dose-response and mechanisms of action of the drugs. Specifically, this study undertook: 1) to determine some possible explanations for the discrepancies observed between the results of platelet survival studies and ex vivo tests of platelet function observed with aspirin, dipyridamole and sulfinpyrazone, and 2) having established the existence of some possible artifacts of ex vivo testing, to use an in vivo test to study i) the dose-response relationship of the drugs and ii) some aspects of the mechanism of action of aspirin and sulfinpyrazone.

The results of this study have 1) provided a better understanding of some of the problems which exist when assessing potential useful antithrombotic agents, 2) introduced a useful in vivo model with which to study the effect of these drugs in vivo, and 3) provided new data concerning the mechanism of action of aspirin and sulfinpyrazone. The following conclusions can be drawn from the results:

1) Screening of drugs in vitro and ex vivo are limited and probably not appropriate in their present form. This is because citrate masks the effect of dipyridamole. Possibly a substitute anticoagulant such as heparin may avoid this problem. Alternatively, such investigations may require the use of washed platelet suspensions in which no anticoagulants are present. But even this type of preparation has its limitations. In addition, by limiting the investigations of the effects of these drugs on platelet function ex vivo and in vitro, any effects produced by drug characteristics such as distribution, metabolism and elimination would be overlooked.

2) The use of an in vivo model such as the one described in this study appears to be a useful test for examining the in vivo effects of these and other drugs. Furthermore, various stimuli can be used in place of collagen (i.e., arachidonic acid, ADP, thrombin) in order to evaluate the effects on or interaction between the drug in question and a variety of stimuli.

3) the relationship between drug-plasma concentration and drug-effect varies with each drug studied. The effect of dipyridamole is

directly related to the concentration of the drug present in the platelet poor plasma. In order to maintain a dipyridamole effect, it may be necessary to increase the frequency of dipyridamole ingestion if the same relationship exists in humans. With aspirin, the effect is influenced by the initial plasma concentration, suggesting that this drug could be taken less frequently. The relationship between sulfinpyrazone-plasma concentration and sulfinpyrazone-effect is complex. The results from this study suggest that further studies must be made before any conclusions can be made about the optimal dose regime.

4) the "synergistic effect" of aspirin/dipyridamole observed clinically may be due, in part, to the alteration of the pharmacokinetics of dipyridamole by the salicylate moiety of aspirin present in the plasma.

5) the prolonged in vivo effect of aspirin is not due to acetylation of megakaryocytes nor exchange of the acetyl group from the plasma proteins onto new platelets entering the circulation. This aspirin effect slowly disappears with time and is consistent with the hypothesis that the appearance of new platelets into the circulation can overcome the aspirin effect.

6) the inhibitory effect of sulfinpyrazone is bi-phasic. Part of the effect is due to sulfinpyrazone per se and part of the effect is due to a metabolite not previously described. If a similar metabolite exists in humans, the current thoughts about sulfinpyrazone

therapy may need revision.

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Dedicated to my wife, Heidi,
whose love and understanding
I could not have done without....
and to Moss, part of our future...

INTRODUCTION

The platelet plays an important role in the genesis and growth of thrombi and the development of atherosclerosis. The platelet adheres to areas of damaged vessel wall and forms aggregates which provide a focal point around which fibrin can form and so stabilize the platelet aggregate. After adhering to certain surfaces the platelet releases a number of constituents which have effects on the vessel wall, other platelets and plasma coagulation components (Deykin, 1974; Luscher, 1956; Mills et al, 1968; Murer, 1972; Mustard and Packham, 1970; Ross and Glomset, 1976; Smith et al, 1973; Stemmerman, 1975; Weiss, 1975). These reactions of platelet adherence, release and aggregation are fundamental to the hemostatic and thrombotic responses.

These platelet reactions can be altered by a number of drugs which therefore have potential as antithrombotic agents. A number of different approaches have been used in the past to study the anti-thrombotic efficacy of such drugs. These include an investigation of 1) the effects of these drugs on platelet adhesion, release and aggregation in vitro, 2) their effects on similar tests ex vivo, 3) their effects in experimental models of thrombosis and 4) their effects in vivo in humans either on platelet survival or inhibition of thrombosis.

This thesis is concerned with the study of three drugs, aspirin, sulfinpyrazone and dipyridamole, which have been shown to have potential as antithrombotic agents in man, using a simple in vivo

method to measure the inhibitory effects.

Platelet adhesion

Platelets adhere to a number of components of the vessel wall which are exposed when the vessel is injured (Baumgartner, 1972, 1974; Baumgartner et al, 1971; Spaet and Zucker, 1964; Stemmerman et al, 1971) and to prosthetic surfaces (Baumgartner, 1974, Mason et al, 1969; Packham et al, 1967; Salzman, 1975). The adhesion of the platelets to basement membrane is augmented by a Factor VIII-related protein (Tschopp et al, 1974; Weiss, 1975; Weiss et al, 1974) and is not followed by the platelet release reaction (Baumgartner et al, 1971, 1976; Stemmerman et al, 1971). On the other hand, platelet adhesion to collagen does not require the VIII-related protein and is followed by the release of contents including adenosine diphosphate (Mustard and Packham, 1970; Weiss, 1975) as well as newly synthesized endoperoxides and thromboxane- A_2 (Malmston et al, 1975; Weiss, 1975). Jamieson has suggested that the adhesion of platelets to collagen depends upon the formation of an enzyme-substrate complex between an incomplete collagen glycoprotein, galactosyl hydroxylysine, and the platelet enzyme, glucose galactosyl transferase (Jamieson, 1974; Jamieson et al, 1971), but other theories have been proposed (Jaffe and Deykin, 1974; Kang et al, 1973; Muggli and Baumgartner, 1974).

The adhesion of the platelet to prosthetic surfaces is not well

understood. The prosthetic materials which are exposed to blood become rapidly covered with plasma protein which may be necessary for platelet adhesion.

The precise biochemical mechanisms of adhesion of platelets to the various biological and prosthetic surfaces has not been completely resolved and these mechanisms may differ with the nature and strength of the underlying stimulus. Thus, drugs which block one of these adhesion mechanisms may not be effective in blocking the other type of adhesion reactions in vivo.

Platelet release reaction

When the platelet adheres to a surface such as collagen, the platelet undergoes shape change and is induced to expel its contents into the surrounding medium (Grette, 1962; Holmson, 1977; Mustard and Packham, 1970; Weiss, 1971, 1975; Zucker, 1975). The products that are released from the platelet which may be important in platelet aggregation include adenosine diphosphate (Weiss et al, 1968; Mills et al, 1968), calcium (Murer, 1972), platelet phospholipids which accelerate blood coagulation (Harada and Zucker, 1971; Kaser-Glanzman and Luscher, 1972) and endoperoxides and thromboxane- A_2 (Hamberg and Samuelsson, 1974; Malmston et al, 1975; Needleman et al, 1976; Smith et al, 1973).

Arachidonic acid is released from platelet membrane phospholipids by the action of phospholipase-A. The arachidonic acid is

then converted by cyclo-oxygenase into prostaglandins and thromboxane- A_2 (Gerrard and White, 1975; Gerrard et al, 1977; Hamberg and Samuelsson, 1974; Hamberg et al, 1974a, 1974b, 1975; Malmston et al, 1975; Needleman et al, 1976; Smith et al, 1973). The currently accepted pathway for the formation of these products is shown in Figure 1 (Samuelsson, 1976; Samuelsson et al, 1976). The endoperoxides and thromboxane- A_2 induce the platelet release reaction as well as influencing vascular smooth muscle tone (Needleman et al, 1976, 1976a; Moncada et al, 1976, 1976a). Some prostaglandins also inhibit the platelet release reaction: Two lines of evidence also suggest that thromboxane- A_2 may cause platelet aggregation independent of the release reaction. Thus, Kinlough-Rathbone et al (1976) have demonstrated that arachidonic acid derivatives cause platelet shape change and aggregation of rabbit and human platelets that had been washed and then degranulated by treatment with thrombin. And, Charo et al (1977) who studied platelet aggregation and platelet secretion simultaneously, found that low concentrations of thromboxane- A_2 and endoperoxides G_2 and H_2 caused platelet aggregation without causing any platelet release.

Thrombin causes platelet aggregation by at least three different mechanisms (Kinlough-Rathbone et al, 1977). It induces the release of adenosine diphosphate which in turn causes platelet aggregation, it stimulates prostaglandin synthesis by the platelet and it also causes platelet aggregation by a mechanism independent of those

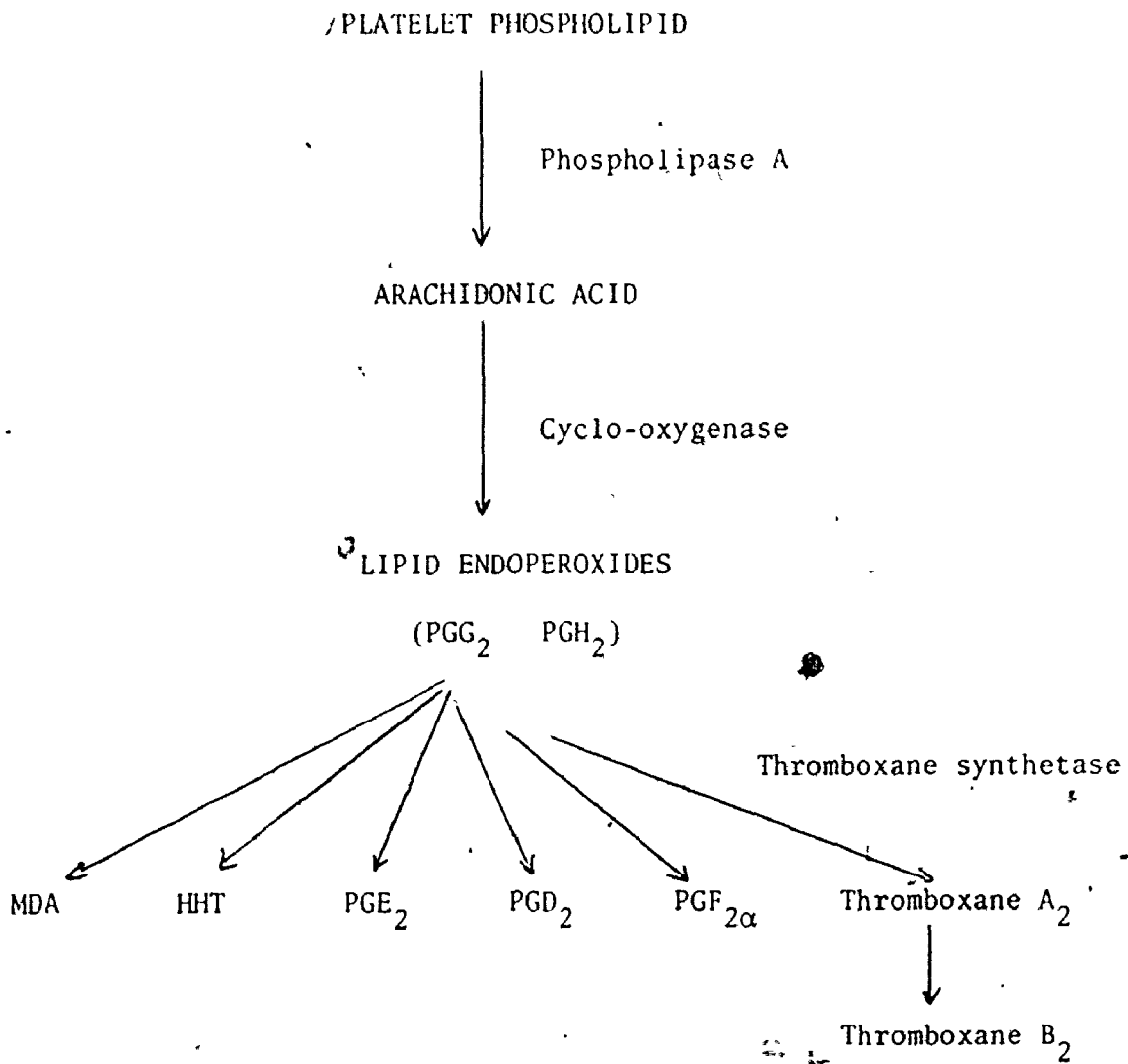


Figure 1. The currently accepted pathway of prostaglandin production by platelets.

two mechanisms (Gerrard and White, 1975; Hamberg and Samuelsson, 1974; Kinlough-Rathbone et al, 1976; Malmston, 1975; Russell and Deykin, 1976). Thus, if substantial amounts of thrombin are generated at the site of thrombus formation the drugs which only inhibit prostaglandin synthesis or even those which only inhibit adenosine diphosphate induced aggregation, may be of limited effectiveness.

Collagen stimulates the platelets to synthesize prostaglandins, release adenosine diphosphate independently of the prostaglandin pathway (Kinlough-Rathbone et al, 1977) and may also activate the intrinsic coagulation system (Akbar and Ardlie, 1976; Walsh, 1975).

The extent to which these mechanisms are affected by collagen is dependent upon the strength of the collagen stimulus. Thus, the effectiveness of drugs which may be useful for the inhibition of any one of these mechanisms may be influenced by the strength of the collagen stimulus.

Other agents which are released from the platelet, such as divalent cations, play a supportive role in the induction of the platelet adhesion and release responses (Harada and Zucker, 1971; Kaser-Glanzman and Luscher, 1972; Mills et al, 1968; Mustard et al, 1975; Chaco et al, 1976) and may also influence the effectiveness of drugs which alter these platelet responses.

Vessel wall response to injury

The injured vessel wall not only acts as an important stimulus for thrombosis but it also synthesizes prostaglandins which may inhibit thrombosis. Recently, a number of investigators have found that the vessel wall synthesizes a potent prostaglandin, PGI_2 , that inhibits platelet aggregation (previously known as PGX or prostacylin) (Moncada et al, 1976, 1976a, 1976b, 1977; Needleman et al, 1975, 1977; Taleson et al, 1977). It has been postulated that in order for PGI_2 to be produced, the platelet must provide the vessel wall with prostaglandins G_2 and H_2 (Moncada et al, 1976), although this view has been questioned. This is because other investigators have demonstrated that isolated perfused vessels in which no platelets are present produce a PGI_2 -like substance which affects both platelet aggregation and the vessel wall calibre (Ellis, 1976; Heyns et al, 1977; Kramer et al, 1976; Weeks, 1976; Weiner and Kaley, 1969). It is likely that PGI_2 provides an important protective mechanism against thrombosis. Thus, drugs which inhibit prostaglandin synthesis have the potential to both inhibit or augment thrombosis. This potential is based on both the relative effectiveness of the drug on prostaglandin synthesis in the platelet and vessel wall which may differ from drug to drug and the relative accessibility of any given drug to the platelet on the one hand and the vessel wall on the other hand.

Prevention of thrombosis.

Thrombosis is an important cause of death and disability in western civilization. Although there are control mechanisms which limit the interaction between the platelet and vessel wall, these are relatively ineffective. For this reason, there has been considerable interest in the pharmacological inhibition of the platelet reactions, adhesion, release and aggregation, which are thought to be important in thrombosis.

The most definitive way of demonstrating the effectiveness of any such agent is by testing it in clinical trials in man. However, outside of any ethical consideration, this is very time consuming and expensive and for this reason various "substitute" screening procedures have been used to evaluate these drugs. The two simplest and most frequently used approaches have been in vitro and ex vivo testing. These methods have limitations which are discussed below.

The efficacy of these drugs has also been assessed in the following clinical situations; in patients with prosthetic heart valves, coronary artery disease, cerebral vascular disease and peripheral arterial thrombosis and ischemia; in patients with venous thrombosis; and in patients with arterial-venous shunts. Very few of the clinical studies adhere to the requirements necessary for the design of valid clinical trial to test the effectiveness of these drugs (Genton et al, 1975) and therefore it has been difficult

to make any concrete appraisal of them.

Mode of action of aspirin

Aspirin inhibits the platelet release reaction induced by adrenaline, collagen, antigen-antibody complexes, gamma globulin coated surfaces and low concentrations of thrombin (Atac et al, 1970; Kinlough-Rathbone, 1975; Mustard and Packham, 1970; Zucker and Peterson, 1970). This inhibitory effect of aspirin can be overcome by increased concentrations of collagen and by weak concentrations of thrombin (Evans et al, 1968; Mustard and Packham, 1970). Although aspirin is cleared rapidly from the circulation, it can inhibit the platelet release reaction for at least five days (O'Brien, 1968; Weiss et al, 1968). This prolonged effect of aspirin suggests that the drug has an irreversible effect on the platelet and recent evidence suggests that this effect is mediated through the inhibition of platelet prostaglandin production. Aspirin has been shown to inhibit the synthesis of prostaglandin endoperoxides and thromboxane-A₂ from arachidonic acid (Roth et al, 1975; Smith and Willis, 1971; Weiss, 1975). Studies by Roth and Majerus (1975) and Valles et al (1976) have demonstrated that this effect is produced through the acetylation by aspirin of the platelet enzyme, cyclo-oxygenase. Other investigators have reported that aspirin also acetylates other platelet membrane proteins and it is possible that the drug may affect platelet function by one of these

mechanisms (Jamieson et al, 1971; Rosenberg et al, 1971). The effect of aspirin is not reflected in any alteration of platelet morphology such as size, cell wall structure, density or mitochondrial activity (Fajardo, 1975). Although the circulating half-life of aspirin is relatively short (2-3 hours), a single dose of aspirin has been shown to prolong the bleeding time for as long as five days in man (Mielke et al, 1969; Nadell et al, 1974). Aspirin has not, however, been shown to have any effect on the prolongation of the reduced platelet survival seen in patients with one of a number of thrombogenic disorders (Harker and Slichter, 1972, 1974).

Mode of action of sulfinpyrazone

Sulfinpyrazone inhibits the platelet release reaction induced by collagen and adrenaline (Packham et al, 1967; Zucker and Peterson, 1970), but does not prolong the bleeding time in human volunteers (Hirsh and Blajchman, 1977). It has been shown to prolong reduced platelet survival seen in patients with gout, prosthetic heart valves, coronary artery disease and recurrent venous thrombosis (Genton and Steele, 1975; Smythe et al, 1965; Steele et al, 1973a, 1975, 1975a; Weily and Genton, 1970; Weily et al, 1974). Ali and McDonald have demonstrated that collagen-induced thromboxane- Λ_2 production is decreased in the presence of sulfinpyrazone (Ali and McDonald, 1977). However, unlike aspirin, this effect appears to be reversible as it can be removed by washing. In addition,

sulfinpyrazone has been shown to inhibit the adherence of platelets to damaged rabbit aorta (Cazenave et al, 1977). On the other hand, it is possible that sulfinpyrazone may exert its effects by other mechanisms not yet described.

There have been no studies to demonstrate any relationship between the amount of sulfinpyrazone circulating within the plasma and its effects in vivo.

Mode of action of dipyridamole

Dipyridamole was initially used as a vasodilator (Nickerson, 1970). In high concentrations in vitro, dipyridamole inhibits primary and secondary adenosine diphosphate induced platelet aggregation and the platelet release reaction induced by collagen and thrombin (Evans et al, 1968; Cucuianui et al, 1971; Mills, 1968; Zucker and Peterson, 1970). Dipyridamole has been reported to slow the rate of uptake of adenosine into the platelet and to decrease platelet adenosine monophosphate levels by inhibiting cyclic adenosine monophosphate phosphodiesterase (Emmons et al, 1965; Cucuianui et al, 1971; Mills and Smith, 1971; Philips et al, 1973; Vigdahl et al, 1971) but it is not known whether or not either one of these two mechanisms are responsible for the inhibitory effect of dipyridamole on platelet aggregation.

An alternative hypothesis to explain the mechanism of the dipyridamole effect was put forth by Aznar and Valles (1974). These

investigators found that the electrophoretic migratory velocity of the platelet was significantly increased after being incubated with dipyridamole in vitro. They postulated that since the platelet electrophoretic migratory velocity was decreased in the presence of platelet aggregating agents, like adenosine diphosphate (Hampton and Mitchell, 1967), that dipyridamole could exert its inhibitory effect on the platelet by altering the electrical potential of the platelet membrane. It is not known whether the electrical potential of the platelet is altered in relation to altered cyclic adenosine monophosphate levels.

Although dipyridamole in high concentrations has inhibitory effects on the platelet when tested in vitro, Harker and Slichter and others failed to demonstrate any ex vivo effects of dipyridamole when given to man in therapeutic doses, and dipyridamole has not been shown to prolong the bleeding time (Harker and Slichter, 1972). Dipyridamole does prolong the reduced platelet survival seen in patients with a number of thrombovascular conditions (Harker and Slichter, 1972, 1974). In addition, these two investigators have demonstrated that a lower dose of dipyridamole can also alter the reduced platelet survival seen in these patients when this dosage is given in combination with one gram of aspirin.

Effects of aspirin, sulfinpyrazone and dipyridamole in man:Transient cerebral ischemia

Some studies indicate that platelet thromboemboli in the cerebral circulation are the cause of transient ischemia (Ashby et al, 1963; Gunning et al, 1964; Russell, 1961). For this reason, drugs which may alter the platelet involvement in this condition have been investigated.

In 1971, Harrison and co-workers reported that aspirin reduced the number of attacks of transient cerebral ischemia in two patients suffering from amaurosis fugax. Mundall et al (1972) reported similar observations in one patient in which the occurrence of transient blindness ceased when aspirin was given and reoccurred when aspirin therapy was stopped. Two multicenter studies have recently been completed and one of these has been published (Fields et al, 1977). In this study aspirin was shown to reduce the episodes of transient cerebral ischemia, stroke and cause specific death in patients with a previous history of transient cerebral ischemic attacks. The effect was limited to patients who had multiple episodes beforehand and in those patients with angiographically appropriate lesions.

In a double-blind crossover trial, Evans found that the incidence of amaurosis fugax was decreased during sulfinpyrazine therapy in 20 patients with transient cerebral ischemia. Each patient was given sulfinpyrazone or a placebo for six weeks and then crossed over to

the alternative therapy for another six weeks. The episodes of transient cerebral ischemia were decreased when the patient was on sulfinpyrazone (Evans, 1973). The effect of sulfinpyrazone on the end point such as stroke or death could not be evaluated because of the brevity of the study.

Blakely and Gent reported on a double-blind study of 291 geriatric patients who were given sulfinpyrazone or a placebo and followed for four years (1975). These investigators found that there was a reduction in death due to thrombovascular causes in those patients given sulfinpyrazone. These investigators did not assess the effects of sulfinpyrazone on the non-fatal events such as stroke or transient cerebral ischemic attacks.

There is no real evidence to demonstrate the lack of or the presence of any effect of dipyridamole on transient cerebral ischemia. One study suggests that dipyridamole does not alter the frequency of these attacks, stroke or death in patients suffering from transient cerebral ischemia or stroke (Acheson et al, 1969). However, the number of patients was small, the length of the study was relatively short and the overall incidence of the two most important endpoints, stroke and death, was low and no real conclusions could be made.

Coronary heart disease

Platelets could be involved in the coronary heart disease in a number of ways. These include 1) incorporation into occlusive

thrombi; 2) platelet aggregates embolizing into the microcirculation, or 3) being involved in the genesis of the atherosclerotic lesion (Chandler et al, 1974; Haerem, 1974; Jorgenson, 1971; Mustard and Packham, 1969). The importance of platelets in coronary artery disease is supported by the findings that up to 50% of those patients with demonstrable lesions in the coronary arteries had reduced platelet survival and this was normalized by both dipyridamole and sulfipyrazone (Harker and Slichter, 1970; Ritchie and Harker, 1977; Steele et al, 1975a). Elwood et al (1974) reported the results of a multicentered, randomized double-blind trial. Men who had suffered a recent myocardial infarction were given 300 mg of aspirin for a period up to 30 months. A life table analysis showed a favourable trend of increased survival in the aspirin-treated group. For those men who had a myocardial infarction within the six week period immediately before entering the trial and given aspirin, the mortality rate was 8% as compared to 13% in the placebo-treated group (Elwood, 1974).

A retrospective study by the Boston Collaborative Drug Surveillance Group (1972) found that the frequency of aspirin ingestion was significantly less in those patients who suffered a myocardial infarction, being 0.9% as compared to 4.9% in the other group. A follow-up survey in Boston (1972) found similar results. Although the potential biases and limitations of a retrospective study limited the conclusions made, the data does suggest that aspirin might be

useful in coronary artery disease. There has been no evidence to suggest that either dipyridamole or sulfinpyrazone is effective in preventing myocardial infarction in similar type patients.

Arterial thrombosis and arterial-venous shunt thrombosis

Although platelets are associated with arterial and arterial-venous shunt thrombosis, only a few studies have examined the efficacy of these drugs in these disorders. The conditions which have been investigated and in which affect the arterial circulation include the thrombotic complications that occur in patients that have diagnostic or therapeutic catheterization, peripheral artery surgery and arterial-venous shunts.

Aspirin was found to have no benefit in preventing arterial thrombosis in patients having brachial or cardiological catheterization (Hynes et al, 1973; Freed et al, 1974).

Sulfinpyrazone was found to be effective in preventing thrombus formation in patients with arterial-venous shunts for hemodialysis (Kaegi et al, 1974). In these patients, thrombus formation which usually occurred in the venous outlet of the shunt presented a serious complication for dialysis. Fifty-two patients were randomly assigned to six months of sulfinpyrazone or placebo therapy. The total number of thrombi and the number of patients with thrombi in each group was measured. A significant decrease in the number of patients with thrombi was found in the sulfinpyrazone-treated group

and there was a significant decrease in the number of thrombi within that group. At the end of six months, the patients were crossed over to the alternate therapy. Again, sulfinpyrazone had beneficial results.

In six patients with arterial-venous fistulas for hemodialysis, who were followed for a year, platelet sensitivity to adenosine diphosphate was increased and 18 thrombi per year found in the fistulas. When the patients received 300-400 mg of dipyridamole daily and followed for another year, both their platelet sensitivity to adenosine diphosphate and the number of thrombi per year in the fistula significantly decreased (Jacques et al, 1976).

Prosthetic heart valves

Thromboembolism is a serious complication in patients with artificial heart valves. Several investigators have demonstrated that platelet survival in patients with prosthetic heart valves is reduced (Harker and Slichter, 1970; Weily and Genton, 1970) and have reported that there is a correlation between shortened platelet survival and the frequency of thromboembolism (Steele et al, 1975a). A number of studies have assessed the usefulness of anti-platelet drugs in patients with prosthetic heart valves and demonstrated some beneficial results.

Sullivan et al (1969) evaluated the benefit of treating patients with prosthetic heart valves with 400 mg dipyridamole daily in

combination with the standard warfarin therapy. During one year of observation, they found that thromboembolic events developed in 14% of the placebo + warfarin-treated patients as compared to 1.3% in the dipyridamole-treated group. Harker and Slichter (1972) reported that the reduced platelet survival seen in patients with prosthetic Heart valves was normalized in those patients given dipyridamole (400 mg daily). In addition, these investigators reported that the combination treatment of 1 gram of aspirin + 100 mg of dipyridamole, daily, also prolonged the shortened platelet survival whereas when each drug was given separately, there was no effect. A Japanese group of investigators also found beneficial results of aspirin + dipyridamole in patients with prosthetic heart valves (Taguchi et al, 1975). These investigators examined the incidence of thrombosis in two groups of patients with prosthetic heart valves and found that the incidence of thrombosis was 1.9% in the first five years in the aspirin + dipyridamole treatment group as compared to 9.1% in the placebo-treated group, and 2.9% in the second five years in the treated group as compared to 14% found in the placebo group.

Genton's group has reported that sulfinpyrazone is also useful in prolonging the reduced platelet survival seen in patients with prosthetic mitral and aortic valves (Genton and Weily, 1972; Weily and Genton, 1970) and have reported some benefit with dipyridamole as well (Steele et al, 1975a).

Venous thrombosis

The traditional view is that venous thrombosis occurs as a result of fibrin deposition (Hirsh and Genton, 1974). This is supported by studies which show both prophylaxis and beneficial treatment of venous thrombosis following anticoagulant therapy. However, there is some histological evidence that platelet aggregates sometimes form the nidus of the venous thrombus (Frédman, 1972; Paterson, 1969; Sevitt, 1969). The reduction in platelet survival seen in patients with recurrent venous thrombosis (Steele et al, 1973a) and the findings of Walsh which showed increased platelet coagulation activity in patients who developed venous thrombi further supports this view (Walsh et al, 1974). Thus, it is possible that drugs which suppress platelet function may be useful in preventing the initial episode of venous thrombosis.

Salzman and associates (1971) assessed clinically the anti-thrombotic effects of four drugs including aspirin and dipyridamole in a group of patients who had undergone hip replacement. They concluded that aspirin was as effective as warfarin and that dipyridamole was not effective.

In another study, Zeckert et al (1973) evaluated the prophylaxis effectiveness of aspirin given randomly to 140/278 patients undergoing surgery for hip fractures. The aspirin treated patients were given 1.5 grams of aspirin daily. Within the two week period following surgery, a total of 12 patients had died, 3 in the aspirin

group and 9 in the placebo group. This was not statistically significant. However, at an autopsy, one pulmonary embolus was found in the aspirin-treated patient and 8 in the placebo-treated patients. This difference was significant at the 0.02 level.

Browse and Hall (1969) evaluated the effect of dipyridamole on the incidence of clinically detectable venous thrombosis in patients undergoing major surgery in a double-blind study. They could find no effect of dipyridamole on the incidence of thrombosis in 315 patients treated with dipyridamole as compared to 334 placebo treated patients, however, the overall incidence of venous thrombosis was very low, even in the placebo-treated group. In another group of patients undergoing general surgery, dipyridamole in combination with aspirin was found to be useful in the reduction of the incidence of post-operative venous thrombosis (Renney et al, 1976).

Inconsistency in the effects of aspirin, sulfinpyrazone and dipyridamole when tested ex vivo and in vivo

The preceding part of this chapter has been an overview of some of the effects of these three drugs on platelet function and in clinical conditions seen in man. When the effectiveness of these drugs in the different test systems were compared with each other, a number of discrepancies were observed. Thus, sulfinpyrazone and aspirin inhibit the platelet release reaction and dipyridamole inhibits both the release reaction as well as adenosine diphosphate

induced platelet aggregation when these drugs were added in high concentrations to platelets in vitro (Cucuianui et al, 1971; Zucker and Peterson, 1970; Mustard and Packham, 1970). In contrast, when these drugs are given to patients at pharmacological doses, the results of tests of platelet function ex vivo are much different. Aspirin has a marked effect on ex vivo platelet release reaction while sulfinpyrazone and dipyridamole showed little or no effect (Warlow et al, 1974; Zucker and Peterson, 1970; Steele et al, 1973). Furthermore, aspirin prolongs template bleeding time in man while sulfinpyrazone and dipyridamole had no such effect (Mielke et al, 1969, 1977; Hirsh and Blajchman, 1977). Clinically, aspirin has no effect in normalizing the reduced platelet survival seen in patients with various thrombovascular disorders whereas sulfinpyrazone and dipyridamole do prolong reduced platelet survival seen in similar type patients (Harker and Slichter, 1970, 1972; Steele et al, 1975a; Genton et al, 1975). The reason for these differences between in vivo and ex vivo effects of these drugs is uncertain. Two explanations are possible. First the discrepancies might be caused by qualitative differences between the nature of the stimulus to which the platelets are exposed in vivo and ex vivo and the differences in the effects of these drugs on such stimuli such as prosthetic heart valves and damaged endothelium in vivo and collagen, adenosine diphosphate and thrombin ex vivo. Secondly, these discrepancies could be due to the effects of ex vivo manipulations such as centrifugation, separation of blood components

and/or the addition of anticoagulants to the blood on the inhibitory effect of these drugs on the platelet release reaction.

Aims of Study

The general objective of this study was to investigate some of the reasons for the different effects of the three drugs. In order to do this, it was necessary to:

- 1) develop a simple model to test the effects of these drugs on platelet function in vivo;
- 2) to make use of this model, to examine the relationship of the plasma-drug levels and their effects, and possible synergistic effects of aspirin and dipyridamole, and
- 3) to examine some of the unresolved issues concerning the mode of action of aspirin and sulfinpyrazone.

In particular, experiments were designed:

- 1) to evaluate the effects of these drugs on the interaction of the platelet with a common stimulus (collagen) used both in vivo and ex vivo. In this way the effects of ex vivo manipulations on the platelet-collagen interaction could be assessed.
- 2) to examine the relationship between dose and effect of these drugs.

No previous studies have reported the relationship of the level of circulating sulfinpyrazone and dipyridamole and their effects on platelet function in vivo. In addition, it has not been established

whether or not the effects of a combination of low dose aspirin + dipyridamole on reduced platelet survival are a result of a "true synergistic" effect or whether this effect is mediated through another mechanism. The answer to these questions may have important ramifications on the dose regime of these drugs used; and

3) to determine whether aspirin exerts its prolonged effect on the platelet by affecting megakaryocytes and therefore altering the function of newly released platelets from them, or by acetylating plasma proteins which may then bind to platelets and interfere with their function.



CHAPTER II

MATERIALS AND METHODS

MATERIALS

A. Platelet function inhibiting drugs and placebos

Acetylsalicylic acid (aspirin) was obtained from Sigma Chemical Corporation, St. Louis, Missouri. The crystals were solubilized slowly by adding sodium carbonate (Fisher Scientific, Toronto, Ontario) to the crystal-glass double distilled water mixture. All aspirin solutions were brought to a final pH of 6.8. During preparation, the pH of the aspirin solution was monitored continuously to ensure that the pH remained below 7.0. This was done to prevent any hydrolysis of aspirin to salicylate which occurs at pHs above 7.0. Batches of aspirin were made up for each series of studies and stored at -70°C . The placebo used with aspirin was 0.55 M sodium carbonate, pH 6.8.

Dipyridamole was kindly supplied by Dr. G. Worsley, Boehringer Ingelheim Canada, Dorval, Quebec. This drug was supplied in 2 ml ampules containing 5 mg/ml. The suspending vehicle which consisted of 1 ml 0.10N HCL, 19 ml water and 30 ml 0.5 M acetate buffer, pH 4.15, was similarly supplied.

Sulfinpyrazone was generously supplied by Ciba-Geigy Canada Limited, Dorval, Quebec. This drug was supplied in 10 ml ampules. The sulfinpyrazone was suspended in a solution consisting of 0.1% benzyl alcohol, 0.01% sodium sulphate, 0.018% ascorbic acid and 0.002% disodium ethylenediaminetetraacetic acid (EDTA).

Indomethacin (Merck, Sharpe & Dohme, West Point, Pa.) was prepared by dissolving the drug with 0.55 M sodium carbonate

(Fisher Scientific, Toronto, Ontario); pH was 8.4.

Sodium salicylate was obtained from Fisher Scientific Company, Toronto, Ontario.

B. Radioactive materials

Sodium chromate (^{51}Cr) was obtained in solution from New England Nuclear, Boston, Mass. It was suspended in saline at a specific activity of 300-400 mCi/mg in a concentration of 1 mCi/ml.

C. Agents used to initiate platelet function in vivo and ex vivo

Acid soluble collagen, used to initiate platelet aggregation, was generously supplied by the Coagulation Laboratory of McMaster University Medical Centre, Hamilton, Ontario. The collagen (Type 1 from bovine achilles tendon, Sigma Chemical Corporation, St. Louis, Mo.) was suspended in 0.522 M acetic acid in a concentration of 2.5 gm/ml, pH 2.8 and stored at 4°C. This solution was stable for at least three months. Each batch was tested on platelet aggregation both in vivo and ex vivo to determine qualitative comparability from one batch to another. When the collagen was used in vivo, it was diluted 1:10 with saline (final pH 3.8) immediately prior to use. The same collagen was used in higher dilutions for in vitro studies.

The n-ethylmalimide (NEM) which was obtained from Sigma Chemical Corporation, St. Louis, Mo., was used to stimulate platelet malondialdehyde production in accordance with the method of Stuart

et al (1975).

Adenosine diphosphate was obtained from Sigma Chemical Corporation, St. Louis, Mo.

D. Anticoagulants

Acid-citrate-dextrose (ACD) was made up according to the method of Aster and Jandl (1964). ACD was used as the standard anticoagulant into which whole blood was collected in order to prepared platelets for i) ^{51}Cr -labelling and re-injection and ii) malondialdehyde assays.

Heparin originating from porcine mucosa was obtained from Connaught Laboratories, Toronto, Ontario.

Sodium citrate (Fisher Scientific, Toronto, Ontario) at an initial concentration of 3.8% was used as the anticoagulant when platelets were prepared for ex vivo and in vitro aggregation studies.

E. Divalent cations

Both calcium chloride and magnesium chloride were obtained from Fisher Scientific, Toronto, Ontario.

F. Anesthetics

In the initial pilot studies, sodium pentobarbital was obtained from both Abbott Laboratories and MRC Laboratories in Ottawa.

Unfortunately, neither drug company maintained any consistency with

their preparation of the sodium pentobarbital solution. In particular, propylene glycol, which was part of the suspending vehicle for the sodium pentobarbital, varied with each solution. This led to a variable degree of hemolysis of blood taken from the anesthetized animals. Therefore, for all the studies reported in this thesis, the sodium pentobarbital, which was obtained in powder form from MTC Pharmaceuticals, Hamilton, Ontario, was dissolved in glass distilled water immediately prior to administration.

METHODS

2

Preparation of platelet suspensions for re-injection

Collection of blood: New Zealand white rabbits were used throughout all experiments. Rabbit platelets were isolated and prepared by a modification of the method of Ardlie, Packham and Mustard (1970). Rabbits were anesthetized with sodium pentobarbital and exsanguinated through a carotid cannula. Six parts blood was collected into syringes containing one part ACD. The blood was centrifuged at 150 g for 15 minutes to sediment the red cells. The platelet rich plasma (PRP) was decanted off and centrifuged at 1200 g for 10 minutes to separate the platelets from the plasma. The platelet poor plasma (PPP) (sic) was decanted and centrifuged at 1200 g for another 10 minutes. The second spin of the PPP provided an additional yield of platelets equivalent to 20-30% of the total platelets collected.

Washing and labelling of platelet suspensions (PS): All platelets, once isolated from the plasma, were suspended in 5 ml calcium- and albumin-free Tyrodes (pH 6.25, osm. 297 mM) containing 100 λ 2% ethyleneglycoltetraacetic acid (EGTA,, K.K. Laboratories, New York, N.Y.) and 90 μ Ci 51 Cr per donor rabbit used. Platelets were transferred to this labelling wash using a siliconized Pasteur pipette. EGTA was included to ensure that any free calcium transferred in the plasma with the platelets was chelated. The platelets

were allowed to stand in this solution for 40 minutes at 22°C. Then the platelets were centrifuged at 1200 g for 10 minutes. The supernatant was discarded and the platelets resuspended in 10 ml calcium-free Tyrodes wash containing 0.35% albumin (bovine, Sigma Laboratories, St. Louis, Missouri). The platelets were allowed to stand in the wash for 5 minutes, re-centrifuged and resuspended in a second calcium-free Tyrode wash. Once again, the platelets were isolated and resuspended in Tyrode albumin (pH 7.35, osm. 297 mM). Five ml Tyrode albumin was used for each donor rabbit. The average platelet count of these suspensions ranged from 2 to $3 \times 10^7/\text{mm}^3$. Three ml of this suspension was immediately injected through a 23 gauge needle into the marginal ear vein of each recipient rabbit.

Preparation of platelet suspension for in vitro tests

The procedure for preparing non-labelled platelet suspensions was similar to the preceding method except for the following changes. Platelets were washed only in calcium-free Tyrode (pH 6.25), the initial wash always contained 100 λ 2% EGTA and the platelets were finally suspended in Tyrode's albumin in a concentration of 3×10^6 platelets/ mm^3 .

In vivo aggregation studies

Rabbits were injected with homologous ^{51}Cr platelets. The

rabbits were anesthetized with sodium pentobarbital (30 mg/kg body weight) and both the left carotid artery and right jugular vein cannulated with polyethylene tubing (Intramedic PE-190). The carotid cannula was positioned such that the tip of the cannula was situated at the orifice of the carotid artery entering the apex of the aortic arch. The tip of the jugular cannula was situated in the common vena cava. The accurate positioning of both cannulae was crucial. The arterial positioning ensured a systemic flow of injected material rather than a coronary flow followed by possible mortality. The venous positioning ensured clear simple blood sampling at predetermined times. One hundred $\mu\text{g}/\text{kg}$ collagen was infused via the carotid cannula over 60 seconds. Serial blood samples (1 ml) were collected from the jugular cannula into syringes containing 0.5 ml ACD. These samples were collected before and 30, 60, 90, 120, 240 and 360 seconds after commencing the collagen infusion. Each sample was placed in a glass tube (12 x 75 mm) and its radioactivity determined in a 1085 gamma scaler (Nuclear Chicago) by counting for 4-10 minutes. In early experiments, a 50 λ aliquot was taken from each sample and diluted in 950 λ 1% ammonium oxalate. Each aliquot was incubated at 4°C for 20 minutes and prepared for visual platelet counting. The pattern of platelet recovery determined by radioactivity counting followed the same pattern as platelet recovery determined by visual platelet count. Since phase microscopic determination of platelet counts was tedious and subject

to counting error, platelet counts were determined by radioactivity in all subsequent studies.

Ex vivo aggregation studies

Platelet aggregation studies ex vivo were performed by a modification of the methods of Born (1962) and Mustard et al (1964). Blood was collected from the experimental animals into either 3.8% sodium citrate (9 parts blood, 1 part citrate) or heparin (2 units/ml). The blood was centrifuged at 150 g for 15 minutes to sediment the red cells. The PRP was decanted off and the remaining red cells-plasma were respun at 1500 g for 10 minutes to obtain any residual PPP. The PRP was diluted with the autologous PPP to a platelet count of $300,000/\text{mm}^3$. One ml aliquots of PRP in siliconized cuvettes were incubated at 37°C for 5 minutes before being tested. Each PRP sample was placed in an aggregation module (Payton Associates, Scarborough, Ontario) which was coupled to a recorder (Rikadenki Uppsala, Sweden). The PRP was stirred by a magnetic stirring device and kept at 37°C . The amount of light transmitted through the suspension from a light source was measured by a photo-electric cell and recorded on the recorder. The addition of 100λ of increasing concentrations of collagen resulted in increased platelet aggregation and an associated alteration of light transmission through the plasma. A typical recording is shown on Figure 2. Maximum aggregation was derived from the continuous recording of

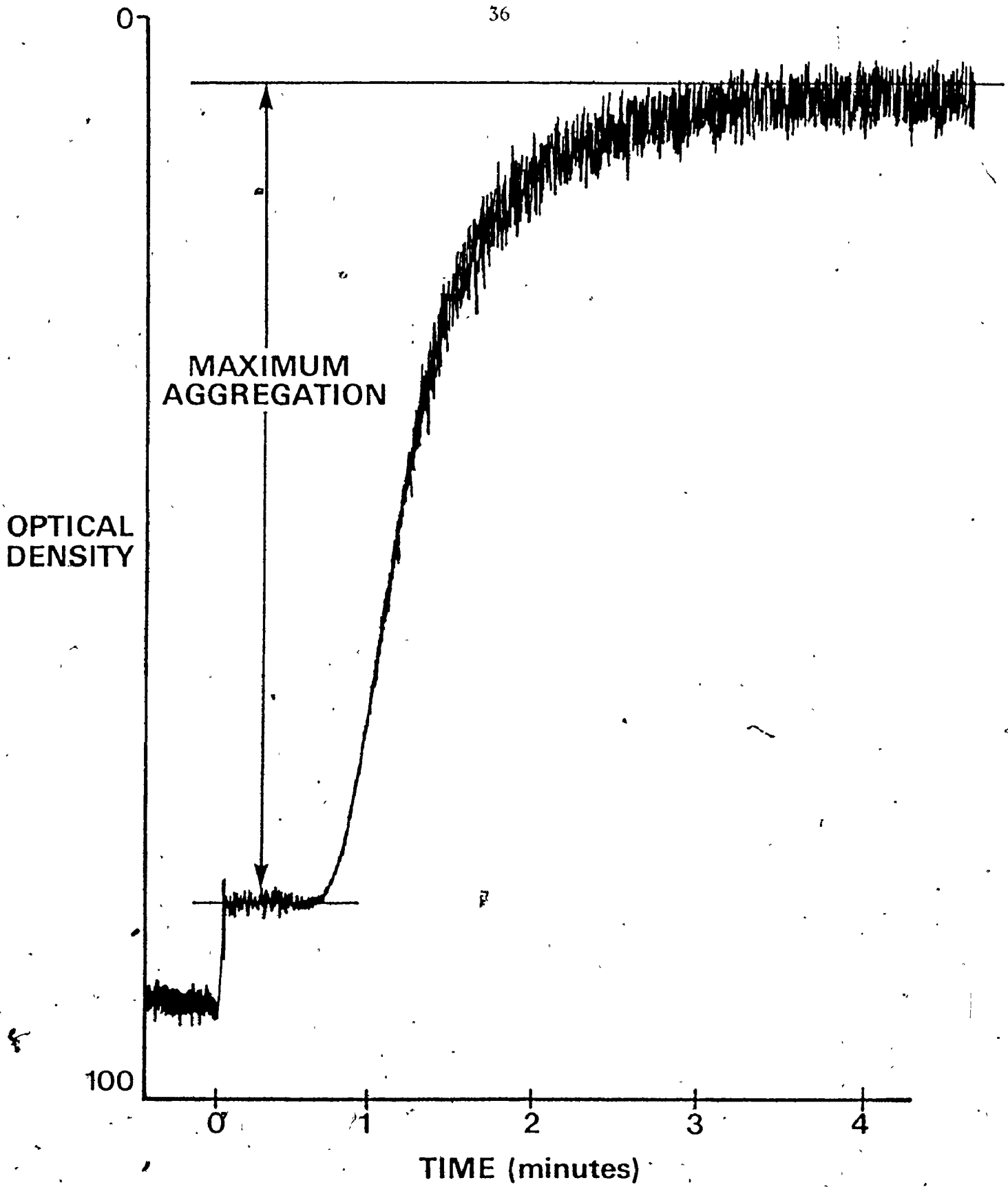


Figure 2. Maximum aggregation derived from the continuous recording of light transmissions in an aggregometer.

the light transmission as indicated on the figure. Studies using platelet suspensions were performed in the same way.

Platelet malondialdehyde (MDA) production determinations

Preparation of platelets for determination of MDA production: Measurement of platelet MDA production has been shown to be a useful index of platelet prostaglandin synthesis (Smith et al, 1976). Determination of MDA production was similar to the method used by Stuart et al (1975) to determine MDA production of human platelets. A volume of 17.6 ml of rabbit blood was collected into 2.4 ml of ACD. The platelets were centrifuged at 150 g for 15 minutes. The PRP was decanted into another tube and respun at 1500 g for 10 minutes. The supernatant was discarded. The tube was inverted for 2 minutes to drain off any excess PPP. Then the platelets were suspended in a 2 ml solution of 1 mM NEM in buffered phosphate saline (0.15 M, pH 7.4) and incubated at 37°C for 60 minutes. A platelet count was performed to determine the total number of platelets present in each sample. This reaction was terminated by the addition of 2 ml 2-thiobarbituric acid (0.53%) (BEH Chemical Limited, Poole, England). This mixture was shaken vigorously in a bench shaker and then boiled for 10 minutes. At that time, the mixture was allowed to cool to room temperature as it was being centrifuged at 800 g for 15 minutes. The supernatant containing the pink chromogen was decanted and read at 532 nm in a

spectrophotometer (Gilford, Model 2400). Results were expressed as nM MDA/ 10^9 platelets.

Preparation of standards: Malondialdehyde bis-(dimethylacetal), (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) was dissolved in ethanol to a concentration of 3.28%. This solution was hydrolyzed overnight with equal volumes of 0.2N HCL. The addition of one part hydrolyzed solution with nine parts buffered phosphate saline yielded an MDA concentration of 10 nM/2 ml when boiled for 10 minutes with 2 ml of 0.53 2-thiobarbituric acid. This freshly made solution was used as the stock solution to make up various dilutions down to a concentration of 0.25 nM MDA. The optical density of each studied sample was plotted against the curve of optical density equivalence of MDA/2 ml of solution variant.

Production of thrombocytopenic rabbits

Rabbits were rendered thrombocytopenic by whole body irradiation by a cesium source (930 rad, 465 rad on each side for 14.5 minutes). During irradiation, the rabbits' necks were shielded by a lead collar in order to prevent any radiation damage to the local underlying blood vessels. The rabbits were given injections of 1 ml penicillin-streptomycin (Derapen C, Ayerst Laboratories, Montreal, Quebec) 24 and 48 hours after irradiation. Seven days later, each rabbit received an intravenous injection of 0.3 ml of sheep antisera

to rabbit platelets 14 to 18 hours before bleeding time studies were to be performed. Sheep anti-rabbit platelet sera was generously prepared and supplied by Dr. Jack Gauldie, Department of Pathology, McMaster University.

Bleeding time (BT) method

Bleeding time studies were performed on the jugular vein of both normal and thrombocytopenic rabbits. Immediately before preparing each rabbit for the BT study, a sample of blood was taken from the marginal ear vein and the platelets in that sample counted by the method previously described. Each rabbit was anesthetized with sodium pentobarbital (30 mg/kg). Both jugular veins were isolated. Flow was occluded distally with a serrafine and each vessel segment was allowed to empty of blood. Then a second clamp was placed proximally on the vessel. The isolated segment was then filled with either aspirin, indomethacin or an appropriate control solution. These agents were injected into the segment by puncturing an inflowing branch of the vessel. The needle was removed and the puncture site closed with a ligature. The test agent was left in the isolated segment for 55 minutes. Both serrafines were then removed and flow restored, allowing the indwelling solution to wash away. Five minutes later, the BT was measured. The vein was arranged above a small aluminum trough. A puncture wound was made into the vein with a 23 gauge needle. The puncture site was

observed until bleeding from the hole stopped. The time for bleeding cessation was recorded. A saline drip directed onto the trough above the jugular vein washed away any blood which may have otherwise accumulated and possibly interfered with the BT. BT was measured on both jugular veins.

Drug plasma concentration determination.

All drug plasma concentration determinations were performed by Dr. Jack Rosenfeld's laboratory at McMaster University.

Aspirin method: Aspirin-plasma concentrations were determined by the method of Trinder (1954). This assay is based upon the principle that free salicylate forms a violet coloured complex with ferric ions which is stable at acid pH, whereas the acetylated salicylate does not form such a complex. Whole blood was collected into citrate. PPP prepared from each sample was stored in glass tubes at -70°C or until assayed. Free salicylate was assayed in an aliquot of each sample and compared to the total salicylate values obtained after basic hydrolysis of another aliquot of the same sample with 0.1 ml 2N sodium hydroxide. All assays were performed in an Hitachi Perkin-Elmer spectrophotometer at 540 μm and compared to standard preparations consisting of sodium salicylate.

Dipyridamole method: Dipyridamole-plasma concentrations were

determined by the method of Zak et al (1963). This method is based upon the principle that dipyridamole fluoresces at alkaline pHs. Dipyridamole was extracted from 0.5 ml PPP into 6.3 ml ether and shaken for 30 minutes at a pH of 8.6. The plasma fraction was then discarded and the dipyridamole re-extracted back into an acid phase of 1 ml 0.1N HCL by shaking for another 30 minutes. The isolated fraction could then be stored at 4°C until analysis. The sample was assayed on an Aminco-Bowman spectrophotofluorometer. Fluorescence excitation was initiated by a light source of 305 μ m and its emission recorded at 500 μ m.

Sulfinpyrazone method: Sulfinpyrazone-plasma concentrations were determined by the method of Rosenfeld et al (1977). Citrated plasma (1 ml) was acidified with 1 ml 0.1N HCL. Sulfinpyrazone was extracted from the plasma into 5 ml benzene by vigorous shaking for 15 minutes. The benzene and aqueous layers were separated by centrifugation for 5 minutes at 900 g. The sulfinpyrazone was extracted from the benzene phase into 5 ml 0.1N NaOH and centrifuged at 900 g for 5 minutes. The organic phase was discarded and sulfinpyrazone was methylated with 0.1 ml MeOH. The methyl sulfinpyrazone was assayed on a Hewlett-Packard 402 gas chromatograph with a flame ionization detector.

CHAPTER III

RESULTS

RESULTS: PART 1

1. An Investigation of the Inconsistencies of the In Vivo and Ex Vivo Effects of Platelet Function Inhibiting Drugs.

(a) Development of an In Vivo Test System

For obvious ethical and economic reasons, the initial evaluation of potentially useful platelet suppressive drugs is best carried out under carefully controlled conditions. The simplest and the least expensive test is an in vitro test. The results obtained in such a test are assumed to reflect the effectiveness of the drug when used as an anti-thrombotic agent in vivo. Thus, any drug which is shown to be effective in that system could then be tested in the in vivo and/or ex vivo systems which, although they are considerably more expensive and demanding, are more relevant to the application of the drug.

The disadvantage of the in vitro approach is that it fails to consider the effects of metabolites which may be important and the effects of artifacts which may be produced because it is performed on anticoagulated blood which is prepared by centrifugation. The first of these theoretical disadvantages is overcome by studying the effects of these drugs ex vivo and both disadvantages can be overcome by developing an appropriate in vivo test.

Review of the literature reveals a number of discrepancies between sulfinpyrazone, dipyridamole and aspirin on platelet function assessed in vivo and ex vivo. For example, sulfinpyrazone and dipyridamole prolong shortened platelet survival seen in some thromboembolic states whereas aspirin does not. On the other hand, aspirin has a marked inhibitory effect on the platelet release

reaction when this is tested ex vivo but sulfinpyrazone and dipyridamole appear to have little effect when tested in standard pharmacological doses. The reasons for these differences between the effectiveness of these drugs when tested in vivo and ex vivo are uncertain, but there appears to be two possible explanations.

Firstly, the discrepancies could be related to qualitative differences between the in vivo and ex vivo stimuli to which the platelets are exposed and to the differences of the relative effectiveness of these drugs on such stimuli. Secondly, these discrepancies could be due to the effects of ex vivo manipulations such as centrifugation and/or the addition of anticoagulants on the inhibitory effects of these drugs on the platelet release reaction. There are a number of examples in the literature demonstrating that the degree of platelet aggregation may be influenced by the nature of the anticoagulant used. Thus, Gordon and MacIntyre (1974) demonstrated that the inhibitory potency of aspirin on collagen-induced platelet aggregation of porcine platelets was much greater in citrated plasma than in heparinized plasma. Mustard et al (1975) examined some characteristics of platelet release reaction and found that adenosine diphosphate induced a secondary wave of aggregation in citrated plasma whereas no secondary wave was achieved in heparinized plasma. They found that adenosine diphosphate-induced platelet secretion varied with the concentration of calcium and ¹⁴C-serotonin release was higher in citrated plasma. This has subsequently been confirmed by

Lages and Weiss (1977). O'Brien et al (1969) have previously demonstrated that adenosine diphosphate-induced aggregation was greater in heparinized PRP although no secondary wave was obtained and that collagen-induced aggregation was less. Fujitani et al. (1976) found that high concentrations of heparin markedly diminished rabbit and guinea pig platelet adherence to glass bead columns.

Collagen-induced platelet aggregation in vitro (or ex vivo) has been widely used as a test to measure platelet adherence, release and aggregation. This is a reasonable approach since collagen is thought to be one of the more important thrombogenic substances of vessel wall. A number of investigators have developed in vivo injury models to study the platelet response to the damaged vessel wall. While this approach is reasonable in theory, it is difficult to quantitate the platelet response. Nishizawa tested platelet inhibitory drugs using a model which involved the intravenous infusion of lethal amounts of collagen to mice (Nishizawa et al, 1972). The animals died because the lungs were occluded with platelet-collagen aggregates. A major drawback of this approach was that this model required a strong collagen stimulus which could mask the possible beneficial effects of the drugs on the platelet. In addition the occlusion of the pulmonary circulation results in many local responses in the lungs which could also mask the effect of any drugs on the platelet-collagen interaction.

We modified Nishizawa's model to make the stimulus more sensitive

to the drug effects.

Anesthetized rabbits with carotid artery and jugular vein cannula were injected with homologous ^{51}Cr platelets. Sixty minutes after the platelet injection the rabbits were infused intra-arterially with 0.25 mg acid soluble collagen (100 $\mu\text{g}/\text{kg}$ body weight), given in 1 ml over 60 seconds. Serial samples for radioactivity were taken before, during and after the infusion. Whole blood radioactivity and visual platelet counts were determined on each sample. The results for each method of platelet recovery were expressed as a percent of the value obtained before the collagen infusion which was defined as 100% recovery. The reduction in circulating whole blood radioactivity was assumed to be due to the platelet collagen aggregation and subsequent sequestration of those aggregates. The fall was transient and the radioactivity returned to within 85% of pre-collagen levels within 5 minutes. This rise in radioactivity was considered to reflect the deaggregation of some of the platelets and their return to the circulation. The platelet response in the in vivo system was defined as maximum platelet aggregation which was quantitated by determining the maximum decrease in platelet radioactivity from the initial 100% baseline as is shown in Figure 3.

In preliminary studies, we examined the pattern of platelet recovery determined both by radioactivity and by direct visual platelet counting. The pattern of platelet recovery determined by radioactivity was the same as the pattern of platelet recovery determined

⁵¹Cr-PLATELET RECOVERY

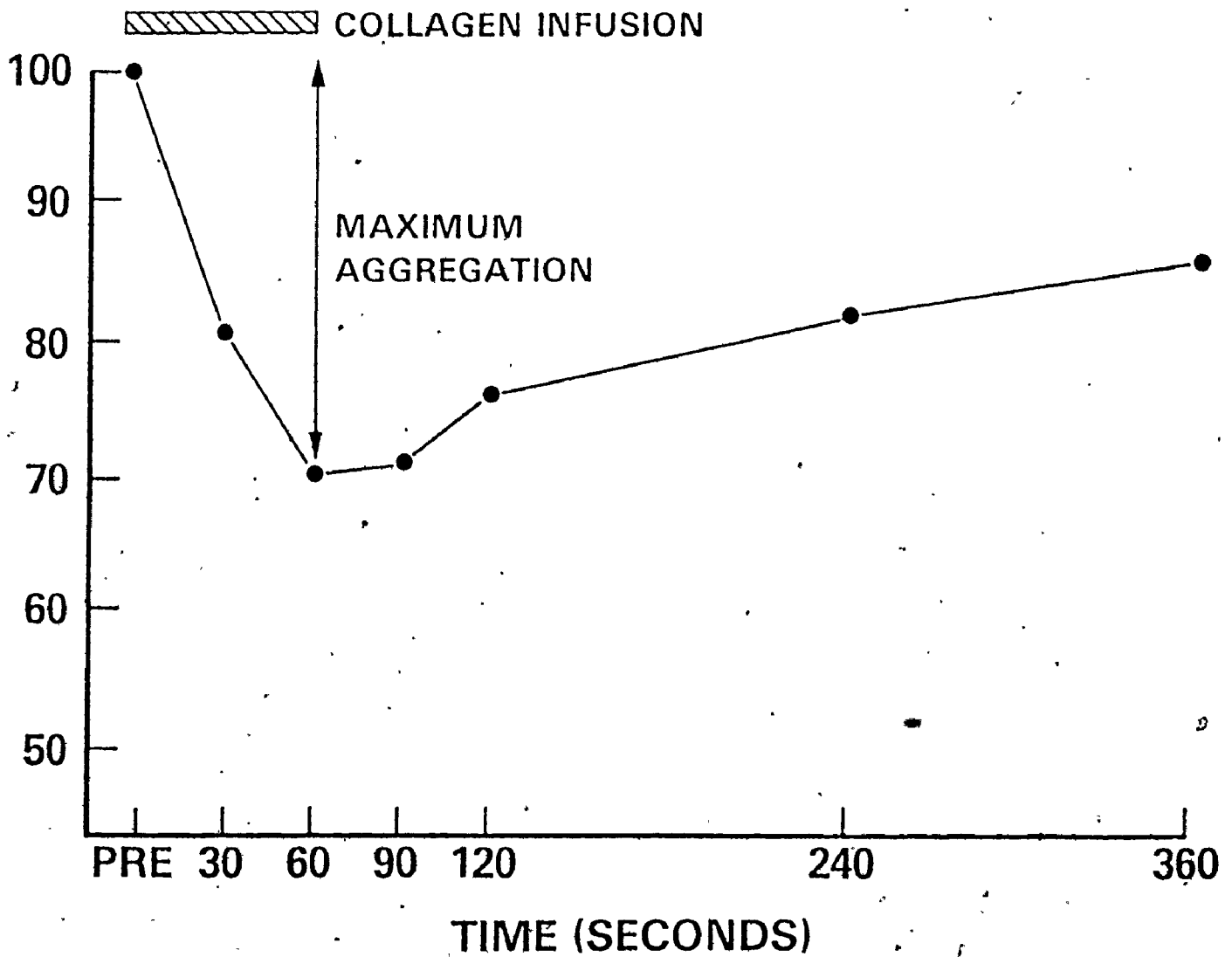


Figure 3. ⁵¹Cr-platelet recovery in 30 rabbits after an intra-arterial infusion of collagen (100 µg/kg). The maximum deflection of the platelet recovery curve from the 100% pre collagen baseline was expressed as maximum aggregation.

visually (Figure 4). Thus, in all subsequent experiments, platelet recovery determined by radioactivity was used.

Dose response to collagen

One hundred $\mu\text{g}/\text{kg}$ body weight of collagen was chosen as the test stimulus because the ratio of collagen to platelets was calculated to be identical with the ratio of collagen to platelets used in the ex vivo studies. However, it was important to determine whether or not the platelet response to this amount of collagen was excessive and to confirm that the platelet response was specific to the collagen infusion and not to its suspending vehicle, acetic acid. Therefore, the following experiments were done to examine the dose response relationship between various collagen concentration and the platelets, and to confirm that this response was a result of a platelet interaction with collagen rather than acetic acid. Serial samples were collected from rabbits which were 1) not infused, or 2) were infused with the suspending vehicle (0.0522 molar acetic acid), 3) 50, 4) 100 or 5) 150 $\mu\text{g}/\text{kg}$ collagen. The maximum deflection of the platelet response curve below the pre-100% baseline which was the maximum aggregation in each group of 6 animals is expressed on Table 1. The radioactivity platelet count varied 2.5% both above and below the 100% baseline and this was attributed to sampling and counting errors. Infusion of the collagen suspending vehicle was not significantly different:

PLATELET
RECOVERY
(%)

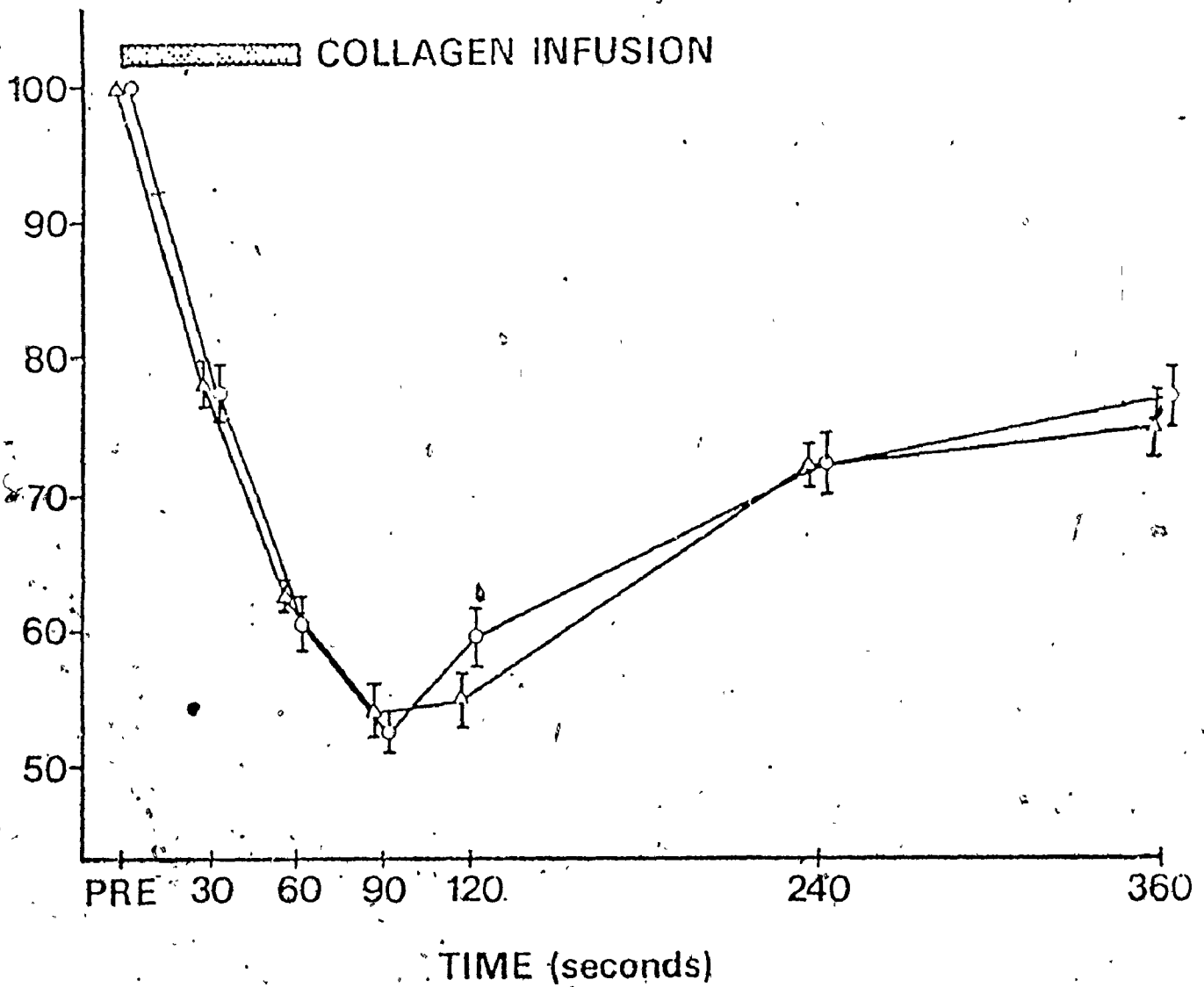


Figure 4. ^{51}Cr -platelet recovery (○) and visual platelet counts (▲) + standard error in six rabbits after a sixty second infusion of collagen (100 $\mu\text{g}/\text{kg}$). Both methods of platelet count determination discriminated the changes in platelet recovery.

Table 1. Dose Response of Collagen: maximum platelet aggregation of ^{51}Cr -platelets in vivo after an intra-arterial infusion of increasing doses of collagen. Data is expressed as % aggregation \pm standard error. The n value for each cell is 6.

		% AGGREGATION
	No Infusion	2.4 \pm 1
	Suspending vehicle	1.8 \pm 1
Dose of	50	20.0 \pm 1
Collagen	100	30.8 \pm 1
Infused	150	40.8 \pm 3
($\mu\text{g}/\text{kg}$)		

However, the collagen infusions were followed by decreases in platelet aggregation. The platelet response was dose related. Thus, the 100 µg/kg collagen stimulus was less than that amount of collagen required to initiate a maximum platelet aggregation response, and was, therefore, used as the stimulus on all future experiments.

Coagulation system involvement in the platelet collagen response

Since it is known that collagen may also activate the intrinsic pathway of coagulation (Nossel, 1975), it was possible that the observed platelet response could be due 1) to a direct interaction with collagen or 2) to an accelerated effect of the coagulation system. Therefore, we investigated the effects of inhibiting the coagulation system on the platelet/collagen response curve. Rabbits were injected with 0, 100 or 200 units/kg heparin. Five minutes after the heparin injection, collagen was infused and maximum aggregation was determined. In addition, partial thromboplastin times (PTT) were performed on the samples collected from the rabbits 5 minutes after the collagen infusion. The results are shown in Table 2. The response of platelets to collagen in rabbits with normal PTT's was not different than the platelet response in those rabbits with PTT's in excess of 150 sec. These results support the hypothesis that the platelet/collagen response is due to a direct interaction.

Table 2. Collagen-induced platelet aggregation in rabbits with prolonged partial thromboplastin times (PTT). Maximum aggregation (% \pm S.E.M.) in rabbits given 0, 100 or 200 units/kg heparin. The mean PTT was 56 seconds in the control animals, was >152 seconds in rabbits given 100 units/kg heparin and >240 seconds in rabbits given 200 units/kg heparin. On the other hand, maximum platelet aggregation did not differ significantly from one group to another. (n = 6)

MAXIMUM PLATELET AGGREGATION

Control	+ 100 units/kg Heparin	+ 200 units/kg Heparin
29.5 \pm 2	26.8 \pm 2	33.3 \pm 2

Effect of Aspirin, Dipyridamole and Sulfinpyrazone on ^{51}Cr Platelets and Autologous Circulating Platelets

Although the response of the ^{51}Cr labelled platelets measured by whole blood radioactivity was similar to the response of normal circulating platelets measured by visual counts, it was necessary to demonstrate that the effect of aspirin, sulfinpyrazone and dipyridamole on both platelet populations was also the same. The possibility that the in vitro handling procedure used to label the homologous platelets altered the platelets and interfered with the drugs' effects, had not been ruled out. Therefore, the following experiment was performed to test the effects of aspirin, sulfinpyrazone and dipyridamole on both populations of platelets. Rabbits were given either 100 mg/kg aspirin or sulfinpyrazone or 10 mg/kg dipyridamole intravenously. Two hours later maximum collagen-induced platelet aggregation was determined both by radioactivity and visual platelet counting. Each drug inhibited the platelet response of both platelet populations equally (Table 3).

Summary

Rabbit platelets aggregate in vivo to collagen. The platelet collagen interaction was quantified by measuring the recovery of the total circulating platelets, by visual counting, or by measuring the radioactivity of the homologous ^{51}Cr -labelled platelets. Determination of a large number of visual platelet counts is tedious

Table 3. Collagen-induced platelet aggregation of homologous and autologous platelets after rabbits were given 100 mg/kg aspirin or sulfinpyrazone or 10 mg/kg dipyridamole. Maximum aggregation was derived from the platelet recovery curves determined by ⁵¹Cr-radioactivity counting and by visual microscopic counting. Data is expressed as percent aggregation \pm S.E.M. (n = 6)

	TREATMENT			
	<u>ASPIRIN</u>	<u>SULFINPYRAZONE</u>	<u>DIPYRIDAMOLE</u>	<u>CONTROL</u>
⁵¹ Cr	17 \pm 2	19 \pm 2	18 \pm 3	26 \pm 2
Visual	17 \pm 2	17 \pm 3	16 \pm 4	30 \pm 2

and prone to errors. Determination of the platelet response by radioactivity count overcomes these problems.

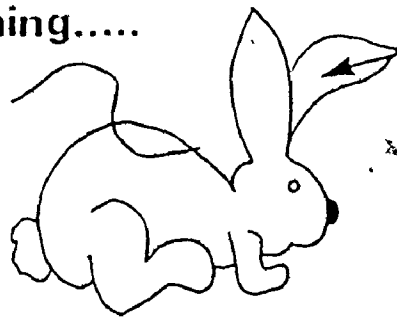
The platelet collagen interaction is not influenced by large doses of heparin suggesting that the effect was not due to the activation of blood coagulation by collagen.

The effect of aspirin, sulfinpyrazone and dipyridamole on platelet aggregation was the same on homologous and autologous platelets.

(b) A Comparison of the Effects of Aspirin, Sulfinpyrazone and Dipyridamole on Collagen-Induced Platelet Aggregation In Vivo and Ex Vivo

Experiments were designed to compare the effects of these drugs in vivo and ex vivo on the same population of platelets using identical concentrations of the collagen stimulus. Anesthetized rabbits with carotid artery and jugular vein cannulae were injected with homologous ^{51}Cr platelets. Fifteen minutes after the platelets were injected, the rabbits were injected with varying doses of either aspirin, sulfinpyrazone or dipyridamole. One hour after treatment, the rabbits were infused intra-arterially for 60 seconds with collagen and serial samples were collected for radioactivity. Immediately before the collagen infusion, blood was collected for measuring collagen-induced platelet aggregation in vitro (Figure 5). The platelet collagen response was referred to as ex vivo collagen

In the beginning.....



Homologus Cr-labelled platelets

15 minutes later.....



Treatment ie: Aspirin
Dipyridamole
Sulfinpyrazone

60 minutes later.....



Collagen infusion (I/A)

Blood sampling

-Radioactivity

Ex Vivo aggregations

Figure 5. Experimental design to study collagen-induced platelet aggregation ex vivo and in vivo on the same experimental animal.

induced platelet aggregation. Fourteen separate experiments were performed to examine the effects of aspirin, dipyridamole and sulfinpyrazone on collagen-induced platelet aggregation in vivo and ex vivo. Table 4 shows the mean in vivo platelet aggregations of the placebo-treated animals for each experiment and the averages for all experiments. When the data was analyzed by Statton-Newman Kinz multi-comparison analysis, there were no significant differences between or within groups, therefore all control data was pooled and the data of the experimental groups were compared with the mean maximum aggregation of the pooled group (Table 4). Similarly, no differences existed between the ex vivo mean aggregation of the control groups and thus all experimental ex vivo data was compared with the mean maximum aggregation of the ex vivo pooled group (Table 5).

Platelet aggregation was maximally inhibited with a dose of 10 mg/kg aspirin both in vivo and ex vivo ($p < 0.001$). Further increases in the dose did not result in any further inhibition of platelet aggregation (Table 6). The pattern of the platelet response to collagen after aspirin treatment is shown in Figure 6 in which the maximum aggregation of the control is expressed as 100% and the results in the treated groups are expressed as a percentage of the control.

When the effect of sulfinpyrazone on the platelet-collagen interaction was tested, a similar pattern of in vivo and ex vivo

Table 4. Maximum aggregation in vivo of the control animals for each experiment. The mean aggregations for each experiment did not differ from one experiment to the next nor did they differ from one treatment group to the next. Therefore, the entire "control" data was pooled and the maximum aggregation of the various treatment groups was compared to the overall control maximum aggregation of 32.5%.

	MAXIMUM AGGREGATION (%)		
	Dipyridamole <u>Controls</u>	Aspirin <u>Controls</u>	Sulfinpyrazone <u>Controls</u>
Experiment 1	37.0	24.3	33.0
Experiment 2	34.3	38.3	26.6
Experiment 3	34.3	33.0	28.7
Experiment 4	33.7	31.0	36.7
Experiment 5		29.0	
Experiment 6		39.5	
	34.8	32.1	30.1
	OVERALL AVERAGE		
	32.5		

Table 5. Maximum aggregation ex vivo of the control animals for each experiment. The mean aggregations for each experiment did not differ from one experiment to the next nor did they differ from one treatment group to another. Therefore, the data from all control animals were pooled. Thus, the maximum aggregations of each treated group of animals was compared to this "overall" control maximum aggregation of 56.5%.

	MAXIMUM AGGREGATION (%)		
	Dipyridamole <u>Controls</u>	Aspirin <u>Controls</u>	Sulfinpyrazone <u>Controls</u>
Experiment 1	59.3	53.7	78.0
Experiment 2	69.7	48.3	68.8
Experiment 3	65.5	49.7	56.0
Experiment 4	44.3	53.7	64.7
Experiment 5		54.0	
Experiment 6		9.0	
	60.2	46.8	66.1
	OVERALL AVERAGE		
	56.5		

Table 6. In vivo and ex vivo maximum platelet aggregation in aspirin-treated rabbits. Maximum aggregation is expressed % mean aggregation. An analysis of variance showed that the lowest dose of aspirin (10 mg/kg) maximally inhibited platelet aggregation in vivo and ex vivo (P <0.001, P <0.001 respectively).

<u>DOSE OF ASPIRIN</u>	<u>MAXIMUM AGGREGATION</u>	
	<u>IN VIVO</u>	<u>EX VIVO</u>
10 mg/kg	26.3	16.7
30 mg/kg	23.8	6.5
65 mg/kg	19.7	23.0
100 mg/kg	23.2	12.0
200 mg/kg	20.8	13.0
Pooled Control	32.5	56.5

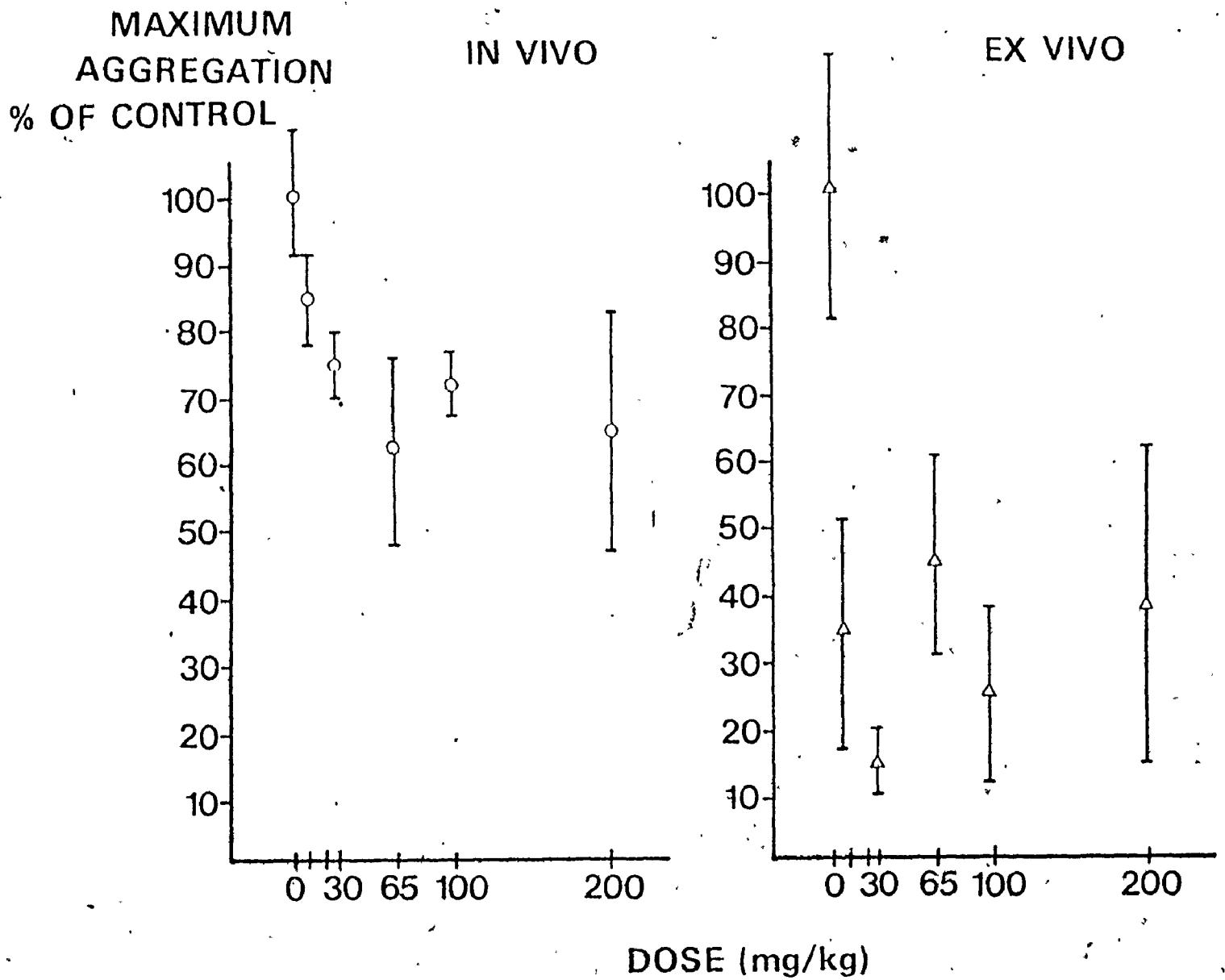


Figure 6. Effect of increasing doses of aspirin on collagen-induced platelet aggregation in vivo and ex vivo. Data is expressed as % of control + S.E.M. The n value of each cell in the treatment conditions is 6. The n value in each control cell is 30.

effects were found, $P < 0.001$ (Figure 7). The effect in vivo reached a plateau at 65 mg/kg whereas ex vivo inhibition continued up to a dose of 200 mg/kg. The mean platelet aggregation values are shown in Table 7.

In contrast, dipyridamole had no effect on platelet aggregation ex vivo whereas all doses of dipyridamole inhibited platelet aggregation in vivo, $P < 0.001$, (Figure 8, Table 8). There was a non-significant trend for the higher doses up to 10 mg/kg to produce a greater effect than the lowest dose. Still higher doses of the drug not shown in this table enhanced aggregation both in vivo and ex vivo. This effect of dipyridamole with higher doses has also been observed in other laboratories (Marquis, Meuleman - personal communications). Thus, when the stimulus used to test the effects of these drugs was kept constant, aspirin and sulfinpyrazone exhibited a similar pattern of inhibition of platelet aggregation in vivo and ex vivo whereas dipyridamole had no effect ex vivo at concentrations which produced inhibition of platelet aggregation in vivo.

The Effect of Blood processing on the Stability of the Drug Effect

Ex Vivo and In Vivo

One possible explanation for the above differences between the effects of dipyridamole in vivo and ex vivo is that the drug loses its potency while the blood is being processed for the ex vivo

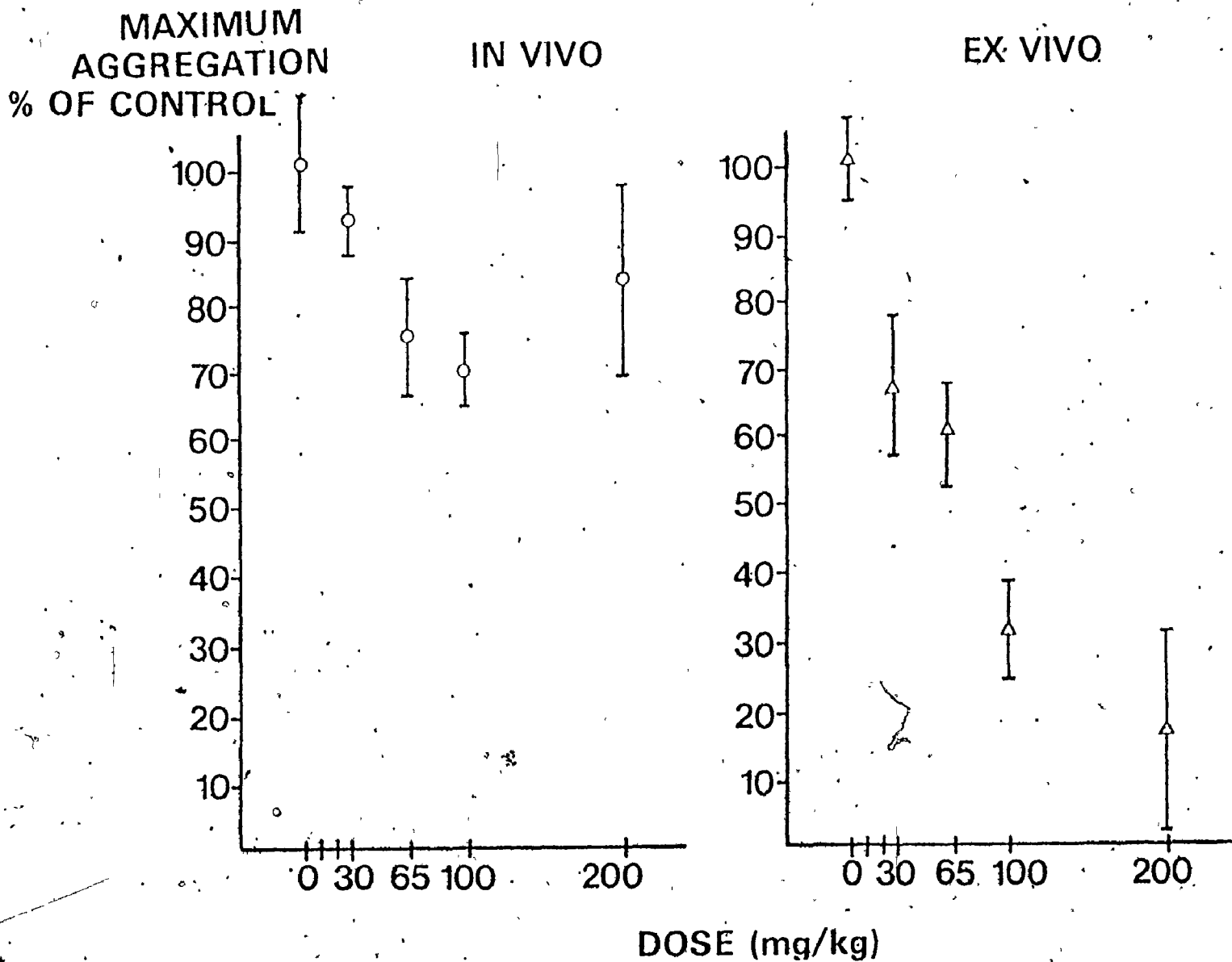


Figure 7. Effect of increasing doses of sulfinpyrazone on collagen-induced platelet aggregation in vivo and ex vivo. Data is expressed as % of control + S.E.M. The n value in each treatment cell is 6, and in each control cell is 30.

Table 7. In vivo and ex vivo maximum platelet aggregation in sulfinpyrazone-treated rabbits. Maximum aggregation is expressed as % mean aggregation. An analysis of variance demonstrated that the lowest dose of sulfinpyrazone maximally inhibited in vivo platelet aggregation whereas ex vivo platelet aggregation was inhibited in a dose dependant manner ($P < 0.001$, $P < 0.001$ respectively).

Dose of Sulfinpyrazone (mg/kg)	<u>MAXIMUM AGGREGATION</u>	
	<u>In Vivo</u>	<u>Ex Vivo</u>
30	26.2	47.8
65	21.3	43.0
100	20.0	22.5
200	26.8	9.5
Pooled Control	32.5	56.5

MAXIMUM
AGGREGATION
% OF CONTROL

IN VIVO

EX VIVO

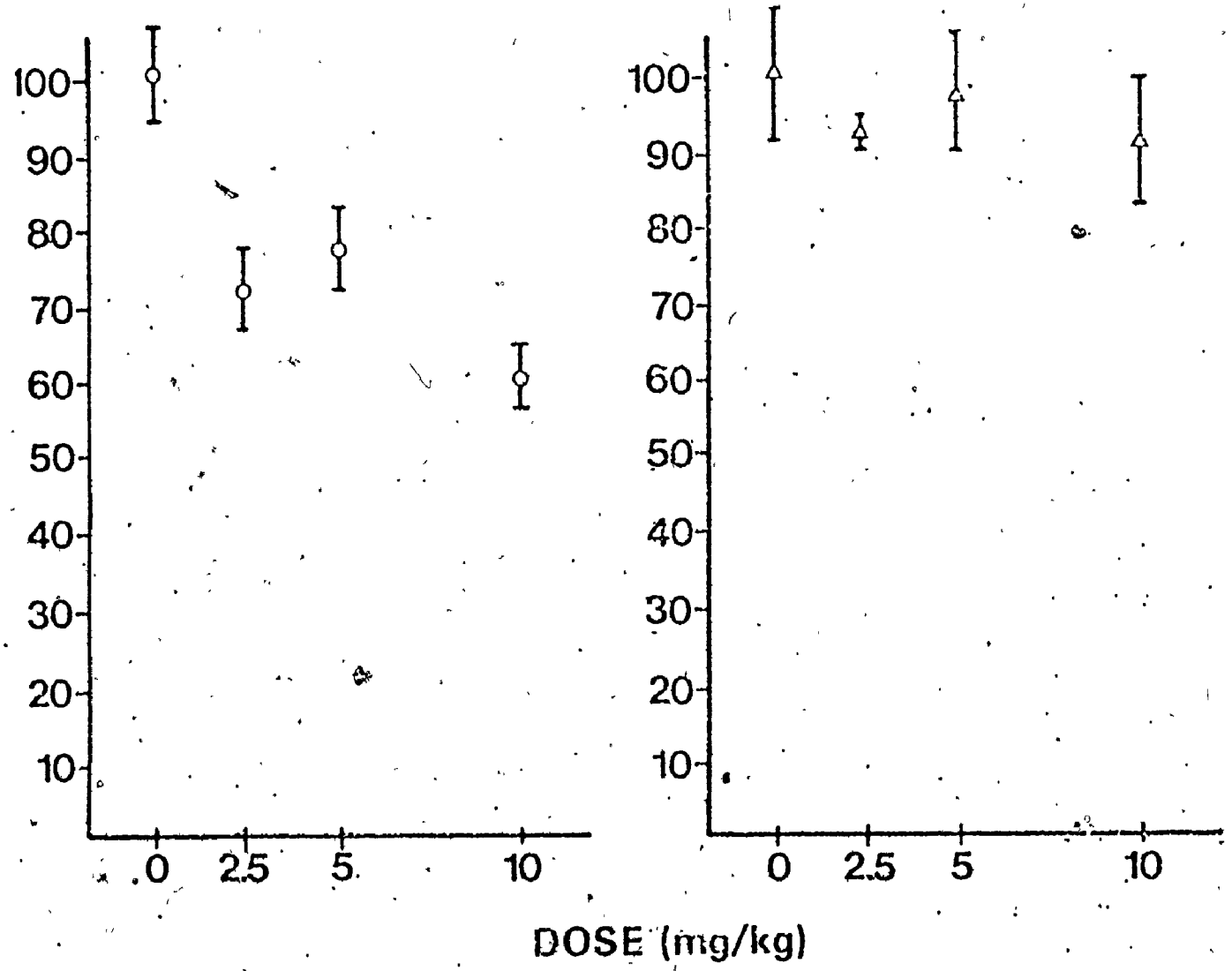


Figure 8. Effect of dipyridamole on collagen-induced platelet aggregation. Data is expressed as % of control. The n value of each treated cell is 6 and of each control cell is 30.

Table 8. In vivo and ex vivo maximum platelet aggregation in dipyridamole treated rabbits. Maximum is expressed as % mean aggregation. An analysis of variance showed that the lowest dose of dipyridamole maximally inhibited aggregation in vivo whereas the drug had no effect on platelet aggregation ex vivo (P < 0.001). There was a non-significant trend that inhibition of platelet aggregation was dose-dependant in vivo.

Dose of Dipyridamole (mg/kg)	<u>MAXIMUM AGGREGATION</u>	
	<u>In Vivo</u>	<u>Ex Vivo</u>
2.5	24.5	60.3
5.0	23.7	52.0
10.0	17.3	53.2
Pooled Control	32.5	56.5

platelet aggregation studies. It required about one hour to prepare the platelet-rich and platelet-poor plasmas, to determine platelet counts and to set up each sample for testing. Therefore, experiments were performed using a high concentration of dipyridamole in vitro ($5 \times 10^{-4}M$) which inhibited platelet aggregation in citrated PRP at 5 minutes. Platelet aggregation studies were repeated 60 and 120 minutes later and the degree of inhibition remained essentially unchanged over the 120 minute duration (Table 9). These results indicate that the potency of dipyridamole is not altered over a 120 minute storage in citrated PRP. Thus, it is unlikely that the difference between in vivo and ex vivo effects of the drug is related to an alteration in potency or stability of the drug during blood processing ex vivo.

Platelet Aggregation in Citrated and Heparinized Plasma

An alternative explanation for the differences between the ex vivo and in vivo effects of dipyridamole is that the drug is altered by the anticoagulant used. Therefore, the following experiments were performed to determine whether the dipyridamole effect could be influenced by the anticoagulant used. To do this, blood was taken from rabbits into the standard citrate anticoagulant and into heparin (2 units/ml) which was used as an alternative anticoagulant. Specifically, two blood samples were collected from a carotid artery cannula from each of 8 rabbits and one sample was

Table 9. Percent inhibition of platelet aggregation \pm S.E.M. in vitro 5, 60 and 120 minutes after incubation at 37°C with 5×10^{-4} M dipyridamole. Data is expressed as a percent of the maximum platelet aggregation \pm S.E.M. obtained before incubation with the drug.

	<u>INCUBATION TIME (minutes)</u>		
	5'	60'	120'
Inhibition of Maximum Aggregation % of Pre Sample	24 \pm 8	24 \pm 9	24 \pm 10

mixed with sodium citrate and the other with heparin. PRP was prepared and the platelet count adjusted to $300,000/m^3$. The rabbits were then treated with 10 mg/kg dipyridamole. One hour later, blood samples were again collected into citrate and heparin. The citrate and heparin PRPs were tested for aggregation using three different concentrations of collagen. Figure 9 shows the percent aggregation of ex vivo collagen-induced platelet aggregation, expressed as % of pre-treatment aggregation, one hour after treatment with the dipyridamole. Once again it was demonstrated that dipyridamole had little or no effect ex vivo in citrated PRP. However, dipyridamole did inhibit platelet aggregation when tested in the heparin-PRP. These results suggest that the lack of an inhibitory effect of dipyridamole on platelet aggregation in citrate-PRP was due to an effect of citrate on the dipyridamole/platelet/collagen interaction. An alternative explanation is that heparin enhances the dipyridamole effect.

To explore this possibility further, experiments were performed to examine the effects of citrate and heparin on the effects of dipyridamole by measuring the effect of dipyridamole on platelet aggregation of washed platelet suspensions containing citrate or heparin.

MAXIMUM
AGGREGATION
% OF CONTROL

CITRATE PRP

HEPARIN PRP

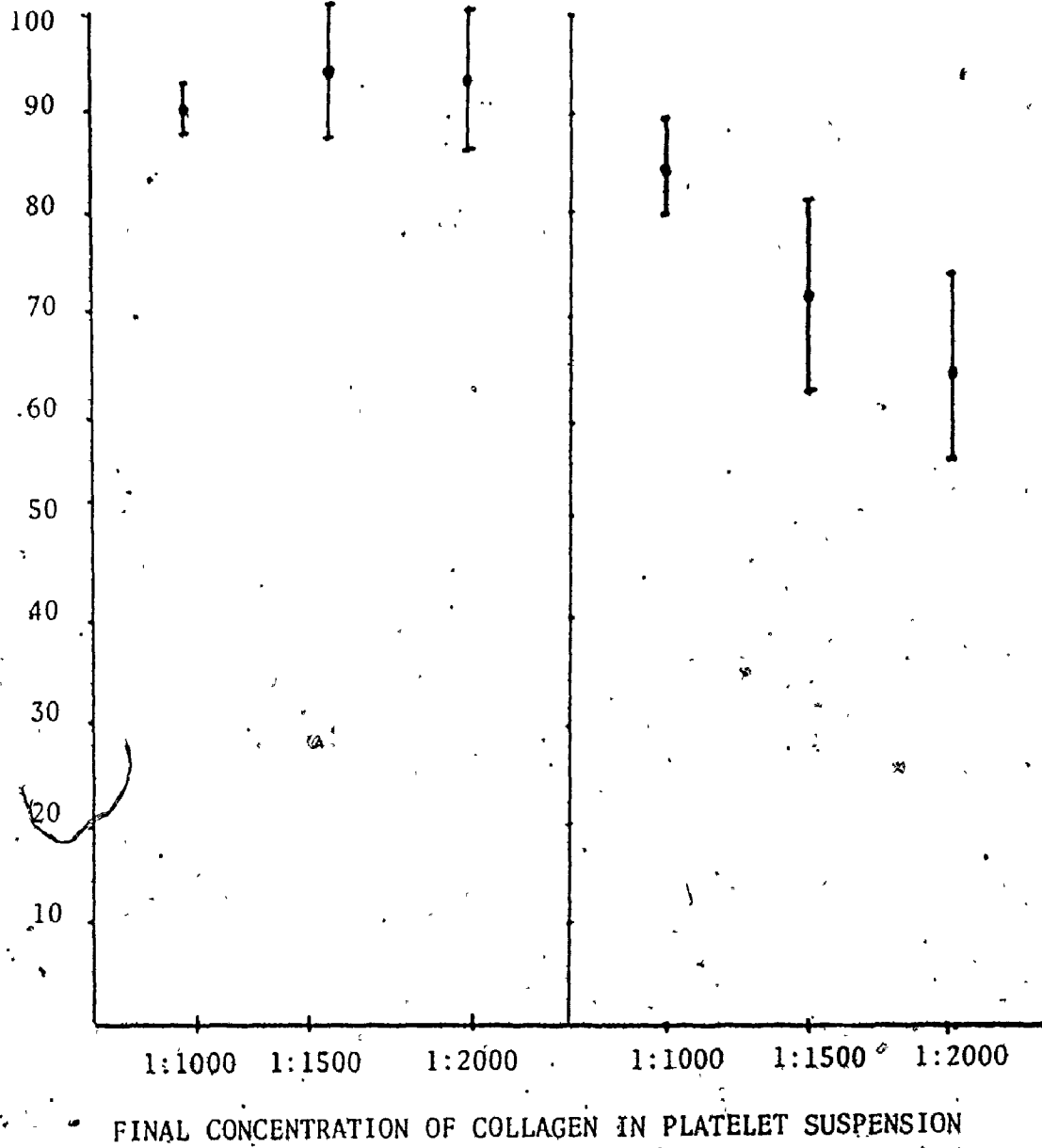


Figure 9. Percent aggregation + S.E.M. of ex vivo collagen-induced platelet aggregation 60 minutes after treatment with 10 $\mu\text{g}/\text{kg}$ dipyridamole. Data is expressed as maximum aggregation % of pre-treatment aggregation.

In Vitro Inhibition of Collagen-Induced Platelet Aggregation of Washed Rabbit Platelet Suspensions by Dipyridamole in the Presence of Citrate or Heparin

In pilot studies, dipyridamole was added to washed rabbit platelet suspensions and the dosage range required to inhibit platelet aggregation was investigated. The concentration of dipyridamole required to produce about 50% inhibition of platelet aggregation was found to be 5×10^{-6} molar. Figure 10 shows the effect of adding two units of heparin on the effect of this concentration of dipyridamole on platelet aggregation. The net inhibitory effect of dipyridamole was not significantly greater than the effect in the absence of heparin.

When citrate was added to the platelet suspensions in a final concentration of 0.38%, platelet aggregation was totally abolished in the control platelet suspensions. Therefore, it was added to the suspension in final concentrations of 0.019% and 0.145%. These two concentrations of citrate had little effect on collagen-induced platelet aggregation in the control suspensions to which plain Tyrodes was added (Figure 11). However, when dipyridamole (5×10^{-7} final) was added to the suspension containing either of the two citrate concentrations, its inhibitory effect was lost (Figure 11).

There are two possible explanation for this result:

- 1) the effect of dipyridamole is decreased as a result of the low

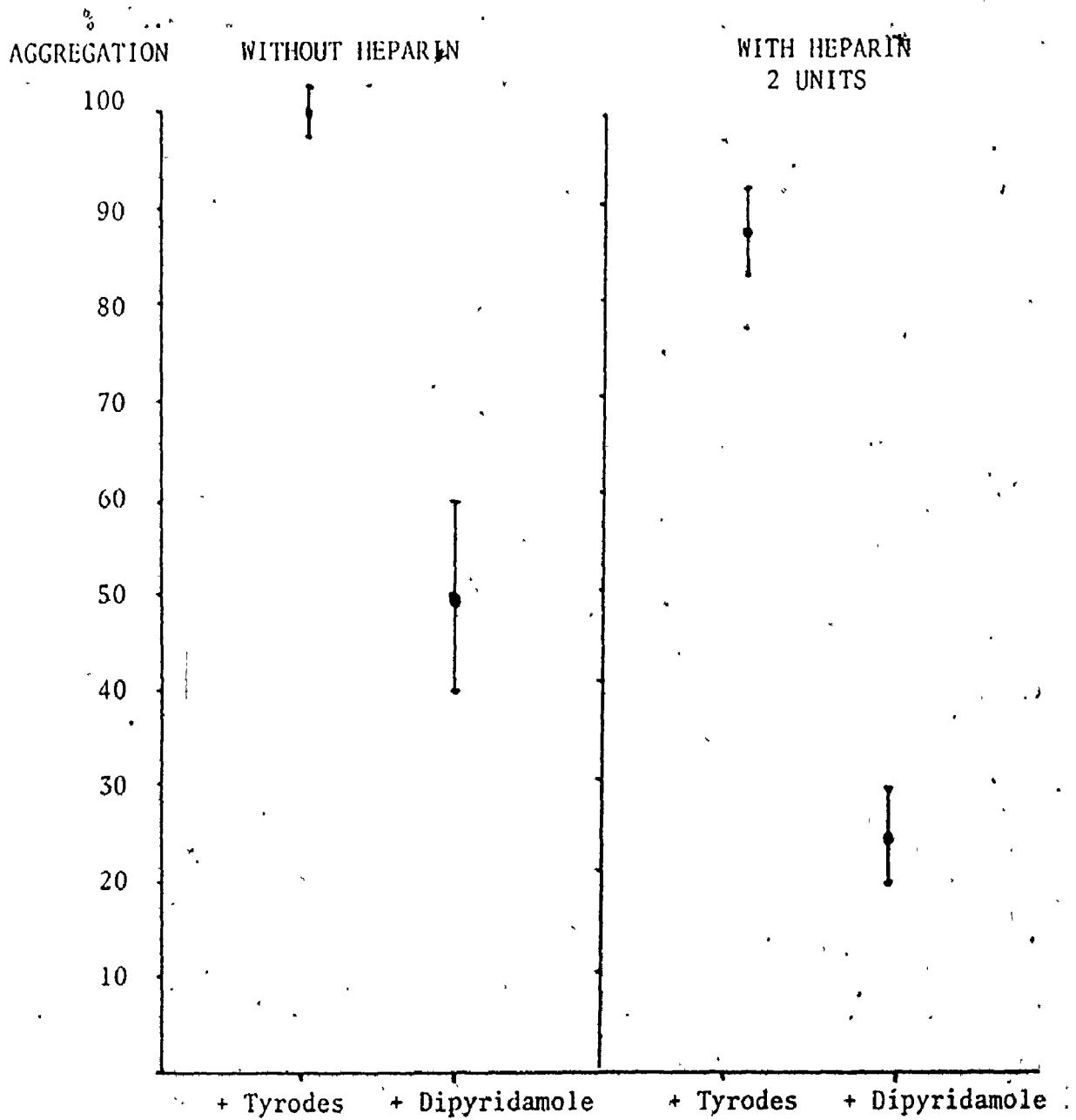


Figure 10. Collagen-induced platelet aggregation + S.E.M. in platelet suspensions incubated with plain tyrodes or dipyridamole ($5 \times 10^{-6}M$) in combination with or without two units heparin.

%
AGGREGATION

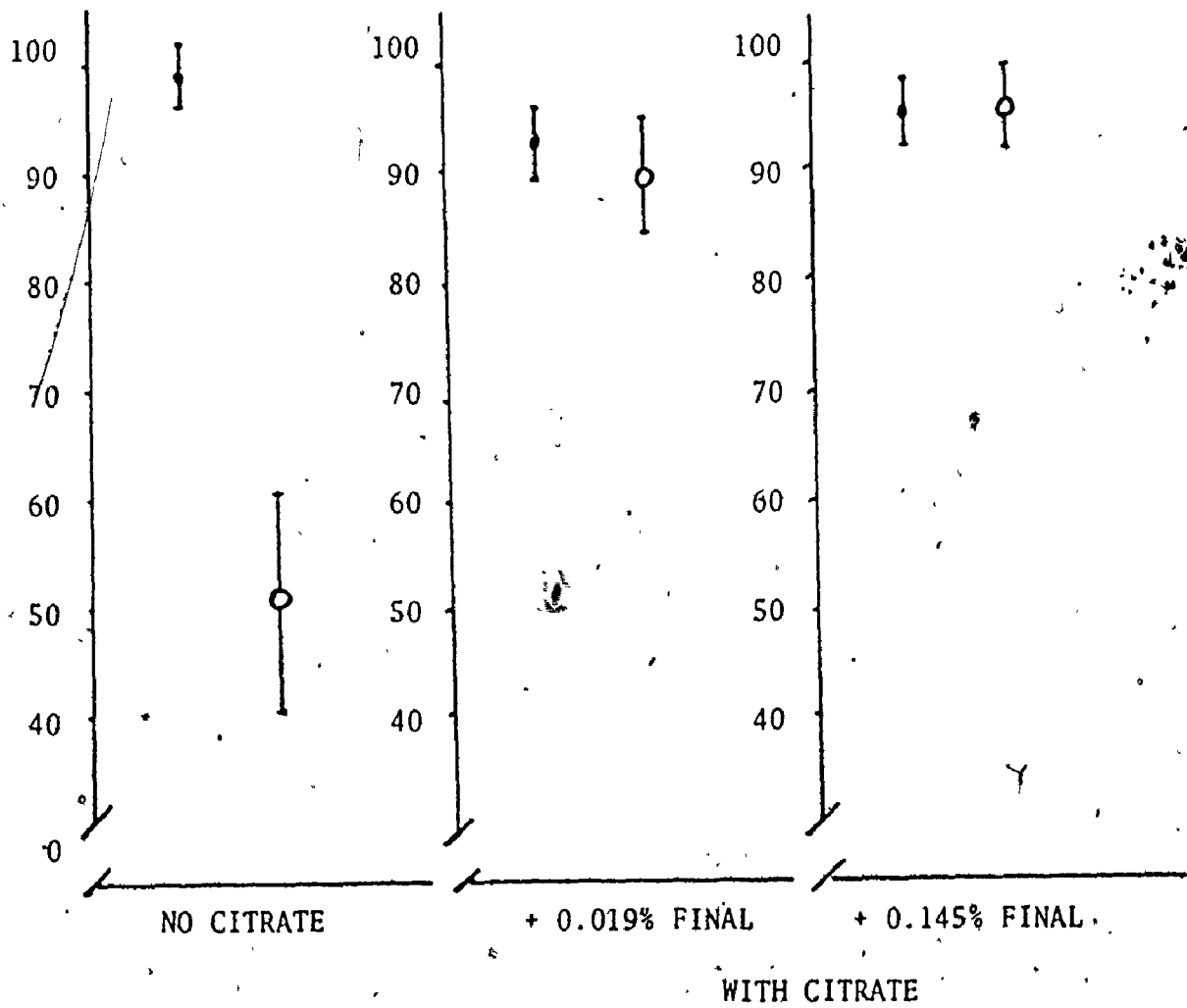


Figure 11. Collagen-induced platelet aggregation of washed platelet suspensions incubated with dipyridamole (5×10^{-6} M) and citrate anticoagulant. Platelet aggregation was tested when plain tyrodes (●) or dipyridamole (○) was added.

concentration of calcium (or magnesium) which was chelated by the citrate; or

2) citrate interferes with the mechanism of action of dipyridamole independent of its chelation effect. Therefore, studies were performed to examine the effect of decreasing the concentration of calcium and magnesium on the inhibitory effect of dipyridamole.

Effect of Decreasing the Divalent Cations on the Inhibitory Effect of Dipyridamole

Platelets were isolated and resuspended in Tyrodes albumin solution in the absence of magnesium ions. Each suspension was divided into five equal aliquots and different concentrations of magnesium ranging from 0.25 mM to 2.0 mM were added to each aliquot. Platelet aggregation was then tested and found to be virtually unaffected by the different concentrations of magnesium in the suspension. A concentration of 5×10^{-6} M dipyridamole inhibited platelet aggregation approximately by 30% in the standard suspension containing 2 mM magnesium. The inhibitory effect gradually increased with decreasing concentrations of magnesium as is shown in Figure 12.

The experiments were repeated substituting suspensions containing decreasing concentrations of calcium. In control platelet suspensions into which plain Tyrodes had been added, platelet aggregation was decreased at calcium concentrations of 0.5 and 0.25 mM (Figure 13). Dipyridamole inhibited aggregation at a calcium concentration of

% AGGREGATION

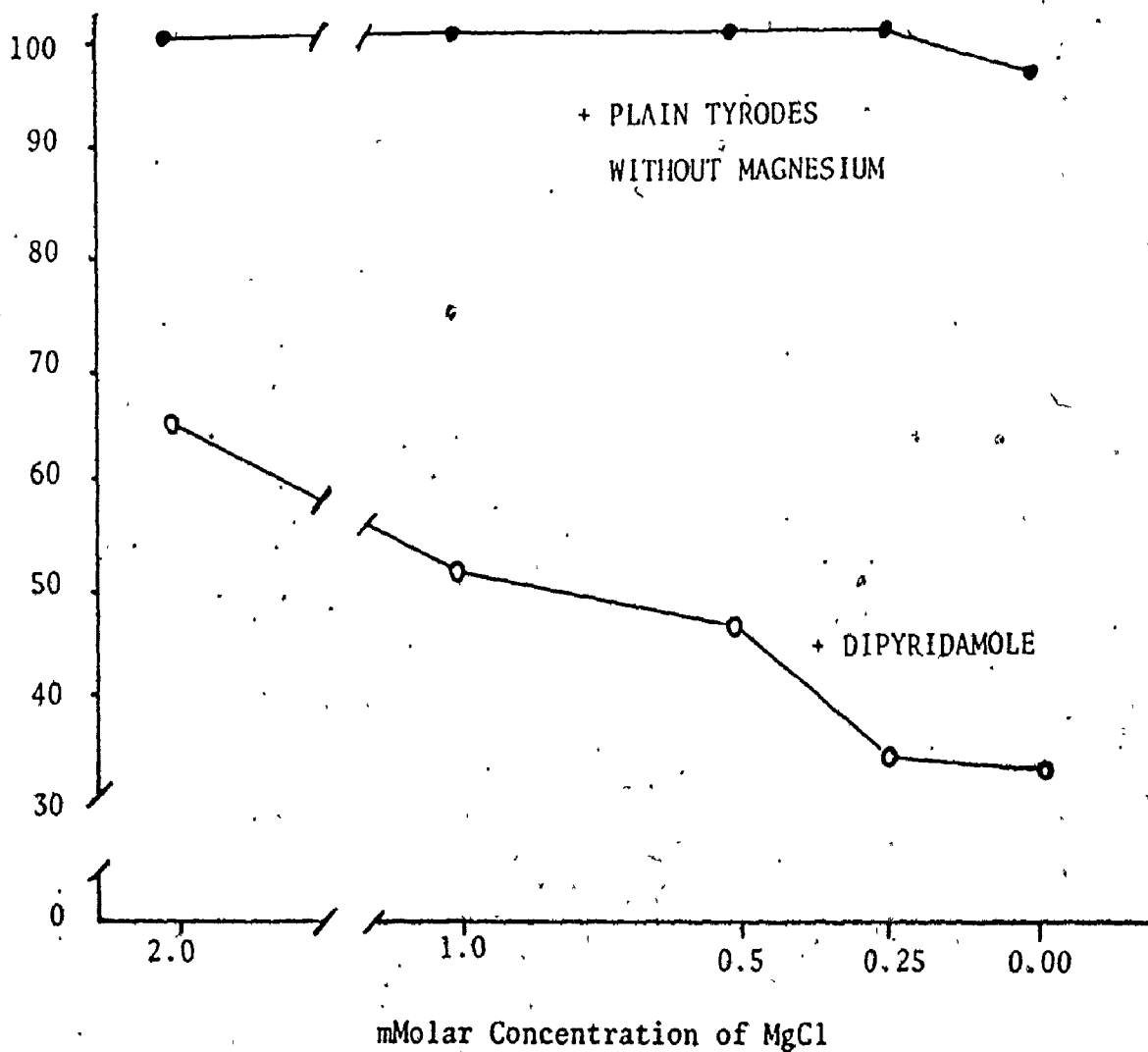


Figure 12. Collagen-induced, platelet aggregation in washed platelet suspensions with decreasing concentrations of magnesium with and without the addition of dipyridamole.

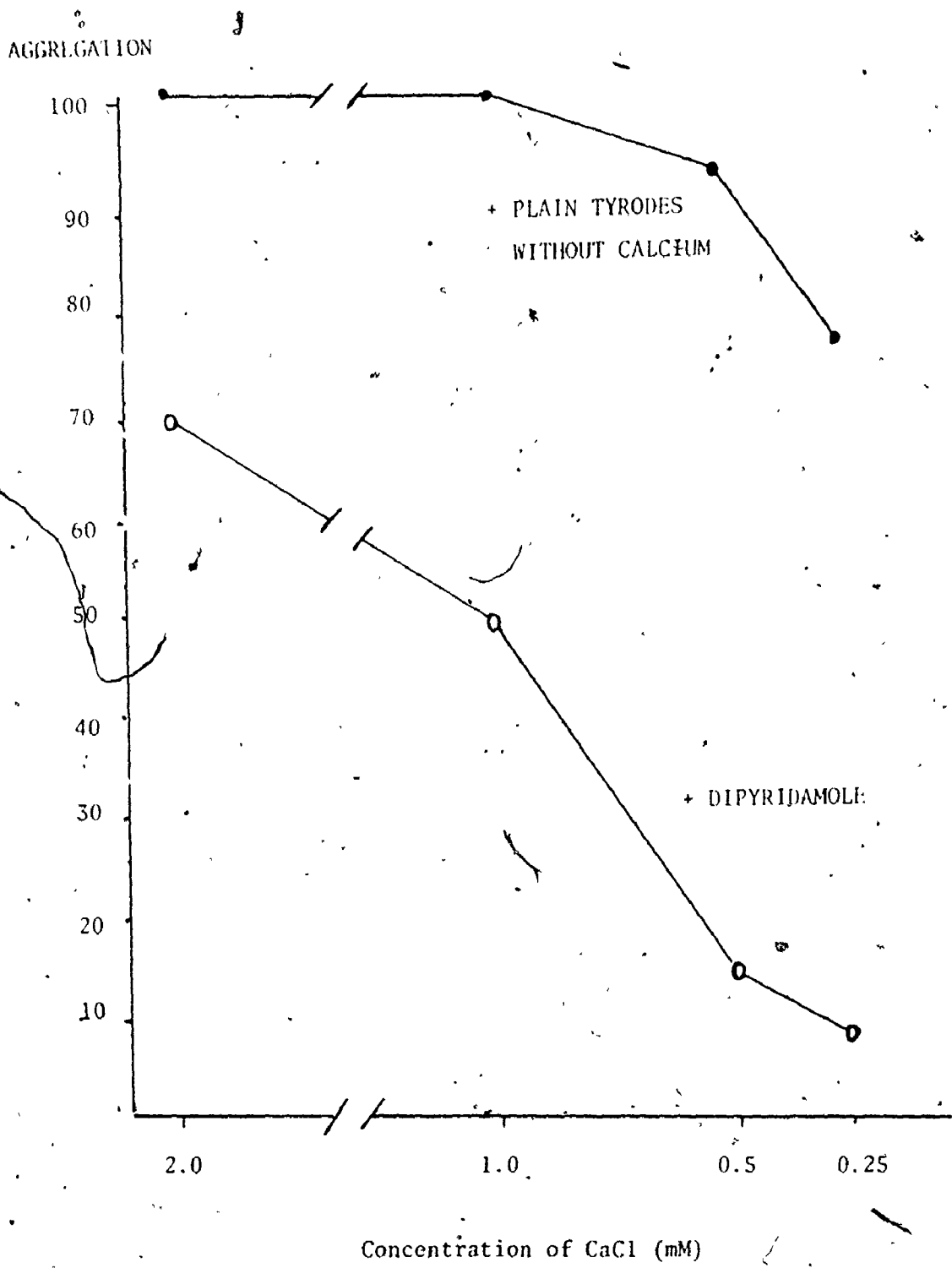


Figure 13. Collagen-induced platelet aggregation in washed platelet suspensions with decreasing concentrations of calcium with and without dipyridamole.

2 mM and this inhibitory effect was increased with decreasing concentrations of calcium.

Part 1b - Summary

It was found that when the stimulus used to initiate platelet aggregation in vivo and ex vivo was kept constant, the patterns of inhibition with aspirin and sulfinpyrazone were similar in both test systems. On the other hand, dipyridamole had no effect ex vivo whereas it was just as effective as aspirin and sulfinpyrazone in vivo. These results suggested that the effects of dipyridamole ex vivo were influenced by the blood processing procedure. Thus, it was shown by using heparin rather than citrate as an anti-coagulant, that dipyridamole also inhibited platelet aggregation ex vivo. The lack of an inhibitory effect of dipyridamole in citrated samples could not be attributed to a decrease in concentration of divalent cations. Thus, citrate, independent of its chelating properties of calcium and magnesium was shown to influence the mechanism of action of dipyridamole.

Part IIa. The Effect of Aspirin on the Platelet

It is generally accepted on the basis of earlier studies carried out by O'Brien and Atac et al, and more recent studies carried out by Roth and Majerus, that the effect of aspirin on the platelet is irreversible and lasts the lifetime of the platelet (O'Brien, 1968; Atac et al, 1970; Roth and Majerus, 1975), and in particular, it has been suggested that a major effect on platelet function is through acetylation of platelet cyclo-oxygenase and hence, the prevention of prostaglandin synthesis (Roth et al, 1975).

Other experiments, however, have been performed which suggest that the prolonged effect of aspirin on platelets may not solely be due to an acetylation effect of aspirin on platelet cyclo-oxygenase but also may be due to an effect of aspirin on the megakaryocyte. O'Brien (1968) examined the duration of the inhibitory effect of aspirin on ex vivo adrenaline- and collagen-induced platelet aggregation of platelets obtained from volunteers who had ingested 1.3 gm aspirin. Platelet aggregation was inhibited for 4 days and returned towards normal on day 5, however, when 10% v/v normal PRP was added to the samples on days 2,3 and 4, platelet aggregation was corrected to normal. These experiments suggested that the 10% of circulating platelets that were released daily from the bone marrow did not behave in the same way in vivo as the addition in vitro of 10% platelets from non-aspirin treated donors.

An effect of aspirin on megakaryocytes has also been suggested by Jafari and associates (1976). These investigators found that PGE synthesis was almost abolished within two hours of ingestion of 600 mg aspirin. Over the next two days, the amount of PGE produced by the circulating platelets was less than the expected 10%/daily increase one would expect to be produced by new non-aspirinated platelets. PGE production then rose sharply towards normal on day 3, reaching a plateau by day 8. Jafari et al likewise suggested that the slow recovery in PGE production during the first two days was due to acetylation of cyclo-oxygenase in the mature megakaryocyte.

In addition to the effect of aspirin on the cyclo-oxygenase of platelets and megakaryocytes, the possibility has been raised that part of the effect of aspirin on platelets may be due to acetylation of plasma proteins which in turn become absorbed onto the platelet and interfere with its function. Thus, Okonkwo and Sise (1971) reported that aspirinated PPP inhibited the second wave of adenosine diphosphate-induced platelet aggregation.

There is also some controversy about whether or not aspirin inhibits platelet adhesion as well as release. Tschopp and Baumgartner (1977) have demonstrated that aspirin inhibits platelet aggregation to damaged vessel wall but does not inhibit platelet adhesion, implying that platelet release is affected whereas platelet adhesion is not. On the other hand, Cazenave and Davis

have reported that aspirin also inhibits adhesion to the damaged vessel wall (Cazenave et al, 1977).

This second part of the study was performed to investigate:

- 1) whether fresh platelets released from the megakaryocytes previously exposed to aspirin function normally;
- 2) whether the aspirinated PPP affects collagen-induced aggregation in vivo; and
- 3) whether aspirin can affect platelet function independently of its inhibitory effect on platelet cyclo-oxygenase.

The effect of aspirin on the platelet released from the megakaryocyte

Experiments were performed to determine whether platelet malondialdehyde production by new platelets released from the megakaryocytes was altered after the megakaryocytes were exposed to large doses of aspirin. Rabbits were injected with 0.5 ml sheep anti-rabbit platelet serum. The platelet count in these animals dropped to $<1000/\text{mm}^3$ within 5 minutes and remained at that level for at least six hours. The rabbits were divided into three groups and treated in the following way. One hour after the administration of the anti-platelet serum, each rabbit in the first group was given 10 mg/kg aspirin, each rabbit in the second group was given 200 mg/kg aspirin and the third group received no treatment and acted as controls). Thirty-five hours later, the platelet count in all of these rabbits had returned to 50,000 -

100,000/mm³. [Normal rabbit platelet counts range from 190 - 250 x 10³/mm³] At this time, each rabbit was exsanguinated and the platelet malondialdehyde production was determined. Platelet malondialdehyde production obtained from rabbits treated with low and high dose aspirin did not differ from the non-aspirin treated rabbits (Figure 14). In addition, malondialdehyde production by the freshly released platelets obtained from the three groups of rabbits which had been previous thrombocytopenic, tended to be higher, although not significantly so, than the malondialdehyde levels achieved from platelets obtained from a fourth group of normal rabbits which had not been made thrombocytopenic. This data argues against an effect of aspirin on the megakaryocytes since platelet malondialdehyde production by newly released platelets from the previously aspirinated animals was normal.

The effect of aspirinated PPP on collagen-induced platelet aggregation in vivo

The following experiments were performed to determine whether aspirinated PPP inhibited collagen-induced platelet aggregation in vivo. Donor rabbits were given 60 mg/kg aspirin. Four or eighteen hours later, these rabbits were exsanguinated and the PPP was prepared from the blood (4 hr ASA/PPP; 18 hr ASA/PPP). One group of rabbits was given 4 ml/kg 4 hr ASA/PPP, a second group was given 4 ml/kg 18 hr ASA/PPP and a third group was given 2.5 mg/kg

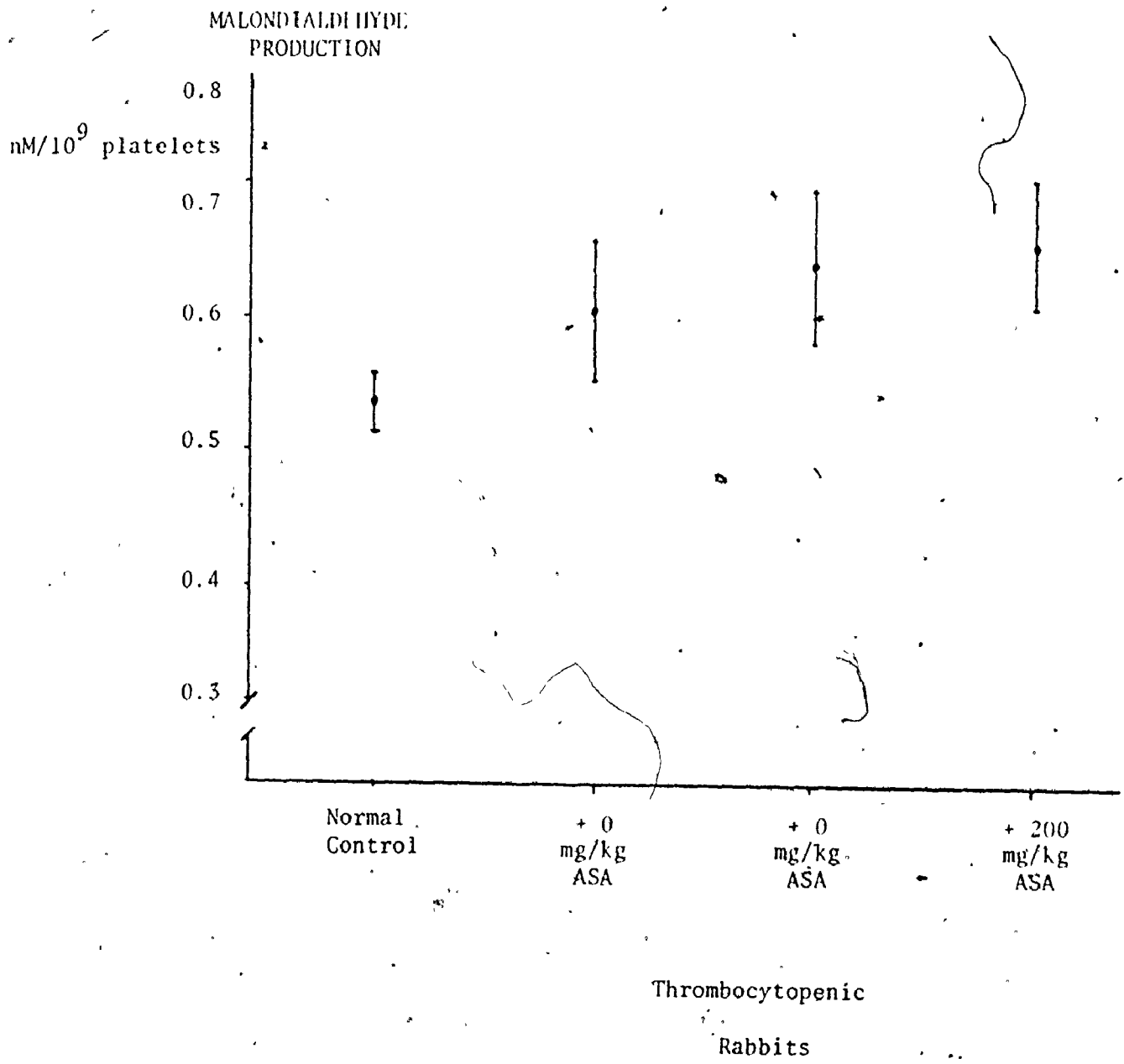


Figure 14. Platelet malondialdehyde production by platelets obtained from normal rabbits or from rabbits made thrombocytopenic 36 hours beforehand and given 0, 10, or 200 mg/kg aspirin one hour later. Data is expressed as nM/10⁹ platelets + SEM.

of the original aspirin used to treat the donor rabbits. Collagen-induced platelet aggregation was determined in vivo one hour after the treatment and the results compared to platelet aggregation observed in a fourth group of rabbits which were given nothing. Immediately before the collagen-infusion, a citrated sample was collected for each rabbit and platelet malondialdehyde production of each sample was determined. The results are shown on Figure 15. Platelet aggregation in the aspirinated/PPP treated rabbits was virtually the same as the platelet aggregation observed in the non-treated rabbits. The lack of any ASA/PPP effect was also reflected by no change in the malondialdehyde production in the same groups of rabbits (Figure 15). Because aspirin in solution is unstable, a control experiment was performed which demonstrated that the aspirin solution in 1/4 of the theoretical dose used for the PPP experiments effectively inhibited in vivo aggregation as well as malondialdehyde production (Figure 15).

These results indicate that aspirinated plasma components do not inhibit platelet function.

Dose-response related effects of aspirin on platelet function

The previous experiments indicate that the prolonged aspirin effect which is observed both in vivo and ex vivo (Mielke et al, 1969, 1977; Stuart et al, 1972, O'Brien, 1968, Atac et al, 1970) is not due to an additional aspirin effect on the megakaryocyte

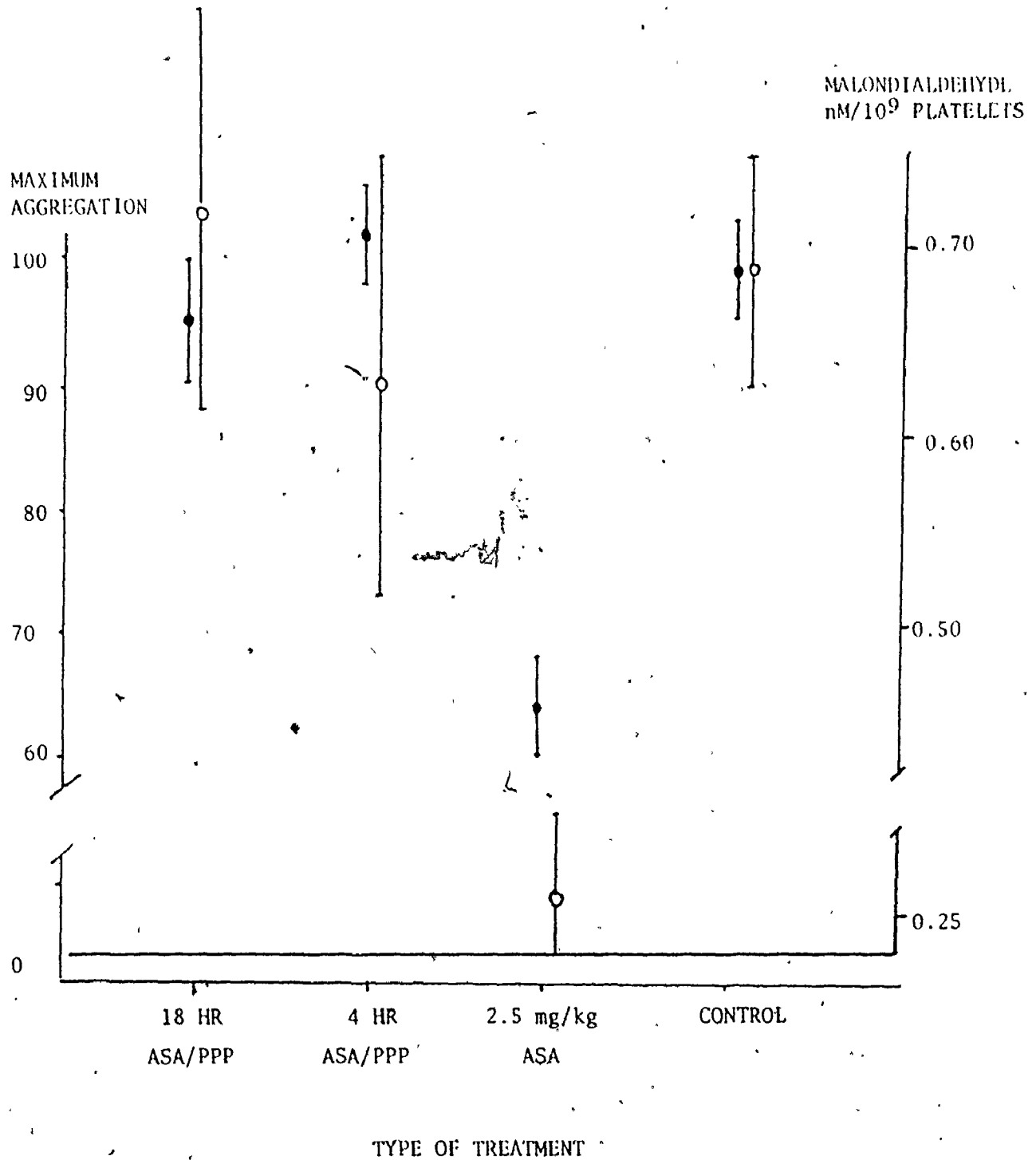


Figure 15. In vivo collagen-induced platelet aggregation + SEM (●) malondialdehyde production + SEM (○) in rabbits given aspirin or aspirinated platelet-poor-plasma one hour beforehand. The n value in each group was 12.

nor due to interference of platelet function by aspirinated plasma components. Therefore, the next series of experiments were performed to investigate the dose response related effects of aspirin on platelet malondialdehyde production and platelet/collagen aggregation.

Rabbits were injected with ^{51}Cr -platelets and then treated with 0, 0.5, 1, 2.5, 10 or 30 mg/kg aspirin. Either 1, 4, 8, 12 or 24 hours later, the animals were infused with collagen and platelet aggregation was determined. A citrated sample was collected immediately before the collagen infusion from the groups of animals that were treated with aspirin and infused with collagen one hour later. Platelet malondialdehyde production was determined on the platelets prepared from these samples. Platelet malondialdehyde was not determined from samples obtained from those animals infused with collagen four or more hours after aspirin treatment since it was possible that the results may reflect in part, malondialdehyde production by new platelets released into the circulation after treatment.

Malondialdehyde production was significantly decreased from control values in platelets obtained from rabbits treated with 0.5 mg/kg aspirin, and was maximally inhibited when the dose of aspirin was doubled to 1 mg/kg ($p < 0.001$). This is shown by the left ordinate and solid line data on Figure 16. When the dose of aspirin was increased to 2.5, 5, 10 or 30 mg/kg, there was no

nM/
MALONDIALDEHYDE
PRODUCTION

%
OF PLATELET
AGGREGATION

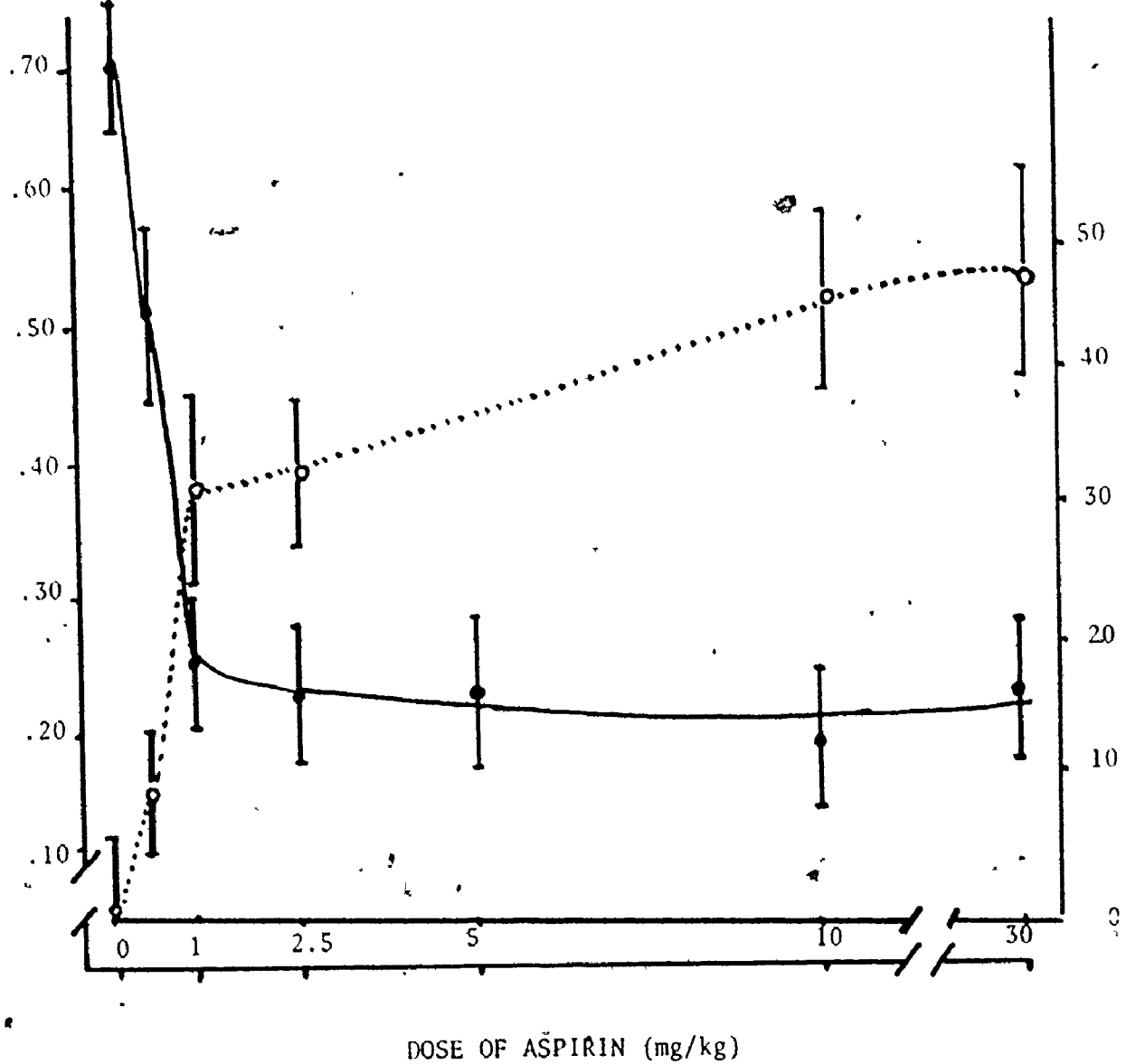


Figure 16. Platelet malondialdehyde production (nM/10⁹ platelet) + SEM (●) and collagen-induced platelet aggregation *in vivo* + SEM (○) in rabbits one hour after being given one of various doses of aspirin.

further inhibition of malondialdehyde production.

In other experiments reported later in the text, 100 or 200 mg/kg ASA did not inhibit platelet malondialdehyde production any more at one hour after treatment than did these lower doses. These results are consistent with the data of Roth and associates (1975) who demonstrated that very low concentrations of aspirin were needed to saturate the cyclo-oxygenase site on the platelet. The dose response effect of aspirin on collagen-induced platelet aggregation showed a different pattern. In non-aspirinated treated rabbits, the mean platelet aggregation was 32.1%. In Figure 16 this is shown as 0% inhibition of platelet aggregation as indicated by the right ordinate and the open circles + S.E.M. at the zero aspirin dose. The decrease in platelet aggregation seen in the treated animals was expressed as the percent inhibition of platelet aggregation as is indicated by the open circles along the broken line. When rabbits were treated with 1 to 2.5 mg/kg aspirin, platelet aggregation was significantly inhibited ($p < 0.001$). When the dose of aspirin was increased from 2.5 to 10 or 30 mg/kg, platelet aggregation was significantly inhibited further ($p < 0.001$). These results indicate that some of the effect of aspirin on the platelet/collagen interaction can be disassociated from the effect of aspirin on platelet malondialdehyde production.

The inhibitory effect of aspirin on collagen-induced platelet aggregation was tested at 1, 4, 8, 12 and 24 hours in rabbits

given either 0.5, 1, 2.5, 5, 10 or 30 mg/kg aspirin. The results are shown in Figure 17 and the results of their analysis in Table 10. At each of the dose levels, the effect of aspirin decreased with the time after its infusion. [The inequality of n values as well as the absence of values in three cells necessitated adjustments in the actual data obtained in order that an analysis of variance and a multiple comparison analysis (Statton-Newman-Kin analysis) could be made. Thus, the statistical analyses were made on predicted values tabulated in Table 11i which was derived from the actual values tabulated in Table 11j].

These results are consistent with the hypothesis that aspirin not only inhibits platelet prostaglandin production in low concentrations by blocking cyclo-oxygenase activity but also inhibits platelet interaction with collagen independently of the prostaglandin pathway when given in higher doses.

Part IIa. Summary

The experiments were performed to examine the effects of aspirin on the megakaryocytes, to examine the effects of aspirinated PPP on platelet aggregation and to examine the dose-related effects of aspirin on platelet malondialdehyde production and in vivo collagen-induced platelet aggregation. The discrepancy between our findings and those reported in the literature which, on the basis of indirect experiments suggested an effect of aspirin on

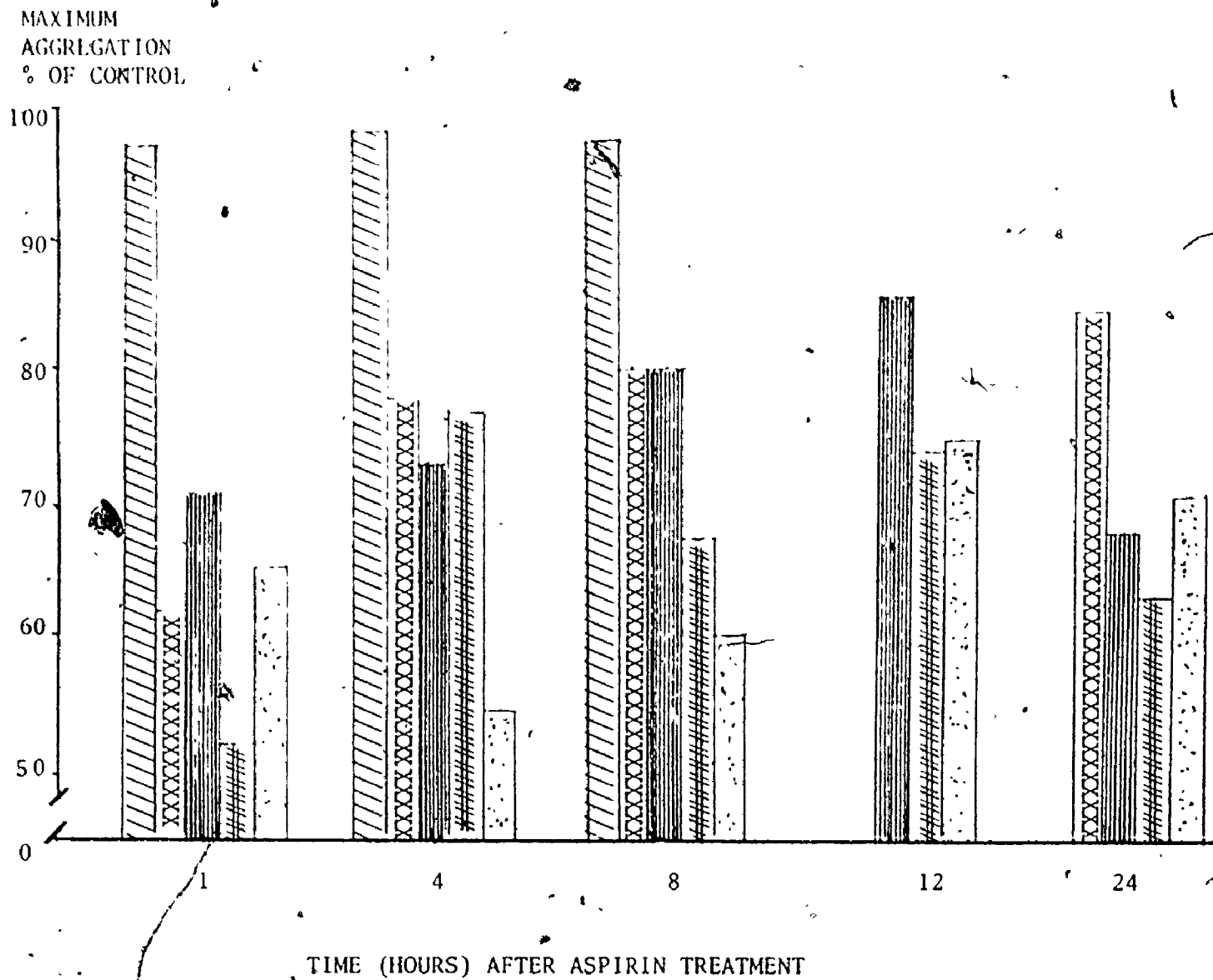







Figure 17. Dose-response of aspirin/maximum aggregation in rabbits given 0.5  , 1  , 2.5  , 10  , or 30 mg/kg aspirin  . At any given interval, the inhibitory effect of aspirin on platelet aggregation was dose-dependent, and the effect of any one dose was time-dependent.

Table 10. Effect of time and dose on ASA/platelet effect

Two-way analysis of variance adjusted for unequal observations per cells. (A) The effect of increasing doses of ASA was examined with the data adjusted for unequal numbers in each dose schedule independent of time. (B) The effect of ASA over time was examined with the data adjusted for unequal numbers in each time period independent of dose variation.

(A)

Source of Variation	Sum of Squares	df	MS	F	P
Dosage	14251.8386	4	3562.96	15.44	<0.001
Time	1025.0857	4			
Dosage-Time Interaction	4344.2825	13	334.18	1.45	ns
Among Cells	19621.2068	21			
Within Cells	<u>25149.3125</u>	<u>109</u>	230.73		
Total	44770.5191	130			

(B)

Source of Variation	Sum of Squares	df	MS	F	P
Time	4298.9654	4	1074.74	4.66	<0.01
Dosage	10977.9589	4			
Dose-Time Interaction	4344.2825	13			
Among Cells	19621.2068	21			
Within Cells	<u>25149.3125</u>	<u>109</u>			
Total	44770.519130				

Table 11: Actual and predicted mean maximum aggregation (% of Control) in rabbits given various doses of ASA and measured different times after treatment. Number in brackets equals n.

ACTUAL

DOSE \ TIME	1 HOUR	4 HOUR	8 HOUR	12 HOUR	24 HOUR
0.5 mg/kg.	97 (6)	98 (3)	97 (3)	0 (0)	0 (0)
1.0 mg/kg	62 (7)	78 (6)	80 (7)	0 (0)	85 (6)
2.5 mg/kg	71 (11)	73 (6)	80 (7)	86 (3)	68 (5)
10.0 mg/kg	47 (7)	77 (6)	66 (6)	74 (9)	63 (6)
30.0 mg/kg	65 (3)	54 (6)	60 (6)	75 (6)	72 (6)

PREDICTED

ii

DOSE \ TIME	1 HOUR	4 HOUR	8 HOUR	12 HOUR	24 HOUR
0.5 mg/kg	93	102	103	113	104
1.0 mg/kg	69	77	78	88	80
2.5 mg/kg	67	75	77	86	78
10.0 mg/kg	55	64	65	75	66
30.0 mg/kg	54	63	64	74	65

megakaryocytes could be explained by Shulman's observations (Shulman et al, 1968) that freshly released platelets spend a period of time in the spleen before being distributed among the circulating platelets.

Our experiments failed to demonstrate an effect of aspirin-PPP on in vivo collagen-induced platelet aggregation indicating that the effect of aspirin on the reaction was a direct one on the platelet.

A different pattern of dose-response relationships was seen when the effect of aspirin on platelet malondialdehyde production was compared to the aspirin effects on in vivo collagen-induced platelet aggregation. Thus, maximum inhibition of platelet malondialdehyde production was seen with a relatively low dose of aspirin (supporting the findings of others) while maximum inhibition of collagen-induced platelet aggregation required considerably higher doses. These results indicate that part of the effect of aspirin on the platelet/collagen interaction is independent of its effects on platelet prostaglandin synthesis.

Part IIb. The effect of aspirin on the vessel wall

Considerably higher concentrations of aspirin than those obtained in Part IIa are usually achieved when aspirin is given therapeutically as an analgesic or as a prophylactic agent against thromboembolism after hip surgery (Salzman et al, 1971; Zeckert

et al, 1973) or on patients with transient ischemia (Fields et al, 1977). These higher concentrations are necessary for its analgesic effects because higher concentrations are required to inhibit prostaglandin production in inflammatory tissue (Patrono et al, 1976). It is possible also that higher concentrations may be required to achieve an optimal anti-thrombotic effect which could be mediated in particular by the inhibition of prostaglandin synthesis by the vessel wall.

The vessel wall has been shown to produce a variety of prostaglandins which both augment and inhibit platelet aggregation and which alter vascular tone (Needleman et al, 1977; Moncada et al, 1976, 1976a, 1976b, 1977; Hadhazy et al, 1976, Gorman et al, 1977). Therefore, drugs which alter prostaglandin production by vessel wall could have an important influence on the thrombosis and hemostasis processes.

This part of the study was performed to determine:

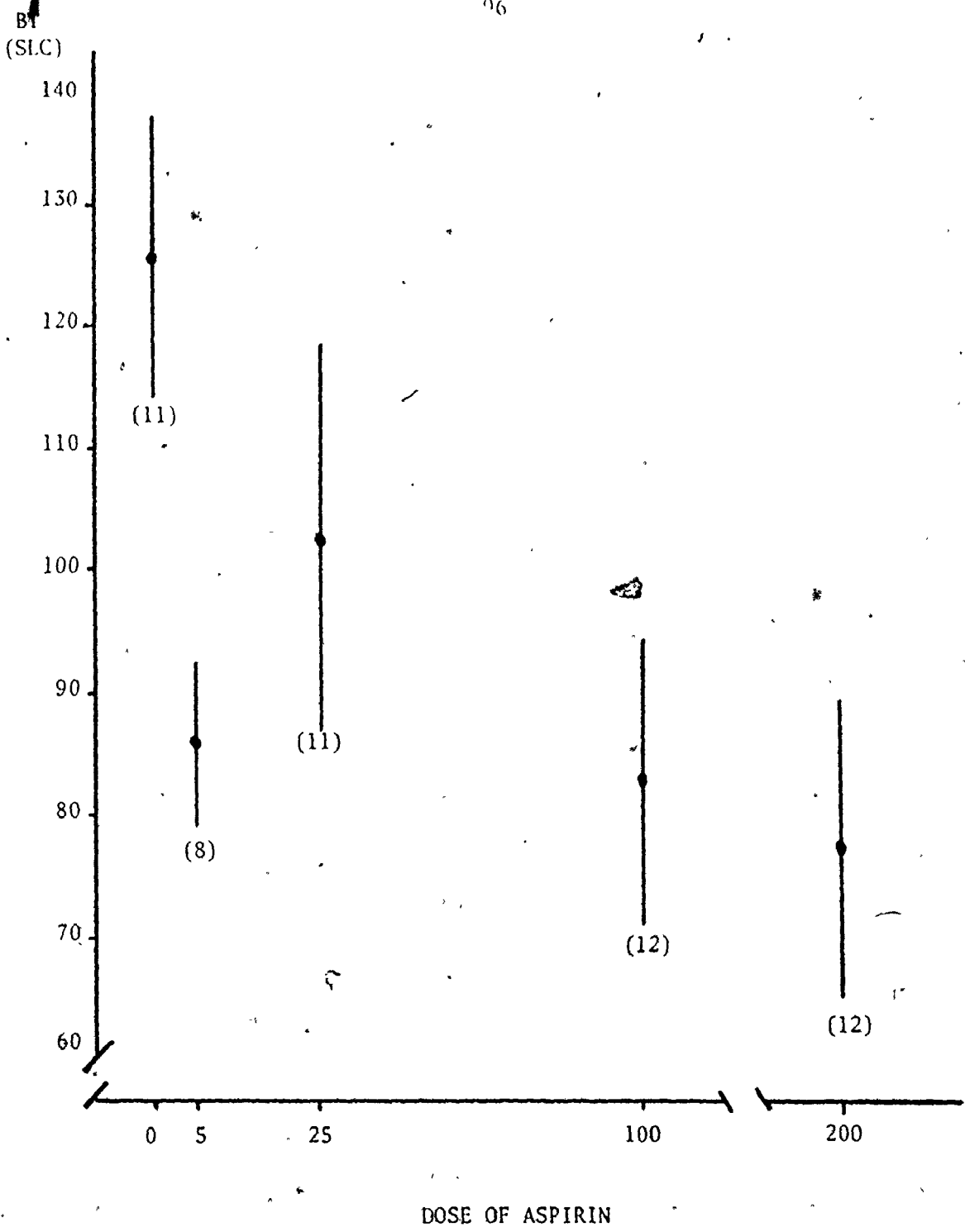
- 1) whether a dose-response relationship existed between aspirin and the jugular vein bleeding time, and
- 2) whether this effect on the vessel wall (unlike on the platelet) was short-lived.

Effect of aspirin on jugular bleeding time and platelet Malondialdehyde production

Normal rabbits were given 0, 5, 25, 100 or 200 mg/kg aspirin intravenously. Sixty minutes later, both jugular veins were

isolated and the bleeding time after a needle puncture in each vessel was determined as described under 'Methods'. The jugular bleeding time was 125 seconds \pm S.E.M. in the placebo-treated rabbits (Figure 18). Platelet malondialdehyde production by platelets obtained from these same animals was $0.59 \text{ nM}/10^9$ platelets. Much to the surprise of the investigator, the bleeding time was decreased in all animals given aspirin (Figure 18). An analysis of the data clearly showed that the bleeding times in all aspirin-treated rabbits was significantly shorter than the control animals, $p < 0.05$ but that there was no dose response effect with increasing doses from 5 - 200 mg/kg, $f = 0.42$ (Table 12). Similarly, the lowest dose of aspirin had a maximal effect on platelet malondialdehyde production. Increase in the dose had no further effect (Figure 18).

It seemed possible that the shortening of the bleeding time by aspirin was related to the inhibition of PGI_2 formation by the vessel wall. This could have two effects. Firstly, it could remove a potent inhibitor of platelet interaction with the damaged vessel wall and secondly, it could remove the vasodilatory effect of PGI_2 and, hence, shorten the bleeding time by producing a sustained vasoconstriction. The lack of a dose-response relationship could be due to a confounding effect of platelets in the system. Therefore, further experiments were performed to study the effect of aspirin directly on the vessel wall in the absence of platelets.



MDA/10 ⁹ PLATELETS (nm/10 ⁹)	0.59	0.23	0.23	0.27	0.23
(+ SEM)	+0.06	+0.05	+0.02	+0.06	+0.05

Figure 18. Jugular bleeding time and platelet MDA production (+ SEM) of rabbits given 0, 5, 25, 100, or 200 mg/kg aspirin i/v 60' beforehand. n = (x)

Table 12: An analysis of variance of the jugular bleeding times in normal rabbits given various doses of aspirin. This analysis failed to show any overall differences among the different doses of aspirin. However, the bleeding times of all treated animals were significantly different from the control animals.

SOURCE OF VARIATION	SUM OF SOURCES	d. f.	M. S.	F	P
Among Animals	95771.6	27	3547.1	1.07	n. s.
Among All Groups	19539.4	4	4884.8	1.47	n. s.
Between Control Treatment	15274.9	1	15274.9	4.51	<0.05
Among Treatments	4264.5	3	1421.5	0.43	n. s.
Among Animals Within Dosages	16232.3	23	3314.4		
Within Animals	2925.5	26			n. s.
TOTAL	98697.1	53			

Jugular bleeding times after intraluminal incubation with aspirin

Rabbits were made thrombocytopenic by a combination treatment of irradiation and anti-platelet serum. Then the rabbits were anesthetized and their jugular veins were isolated. The veins were emptied of their blood contents and then a segment of each was isolated with a distal and proximal clamp. One vessel was filled with either aspirin suspended in 0.55M sodium carbonate, pH 6.8, or lysine aspirin suspended in saline, pH 3.5. In each case, the aspirin concentration was 100 mg/ml. The other vessel was filled with the appropriate control (0.55M sodium carbonate, pH 6.8, or saline, pH 3.5). After one hour incubation, the clamps were removed and blood flow restored, allowing the blood to wash away the vessel segment contents (volume 200 - 300 λ). Five minutes later, each vessel was punctured by a 23 gauge needle and the time taken for cessation of bleeding was recorded. Studies were made in both the normal and the thrombocytopenic rabbits.

The results are shown on Table 13. Since there was no significant difference between the effects of aspirin suspended in saline or sodium carbonate, the data from both were pooled. A paired t-test analysis of the bleeding times in aspirin and control treated vessels in non-thrombocytopenic rabbits showed that intraluminal installation of aspirin produced a non-significant shortening of the bleeding time ($p < 0.10$). However, in rabbits with platelet counts $< 20,000/m^3$, this difference was significant with a bleeding

Table 13: Jugular bleeding time in normal and thrombocytopenic rabbits after their jugular veins were topically exposed to aspirin, saline or sodium carbonate. In each rabbit, one jugular vein was isolated and filled with lysine or Sigma ASA (100 mg/ml)(pH 7.5, 6.8, respectively). The opposite jugular was appropriately filled with either NaCl pH 3.5 or 0.5 M NaCO₃ pH 6.8. Each vessel remained filled for 55 minutes at which time flow was restored. Bleeding time was assessed 5' later. Since there were no differences in effects between the lysine or Sigma ASA, the data was pooled and treated as the same.

	<u>NORMAL RABBITS</u>	
<u>PC X 10³</u>	<u>ASA</u>	<u>CONTROL</u>
230.9	52.9 ± 11	72.2 ± 10
	$\bar{x} - \bar{y} = 19.3 \pm 12$	
	n = 11	
	p < 0.10	

	<u>THROMBOCYTOPENIC</u>	
<u>PC X 10³</u>	<u>ASA</u>	<u>CONTROL</u>
<20.5	308.5 ± 90	699 ± 71
	$\bar{x} - \bar{y} = 390.8 \pm 99$	
	n = 11	
	p < 0.005	

time reduction from 699 seconds to 309 seconds ($p < 0.001$). The control bleeding times recorded from the normal (72 seconds) and thrombocytopenic rabbits (699 seconds) were similar to the bleeding times observed in normal and thrombocytopenic rabbits whose veins had not been exposed to the suspending fluids (Blajchman et al, 1977). This suggests that neither osmolarity nor pH were important contributing factors in these observations.

If the hypothesis that the shortening of bleeding time by aspirin was due to its effect on vessel wall PGI_2 synthesis was correct, a similar effect would be expected to be observed with other prostaglandin inhibitors. Therefore, the experiments were repeated with indomethacin.

Effect of indomethacin on jugular vein bleeding times

The jugular veins of normal and thrombocytopenic rabbits were filled with either indomethacin (15 mg/ml) or sodium carbonate (0.5M, pH 8.4). The results are shown on Table 14. Bleeding time was reduced from 64 seconds to 29 seconds in the normal rabbits ($p < 0.05$) and from 864 to 244 seconds in thrombocytopenic rabbits ($p < 0.005$).

The data obtained from both the aspirin and indomethacin experiments support the explanation that the shortening in jugular vein bleeding time was a result of an inhibition of vessel wall PGI_2 production.

Table 14: Jugular bleeding time in normal and thrombocytopenic rabbits after their jugular veins were topically exposed to indomethacin or Na Carbonate (pH 8.4). One vessel was filled with indomethacin (15 mg/ml, pH 8.4), the other with 0.5 M NaCO₃, pH 8.4 for 55'. Flow was restored in each vessel and bleeding time was measured 5 minutes later.

NORMAL RABBITS BT (SEC)

<u>PC X 10³</u>	<u>INDOMETHACIN</u>	<u>CONTROL</u>
254.8	28.8 ± 6	63.8 ± 13
	$\bar{x} - \bar{y} = 35 \pm 12$	
	n = 4	
	p < 0.05	

THROMBOCYTOPENIC BT (SEC)

<u>PC X 10³</u>	<u>INDOMETHACIN</u>	<u>CONTROL</u>
4.6	244.2 ± 134	864.2 ± 36
	$\bar{x} - \bar{y} = 620 \pm 137$	
	n = 6	
	p < 0.005	

Duration of the aspirin effect on vessel wall

The platelet is an anucleated cell and cannot resynthesize cyclo-oxygenase. Thus, once the platelet cyclo-oxygenase has been acetylated, the effect is permanent. The cells of the vessel wall, however, are nucleated and potentially have the possibility to synthesize and replace the depleted or inactivated materials. If this were so, one would predict that the aspirin effect on the vessel wall would be relatively short-lived as more cyclo-oxygenase was synthesized by these cells. Information related to the duration of aspirin effect on vessel wall could be of considerable practical importance when considering the timing of aspirin administration as an antithrombotic agent.

Jugular veins were isolated and filled with aspirin or placebo in the same manner as before. Bleeding times were performed on the vessels 30, 60 or 120 minutes after restoration of flow. These times were chosen because the acetylation effect of aspirin is completed within 20 to 60 minutes (Levy, 1976). Therefore, it would be expected that the vessel wall would be able to overcome the aspirin effect in greater than 60 minutes. The bleeding times are shown on Figure 19. At 30 minutes, there was a significant decrease in bleeding times in the aspirin-treated vessels (Table 15). This effect was maintained at 60 minutes. At 120 minutes, there was no apparent difference between the aspirin- and placebo-treated vessels.

BLEEDING TIME (SEC)

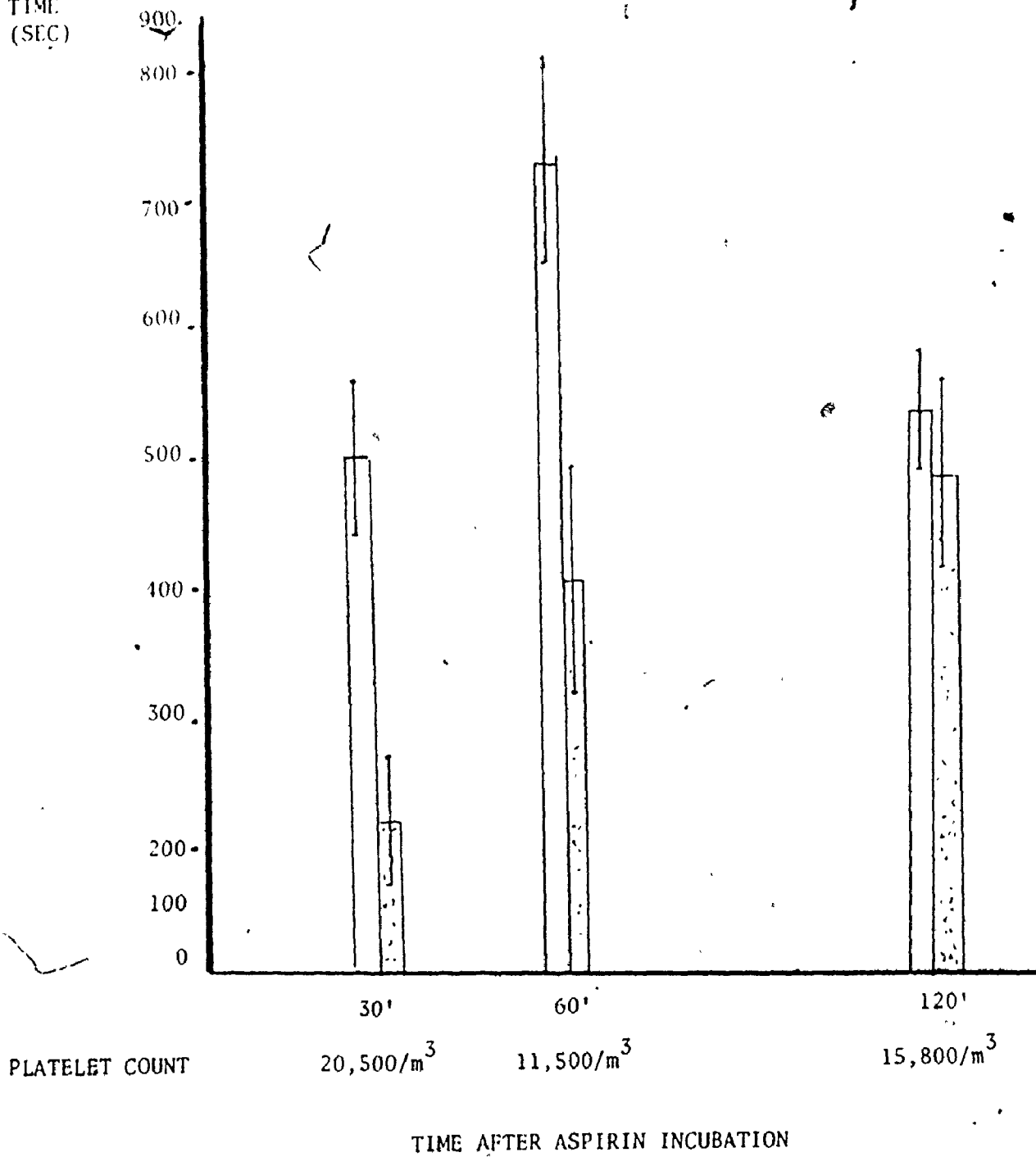


Figure 19. Jugular bleeding time in thrombocytopenic rabbits after local jugular incubation for 60' with 100 mg/ml aspirin or saline.

Saline Treated Vessel  ASA Treated Vessel 

Table 15: Jugular bleeding time in rabbits (made thrombocytopenic with APS) after local vein incubation with ASA or Saline for 60 minutes. Bleeding times were determined 30, 60 and 120 minutes after treatment and restoration of flow.

	<u>30' POST R</u>			<u>60' POST R</u>			<u>120' POST R</u>		
	<u>PC X 10³</u>	<u>ASA</u>	<u>SAL</u>	<u>PC X 10³</u>	<u>ASA</u>	<u>SAL</u>	<u>PC X 10³</u>	<u>ASA</u>	<u>SAL</u>
M	20.8	37.1	402.6	11.5	298.1	630.4	15.8	386.3	437.3
SE		35.9	63.8		100.6	85.5		80.3	51.5
n		8			8			8	
P		<0.005			<0.025			ns	
SAL/ASA		2.9			2.1			1.1	

These experiments were repeated in another group of thrombocytopenic rabbits in which the aspirin was applied topically to the outside of the vessel every fifteen minutes for one hour. The results were similar (Figure 20). Thus, at 60 minutes after the last aspirin treatment, the bleeding time was markedly shorter than in those vessels which were exposed to sodium salicylate. By two hours, the aspirin effect had disappeared. More recent experiments carried out by Blajchman et al indicated that the effect of intraluminal aspirin is associated with a reduction in the size of the needle puncture hole which is presumably the consequence of the inhibition of PGI_2 production and subsequent vasodilatory effects (Blajchman et al, 1977a).

These results are consistent with the hypothesis that the vessel wall can resynthesize new cyclo-oxygenase when this enzyme is acetylated by aspirin.

Part 2. Summary

This investigation of the different effects of aspirin on the platelet and the vessel wall demonstrates that:

- 1) aspirin inhibits the function of those platelets circulating at the time of the aspirin treatment but does not inhibit the function of those platelets released into the circulation following the aspirin treatment. Thus, it seems likely that any aspirin effect on megakaryocytes, if any at all, is reversible since these cells may replace the

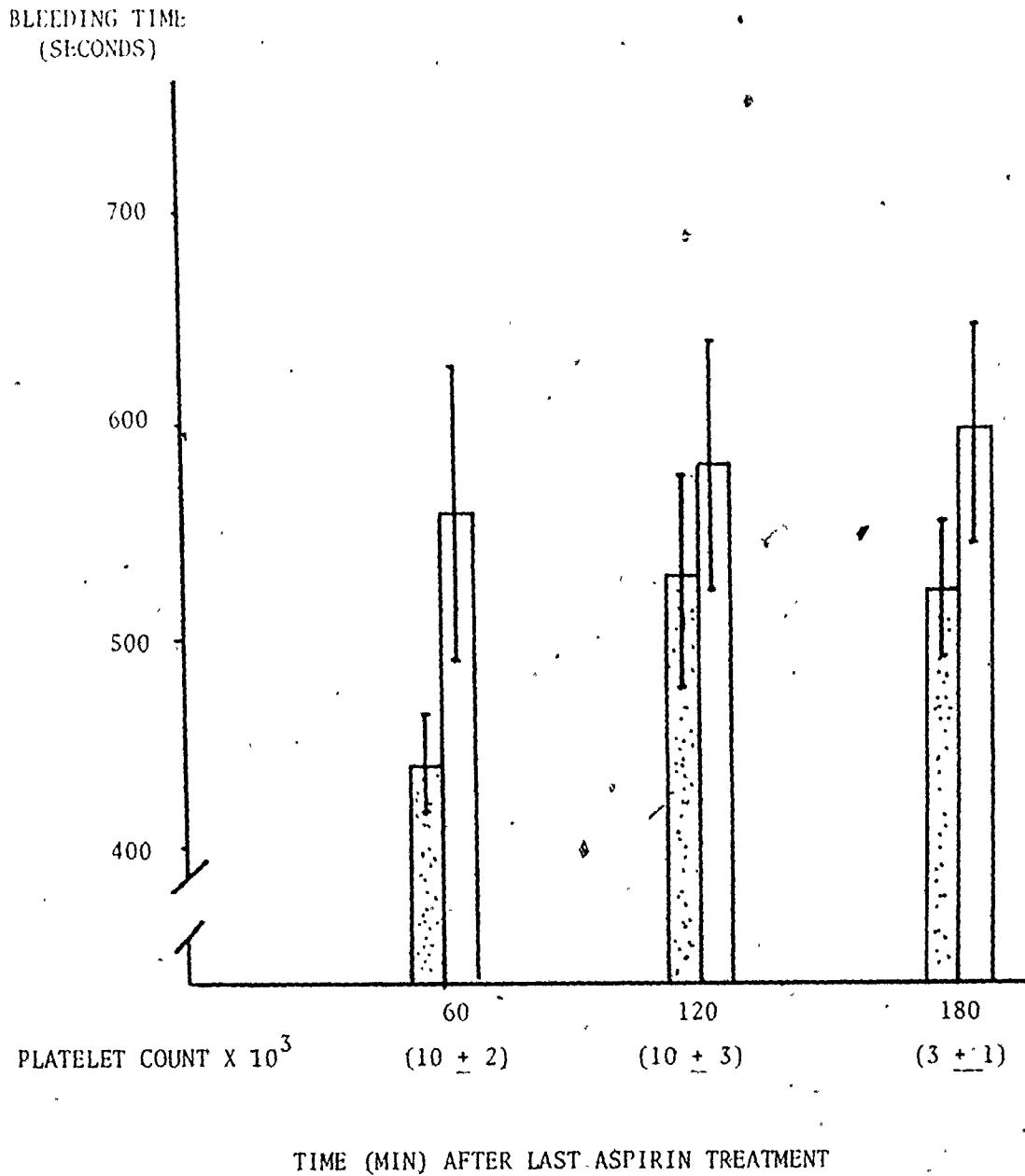


Figure 20. Jugular bleeding times \pm SEM in thrombocytopenic rabbits 60, 120, and 180 minutes after a topical application of 5 ml aspirin (100 mg/ml) [stippled] or 5 ml sodium salicylate 100 mg/ml [white], applied every 15 minutes for 60 minutes. (n = 8)

depleted enzyme as is suggested by the normal production of MDS by platelets freshly fragmented from the megakaryocytes;

2) that acetylated plasma proteins do not have the ability to acetylate or block platelet prostaglandin synthetase;

3) that mechanisms other than the inhibition of platelet prostaglandin synthesis activity do contribute to the inhibition of the platelet-collagen interaction in vivo since the extent of inhibition of the reaction in vivo continues with a dose response relationship well beyond that dose which produces maximum inhibition of platelet malondialdehyde synthesis;

4) that aspirin has an effect on vessel wall responses and that this effect is manifest by a shortening of vessel wall bleeding time;

5) that the effect on vessel wall bleeding time appears to be a result of the inhibition of vessel wall dilation by PGI_2 ; and

6) that the aspirin effect on the vessel wall is reversible primarily because the vessel wall can synthesize new products to replace those affected by aspirin.

Part III. Effect of dipyridamole on platelet function

Dipyridamole has been shown to be an effective anti-thrombotic agent (Sullivan et al, 1969). The mode of action is uncertain. It has been shown to inhibit platelet adenosine uptake and it increases platelet cyclic adenosine monophosphate by inhibiting phosphodiesterase, but it is uncertain whether either of these two effects are

important in its effectiveness as an inhibitor of platelet function (Lmmons et al, 1965; Cucuiianui et al, 1971, Mills and Smith, 1971; Philps et al, 1973).

There are a number of unresolved issues related to the effectiveness of dipyridamole as a drug which suppresses platelet function. The first is its lack of effect on platelet function ex vivo in doses which produce an effect on platelet survival in vivo (Harker and Slichter, 1970; Warlow et al, 1974; Zucker and Peterson, 1970). This problem has been discussed and a possible explanation offered as a result of experiments performed in Part I of this thesis. The second is the observation of an apparent synergism between aspirin and dipyridamole. Harker and Slichter (1970,1972) have shown that a low dose of dipyridamole (100 mg/day) in combination with 1 gm aspirin/day was as effective as a higher dose of dipyridamole (400 mg/day) in prolonging the reduced platelet survival seen in a number of patients with prosthetic heart valves. There are two explanations for these results.

The first is that the synergism between these drugs is real and due to the additional effect of aspirin on platelet function. The second is that the synergistic effect is only apparent and due to an effect of aspirin on the plasma level, distribution and/or clearance of dipyridamole. For example, dipyridamole has been shown to bind to high affinity sites on the platelet and this binding is inhibited by a plasma protein, an α_1 acid glycoprotein, which competes with the

platelet for dipyridamole (Niewiarowski et al, 1975; Subbarao et al, 1977,1977a). Thus, it is possible that aspirin could displace dipyridamole from the protein and hence, increase its availability for platelet binding. A second way in which aspirin could influence dipyridamole levels is by altering the rate of clearance of dipyridamole since both drugs are cleared from the plasma by the glucuronide pathway (Cohen, 1976; Levy, 1975; Levy and Tsuchiza, 1972; Zak et al, 1963).

The experiments in this part of the study were performed to investigate the apparent synergism between aspirin and dipyridamole. These experiments were performed by:

- 1) determining the dose response effects of dipyridamole on in vivo collagen-induced platelet aggregation and relating them to the dipyridamole-plasma levels;
- 2) determining the added effects of aspirin on the effects of dipyridamole on platelet aggregation and on dipyridamole-plasma levels; and
- 3) determining the effect of aspirin and salicylate on the pharmacokinetics of dipyridamole.

The relationship between dipyridamole-platelet effect and dipyridamole-plasma levels

Rabbits previously injected with ^{51}Cr -platelets were given 3 mg/kg dipyridamole, intravenously. At intervals ranging from 1/4 hour to 4 hours after treatment, collagen-induced platelet

aggregation was determined in vivo. Immediately before the collagen infusion, a citrated sample was collected from each rabbit and the concentration of dipyridamole within that sample determined.

The pattern of platelet aggregation at intervals after the dipyridamole/injection is shown on Figure 21. Maximum platelet aggregation was $32 \pm 1\%$ in control rabbits (this is expressed as 100% aggregation in Figure 21). Within the first hour of treatment, platelet aggregation was reduced to 19% aggregation (about 65% of the control group). This effect was less at two hours and was completely lost at four hours.

There was a relationship between the dipyridamole-plasma level and its effect on aggregation (Figure 22) indicating that the effect of dipyridamole on collagen-induced platelet aggregation is directly related to the concentration of dipyridamole circulating within the plasma.

The effect of aspirin on the dipyridamole effect

In previous experiments, it was shown that a dose of aspirin >1 mg/kg inhibits collagen/platelet aggregation. Therefore, for these experiments, a dose of aspirin (0.5 mg/kg) which itself did not inhibit platelet/collagen aggregation, was selected to explore the possibility of a synergistic effect between aspirin and dipyridamole. At this dose of aspirin, aspirin did not increase the effect of 3 mg/kg dipyridamole on in vivo collagen-induced platelet aggregation (Figure 23). Therefore, experiments were then performed

MAXIMUM
AGGREGATION
% OF CONTROL

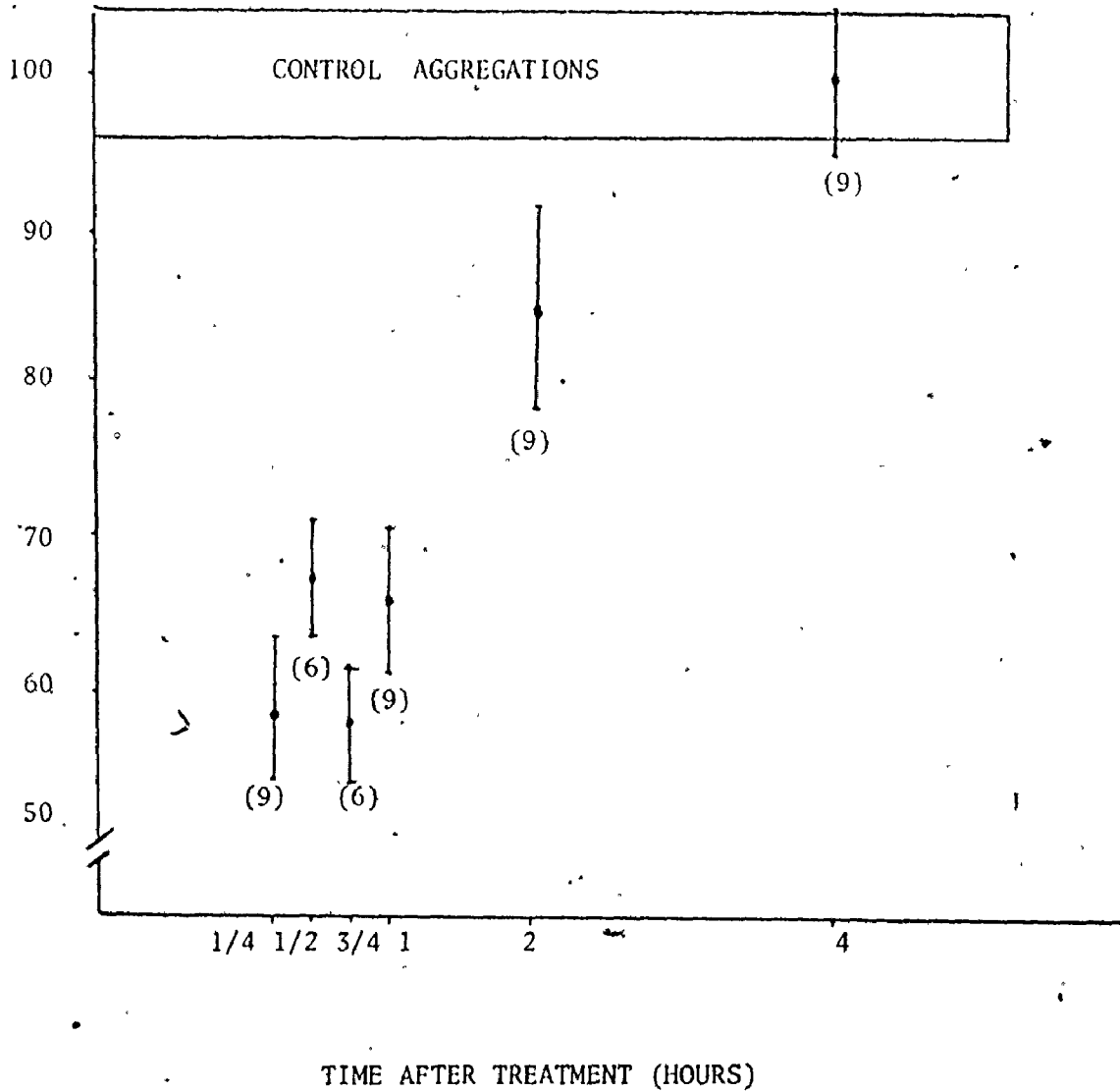


Figure 21. Maximum collagen-induced platelet aggregation in vivo in rabbits given 3 mg/kg dipyridamole. Data is expressed as % of Control. Maximum aggregation \pm SEM of 14 control rabbits was 32 ± 1.3 . Values in brackets equal the n value of each group.

MAXIMUM
AGGREGATION
(% of control)

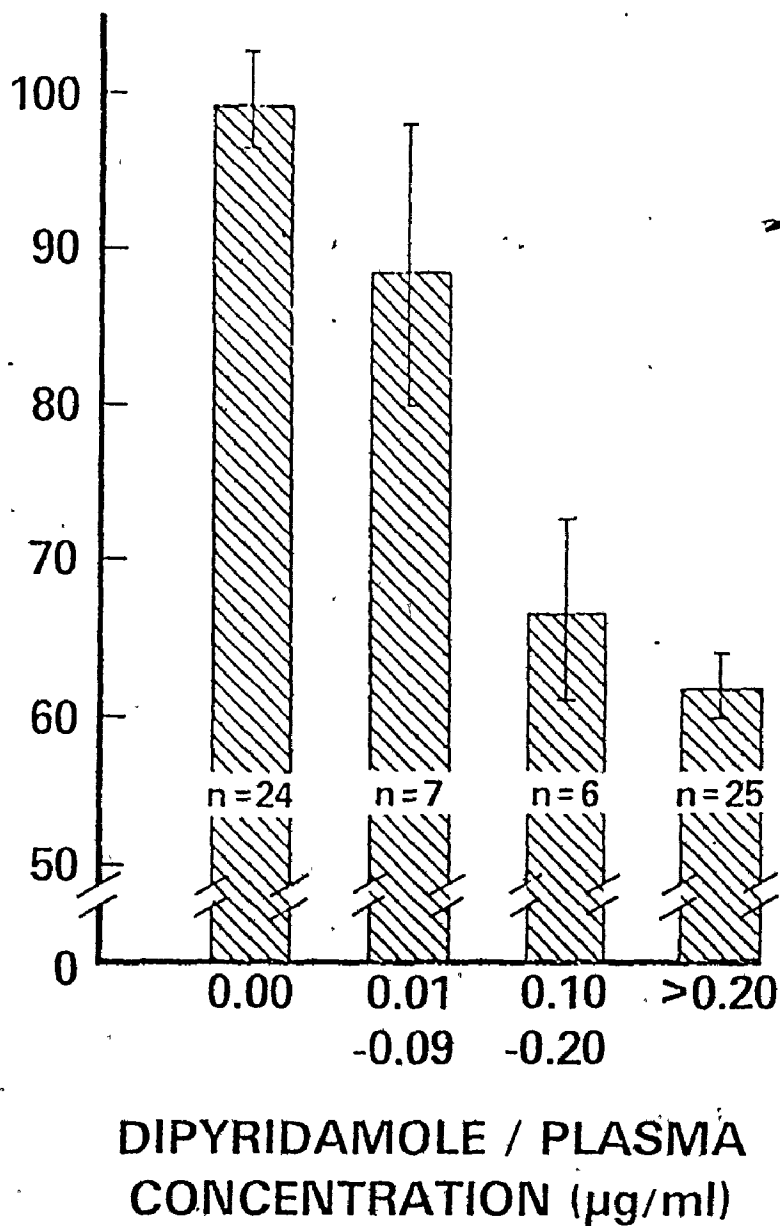


Figure 22

The relation between maximum collagen-induced platelet aggregation ($\% \pm \text{SEM}$) and dipyridamole/plasma concentration in rabbits given 3 mg/kg dipyridamole

MAXIMUM
AGGREGATION
(% of control)

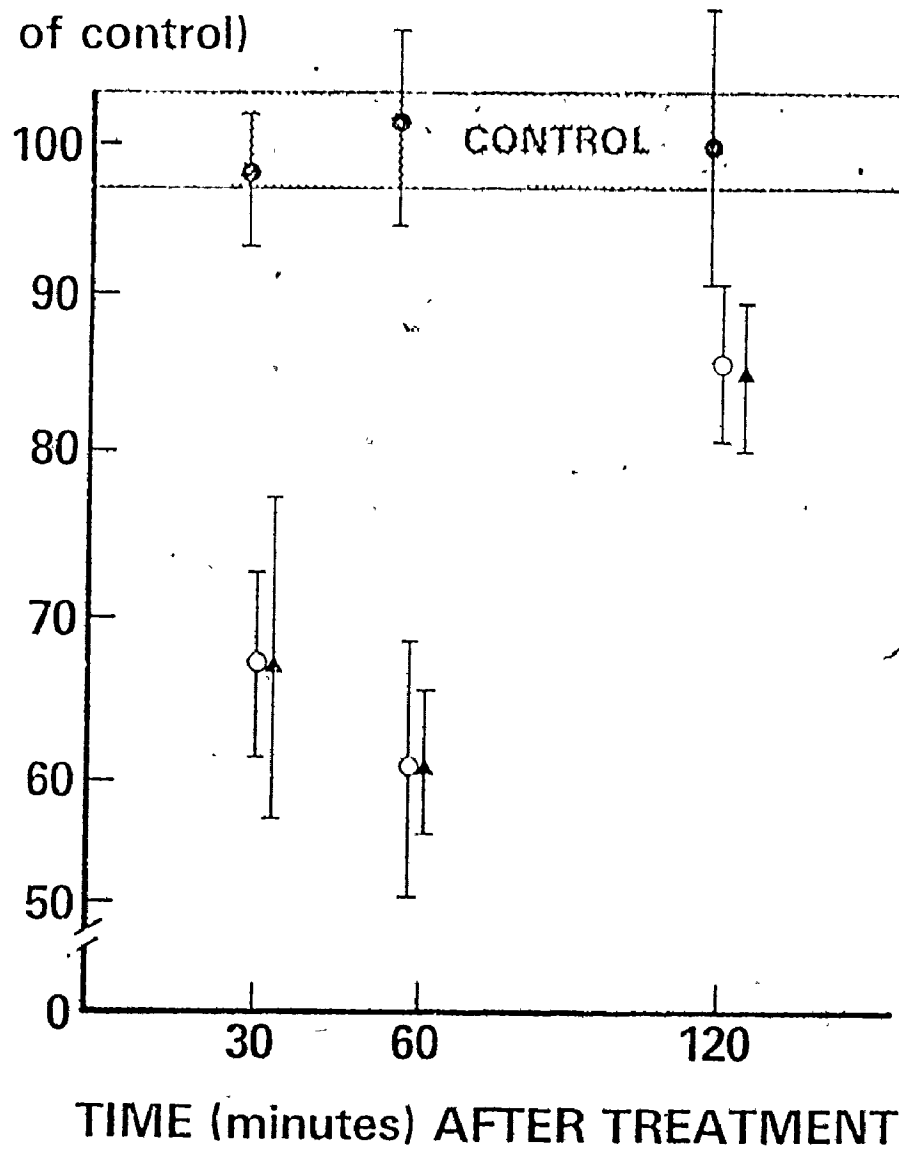


Figure 23.

Maximum collagen-induced platelet aggregation in vivo in rabbits following treatment with

- 1) 0.5 mg/kg aspirin (●)
- 2) 3.0 mg/kg dipyridamole (○), or
- 3) a combination of both 1) and 2) (▲)

(n=8)

to examine the effects of higher doses of aspirin on the dipyridamole-plasma concentration.

Rabbits were given either 3 mg/kg dipyridamole or a combination of 30 mg/kg aspirin followed 5 minutes later with 3 mg/kg dipyridamole. A sample of blood was collected from each rabbit either 30, 60 or 120 minutes later. The dipyridamole concentration was determined in each sample. The results are shown on Figure 24. At each of these times the dipyridamole-plasma concentration in the rabbits treated with aspirin + dipyridamole was at least 30% higher than the level of dipyridamole seen in those animals given dipyridamole alone ($p < 0.01$). This data is compatible with the hypothesis that aspirin acetylates dipyridamole binding sites on the plasma proteins or tissues, potentially altering the distribution and/or clearance rather than that aspirin and dipyridamole affect platelet function synergistically. Since salicylate itself has a number of characteristics in common with aspirin, it is possible that salicylate, i.e. sodium salicylate, could also alter dipyridamole in a manner similar to aspirin. Therefore, the next set of experiments were performed to study the effect of salicylate and dipyridamole rather than aspirin and dipyridamone on in vivo collagen-induced platelet aggregation.

DIPYRIDAMOLE-PLASMA
CONCENTRATION
ug/kg

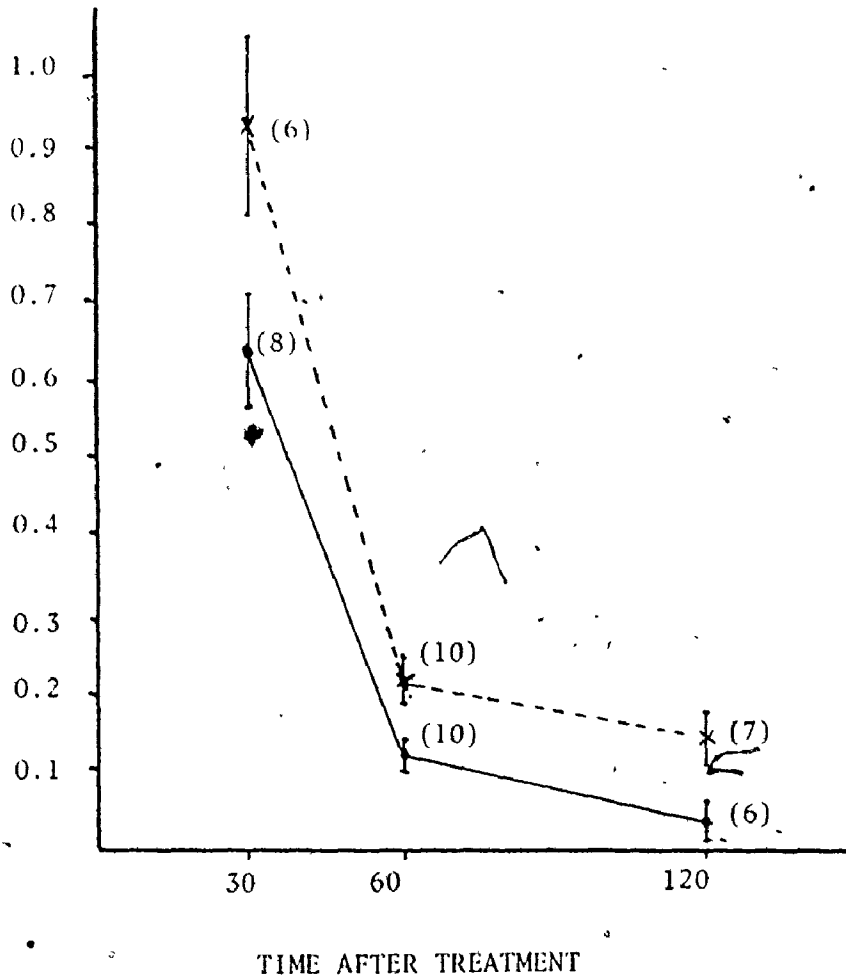


Figure 24. Dipyrđamole concentrations in plasma of animals treated with 3 mg/kg dipyrđamole alone (●, solid line) or given a combination of 30 mg/kg aspirin + 3 mg/kg dipyrđamole (x, broken line). Data is given in ug/ml \pm S.E.M. Number in () equals n.

The effect of a combination of dipyridamole and salicylate on collagen-induced platelet aggregation in vivo

Rabbits were given one of the following treatments: 3 mg/kg dipyridamole; 30 mg/kg sodium salicylate; 30 mg/kg sodium salicylate followed 5 minutes later by 3 mg/kg dipyridamole; and a fourth group which received no treatment served as controls. Three hours later, platelet aggregation to collagen was determined in the four groups. A time of three hours was selected because in previous experiments it had been shown that at this dose, the effect of dipyridamole was no longer evident. The results are shown in Figure 25. In the groups given dipyridamole or salicylate alone, platelet aggregation was the same as in the control animals as indicated by the hatched area marked 'control'. However, when rabbits were given salicylate before dipyridamole, platelet aggregation was reduced. The concentration of dipyridamole in the plasma at 3 hours of these rabbits receiving salicylate plus dipyridamole was significantly higher than the concentration seen in those rabbits given dipyridamole alone $p < 0.01$, (Figure 25). These results support the hypothesis that the salicylate moiety of aspirin increases the effect of dipyridamole on collagen-induced platelet aggregation by altering the pharmacokinetics of dipyridamole.

Part III. Summary

It has been shown that the effect of dipyridamole is directly

MAXIMUM
AGGREGATION
(% of Control)

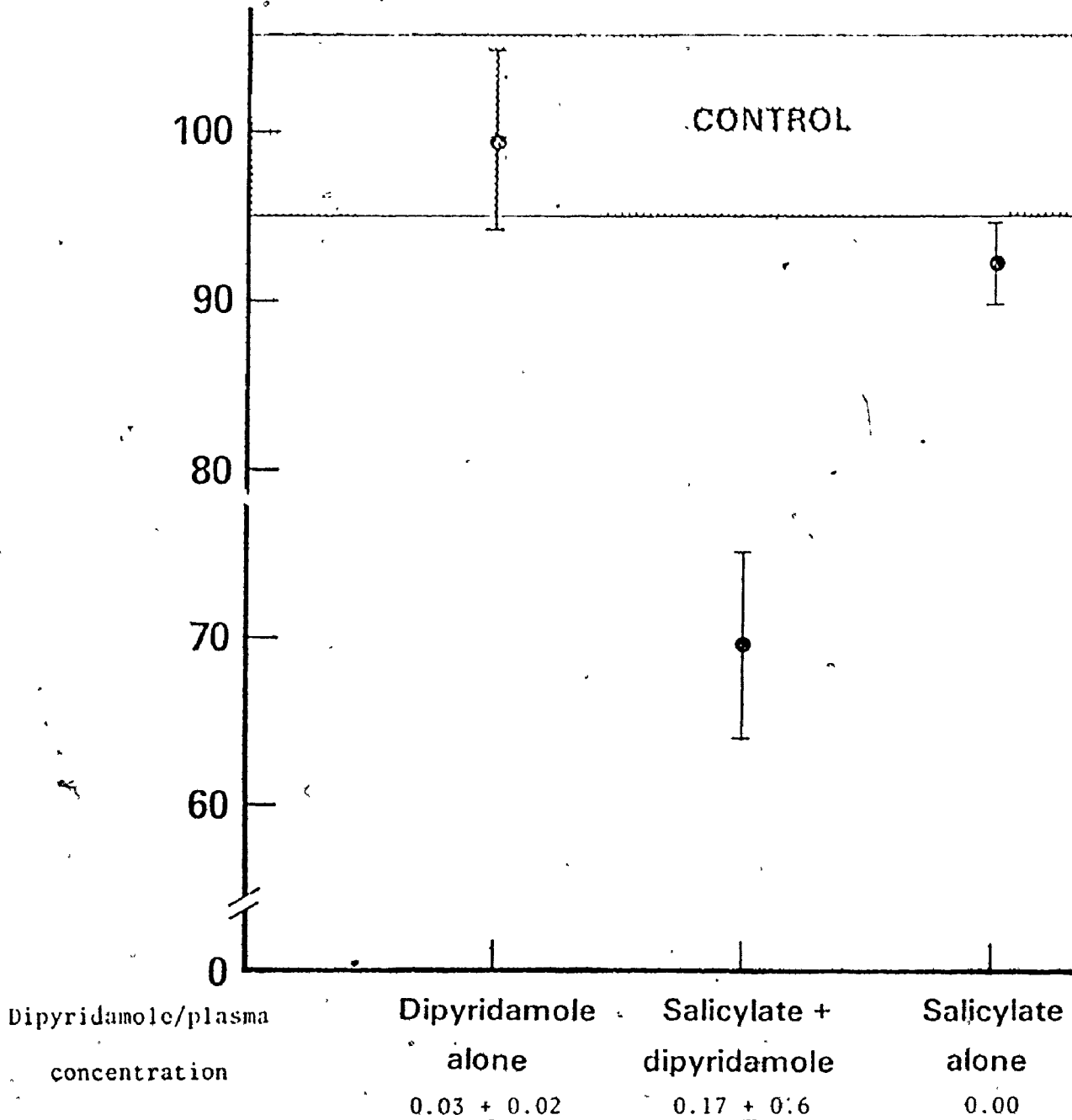


Figure 25

Maximum collagen-induced platelet aggregation in vivo \pm SEM in rabbits 3 hours after being given 3 mg/kg dipyridamole, 30 mg/kg sodium salicylate, or a combination of both (n=12)

related to the level of dipyridamole circulating within the plasma and that this effect is short-lived lasting for less than four hours in the rabbit following a single bolus of dipyridamole. The effect of dipyridamole on platelet aggregation in vivo is directly related to the concentration of dipyridamole within the plasma. This effect can be prolonged when dipyridamole is given in combination with salicylate which results in an increased dipyridamole level.

Part IV. Effects of Sulfinpyrazone

A number of investigators have shown that sulfinpyrazone inhibits the platelet release reaction (Packham et al, 1967; Zucker and Peterson, 1970). However, when given in therapeutic doses, sulfinpyrazone did not inhibit platelet function ex vivo (Packham and Mustard, 1974). Recently, however, Ali and MacDonald were able to demonstrate that sulfinpyrazone given in therapeutic doses inhibited the collagen-induced release of SHF from platelets (Ali and MacDonald, 1977). In these experiments, a weak collagen stimulus was used and they postulated that the failure of other investigators to demonstrate an ex vivo effect was related to the fact that they used a strong collagen stimulus.

The specific mechanism of action of sulfinpyrazone is not well understood. Ali and MacDonald (1977) demonstrated that the production of thromboxane B₂ by platelets exposed to arachidonic acid was decreased by sulfinpyrazone, indicating that the drug, like other non-steroidal anti-inflammatory drugs, inhibits prostaglandin synthesis. But it is uncertain whether the antithrombotic effect of sulfinpyrazone can be attributed solely to this mechanism.

It has been suggested that there is a considerable delay before the effect of sulfinpyrazone is evident (Mustard et al, 1967). Thus, Mustard and co-workers found that platelet survival was more prolonged in rabbits given sulfinpyrazone for more than

30 days as compared to the platelet survival in rabbits given sulfinpyrazone for less than 15 days. More recently, Kaegi et al (1974) reported that the effect of sulfinpyrazone was evident within a week of the drug's administration to man in therapeutic doses. However, this study did not exclude the possibility that sulfinpyrazone exerted its antithrombotic effect in less than a week.

The clearance of sulfinpyrazone from the circulation is fairly rapid with 85-95% of the ingested drug being excreted within 24 hours (Aldridge and Johnson, 1972; Dieterle et al, 1975). Recently, Rosenfeld et al have shown that sulfinpyrazone-plasma levels in patients treated with sulfinpyrazone for at least a year were not any higher than those levels observed in volunteers who had taken sulfinpyrazone for either one day or one week (Rosenfeld et al, 1977).

The objective of this part of the thesis was to investigate the mechanism of action of sulfinpyrazone by examining the relationship between the inhibitory effect of the drug on collagen-induced platelet aggregation and the plasma-drug levels and its effects on platelet prostaglandin synthesis.

The relationship between platelet aggregation and sulfinpyrazone plasma levels

The first set of experiments examined the relationship

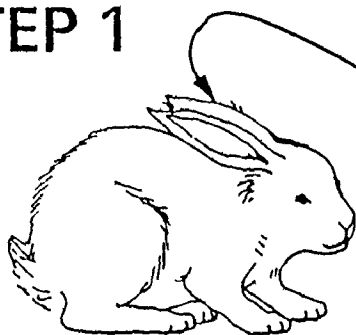
between the sulfinpyrazone-plasma concentrations and platelet aggregation in vivo. The experimental design is shown in Figure 26. Rabbits were injected with ^{51}Cr platelets and then given 100 mg/kg sulfinpyrazone intravenously at different times as indicated. In vivo platelet aggregation was performed 19 hours after ^{51}Cr -platelet injection. Immediately before the collagen infusion, a sample of blood was collected for SUL-PPP concentration determinations which were performed by gas chromatography.

Figure 27 shows the relation between the sulfinpyrazone-plasma levels and the in vivo collagen-induced platelet aggregation. Sulfinpyrazone levels were 400 $\mu\text{g}/\text{ml}$ one-half hour after injection of sulfinpyrazone. This fell rapidly so that at four hours it was less than 30 $\mu\text{g}/\text{ml}$ and at eight hours was not detectable. The effect of sulfinpyrazone on collagen-induced platelet aggregation showed a different pattern. At one-half hour platelet aggregation was reduced from 33% (control aggregation) to 19%. At 4 hours, aggregation showed less inhibition which at 8, 12 and 18 hours was progressively increased despite the absence of any sulfinpyrazone-plasma levels.

There appear to be two explanations for this effect. These are:

- 1) that the late effect on the platelet response was due to a non-specific effect of the initially high concentrations of sulfinpyrazone in the plasma at the beginning of the experiments, and
- 2) that a sulfinpyrazone metabolite is formed in vivo which is

STEP 1



HOMOLOGOUS
 ^{51}Cr -PLATELETS

STEP 2



100 mg/kg SUL
GIVEN EITHER
18, 12, 8, 4 OR $\frac{1}{2}$ HOUR
BEFORE STEP 3

STEP 3



COLLAGEN

^{51}Cr RADIOACTIVITY

SUL-PPP
CONCENTRATION

Figure 26 - Experimental design to study the relationship between sulfinpyrazone plasma concentration with platelet aggregation in vivo

MAXIMUM
AGGREGATION
(%)

SUL-PPP
CONCENTRATION
($\mu\text{g/ml}$)

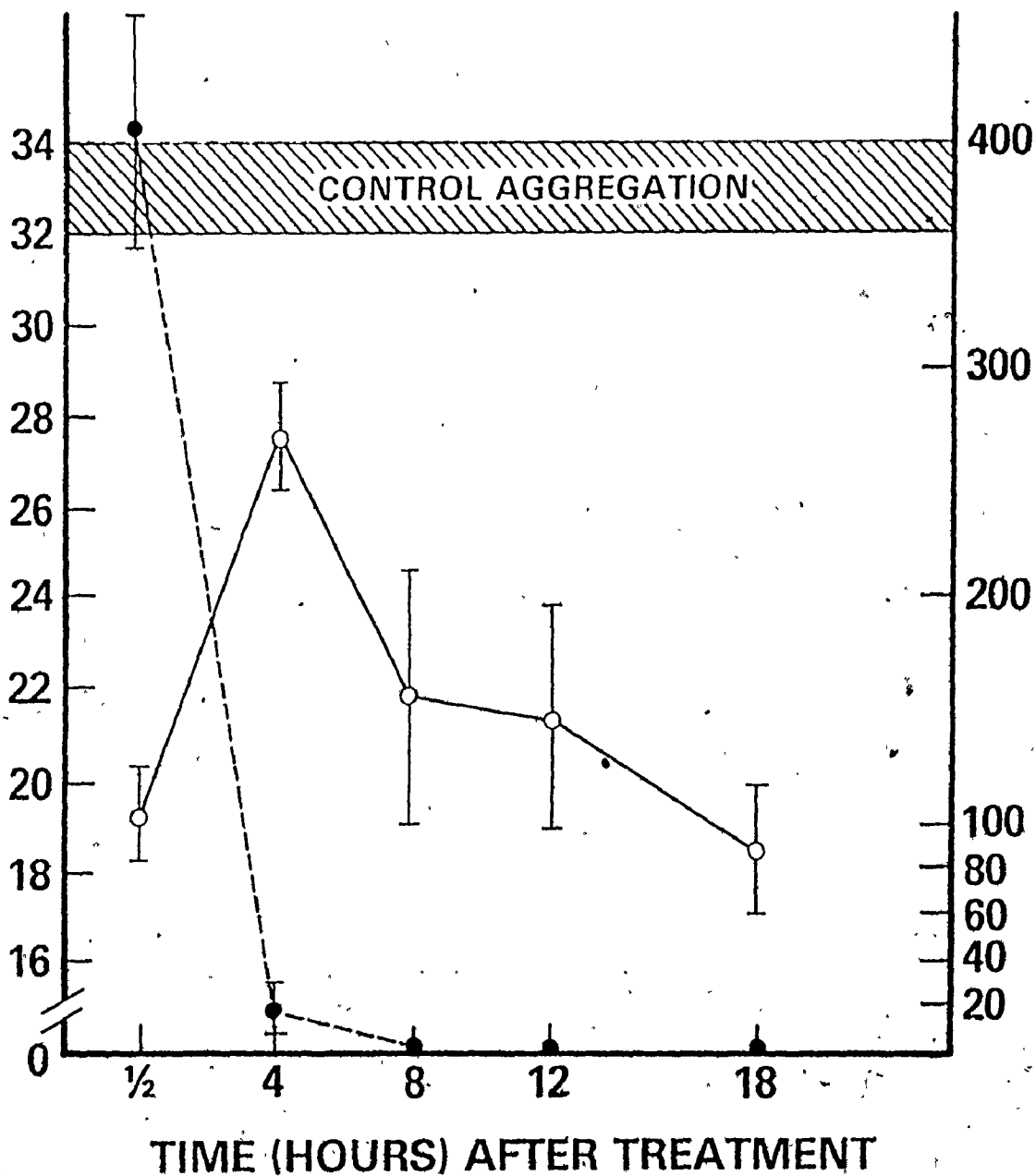


FIGURE 27

**MAXIMUM PLATELET AGGREGATION \pm SEM (o-o)
AND SUL-PPP CONCENTRATION \pm SEM (•-•) IN
RABBITS TREATED WITH 100 mg/kg SUL**

responsible for the continuing inhibition after 4 hours.

To test the possibility that the initially high sulfinpyrazone-plasma concentrations may have had a toxic effect on the platelet which became manifest after 4 hours, the following experiments were performed.

Effect of high in vivo concentrations of sulfinpyrazone on platelet aggregation in vivo

Rabbits were given 100 mg/kg of sulfinpyrazone either as an intravenous bolus injection or as an intravenous infusion given over 1 hour. Infusion was administered by a Harvard infusion pump (Model 942, Harvard Apparatus Company, Inc., Mass., USA). Immediately following the infusion, samples were collected for SUL-PPP concentration determinations. Eighteen hours later, maximum in vivo platelet aggregation was determined in all rabbits. In the bolus-treated rabbits in which the initial sulfinpyrazone exceeded 400 µg/ml, maximum platelet aggregation was 22.8%. This was not significantly different from the maximum platelet aggregation of 19.8% seen in those rabbits given sulfinpyrazone as an infusion and in which the initial sulfinpyrazone concentrations did not exceed 250 µg/ml (Table 16). Furthermore, when ADP-induced aggregations were performed on aliquots of those samples tests ex vivo, the aggregations were normal.

Furthermore, the non-specific effect of sulfinpyrazone in

Table 16 : Platelet aggregation and maximum SUL-PPP concentration in rabbits given 100 mg/kg SUL:

- i) as a bolus treatment, or
- ii) given as an infusion over 60 minutes.

	BOLUS	INFUSION
MAXIMUM AGGREGATION (%)	22.8	19.8
MAXIMUM SUL-PPP CONCENTRATION	>400 µg/ml 1/2 hr post bolus	<250 µg/ml immediately post infusion

vitro only begins to occur at concentrations greater than 2 mM. The results of these experiments did not, therefore, support the hypothesis that the prolonged effect was due to a non-specific effect of the initially high concentration of the drug at the beginning of the experiment. Therefore, the next step was to investigate the possibility that a metabolite was produced in vivo which exerted its effect on platelet function for up to 18 hours after sulfinpyrazone injection.

Effect of sulfinpyrazone-treated plasma on platelet malondialdehyde production

Aliquots of platelets obtained from normal rabbits were incubated for 60 minutes in plasmas obtained from, i) normal rabbits, ii) rabbits treated one hour beforehand with 10 mg/kg of sulfinpyrazone and which contained detectable amounts of sulfinpyrazone, or iii) in plasma obtained from rabbits treated 18 hours beforehand with 100 mg/kg of sulfinpyrazone and which contained no detectable sulfinpyrazone. Following incubation, platelet malondialdehyde production was determined by the method of Stuart and associates (1975). Platelets obtained from the donor rabbits were also incubated in the plasmas obtained from i), ii) and iii) and malondialdehyde production was determined.

When normal platelets were incubated in homologous plasma containing no sulfinpyrazone, malondialdehyde production was

0.53 nM/10⁹ platelets. When these platelets were incubated in the PPP's obtained from the sulfinpyrazone-treated donor rabbits, there was a significant reduction in malondialdehyde to 0.46 nM in the one hour PPP containing 4.4 µg/ml sulfinpyrazone and a further reduction to 0.36 nM in the 18 hour PPP containing no detectable sulfinpyrazone, $p < 0.001$ (Table 17).

Malondialdehyde production in platelets harvested from donor rabbits treated with 10 mg/kg sulfinpyrazone, and incubated in normal PPP was 0.44 nM (Table 18). When these platelets were incubated in the one hour PPP, malondialdehyde production was 0.39 nM and when the platelets were incubated in the 18 hour PPP, malondialdehyde production was 0.36 nM. These differences were not statistically significant. When the 18 hour platelets were incubated in the three different PPP's, similar results were observed (Table 19).

These series of experiments are summarized on Figure 28 which shows that in all cases, maximum reduction in malondialdehyde occurred when the platelets were incubated in the 18 hour PPP. A comparison (Statton-Newman-Kinzig analysis) of the amount of malondialdehyde produced by platelets harvested from normal PPP, one hour sulfinpyrazone PPP and 18 hour PPP, and incubated in normal PPP, showed a significant reduction in malondialdehyde production in both treated groups ($p < 0.01$) and a significant difference between the two treated groups ($p < 0.05$).

Table 17: MDA production (nM/10⁹ platelets) of normal rabbit platelets incubated in plasmas obtained from normal rabbits or from rabbits treated one hour beforehand with 10 mg/kg SUL or 18 hours beforehand with 100 mg/kg SUL. An analysis of variance demonstrated a significant difference between plasma conditions.

TYPE OF PLASMA IN WHICH PLATELETS

WERE INCUBATED

Normal Homologous Plasma	10 mg/kg - 1 Hr Plasma with 5 µg/ml SUL	100 mg/kg - 18 Hr Plasma <u>no SUL</u>
--------------------------------	---	--

0.53

0.46

0.37

ANALYSIS OF VARIANCE

Source of Variation	F Values	P Values
Among Plasma Treatment	35.9	<0.001

Table 18: MDA production (nM/10⁹ platelets) by platelets obtained from rabbits treated with 10 mg/kg SUL one hour beforehand and incubated for 60 minutes in the three types of plasma.

TYPE OF PLASMA IN WHICH PLATELETS
WERE INCUBATED

Normal Homologous Plasma	10 mg/kg - 1 Hour Plasma with 5 µg/ml SUL	100 mg/kg - 18 Hours Plasma <u>no SUL</u>
0.44	0.39	0.36

ANALYSIS OF VARIANCE

Source of Variation	F Value	P Value
Among Plasma Treatments	3.53	n.s.

Table 19: MDA production (nM/10⁹ platelets) of platelets which were obtained from rabbits treated with 100 mg/kg SUL 18 hours beforehand and then incubated in the three types of plasmas.

TYPE OF PLASMA IN WHICH PLATELETS WERE INCUBATED		
Normal Homologous Plasma	10 mg/kg - 1 Hour Plasma with 5 µg/ml SUL	100 mg/kg - 18 Hours Plasma <u>no SUL</u>
0.40	0.37	0.36

Source of Variation	F Value	P Value
Among Plasma Treatment	4.02	n.s.

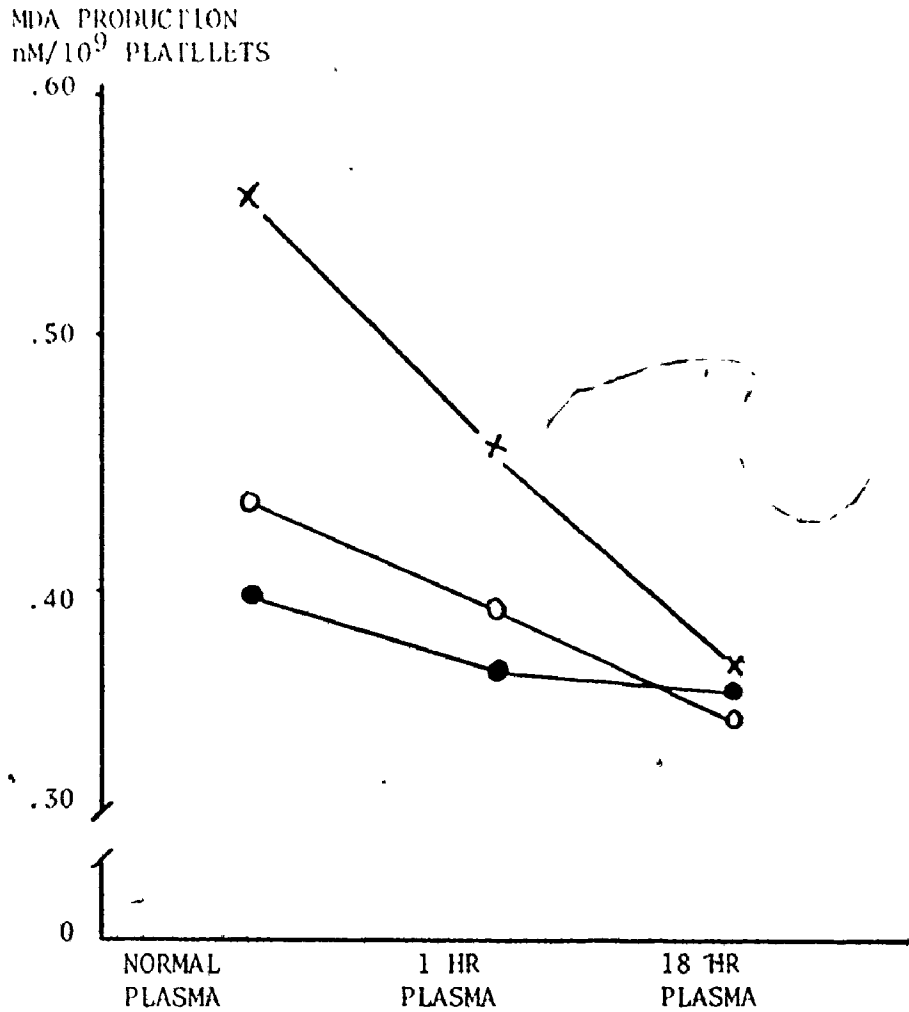


Figure 28. Mean MDA production of platelets obtained from normal rabbits or rabbits treated one hour beforehand with sulfipyrazone (10 mg/kg) or rabbits treated 18 hours beforehand with 100 mg/kg sulfipyrazone and incubated for 60' in plasmas obtained from the same rabbits.

(●-●) 18 Hour Treatment

(O-O) 1 Hour Treatment

(X-X) Control

Further experiments were performed to determine whether the prolonged effect on in vivo collagen-induced platelet aggregation was also seen with lower doses of sulfinpyrazone.

Dose response curve of prolonged effect of sulfinpyrazone

Rabbits were given 10, 30 or 65 mg/kg sulfinpyrazone. Maximum platelet aggregation was determined 1, 4, 8, 12, or 18 hours after treatment. The lowest dose of sulfinpyrazone had little or no effect on aggregation as is shown on Figure 29. At one hour, platelet aggregation was inhibited to 24% after treatment with 35 mg/kg sulfinpyrazone and to 18% after treatment with 65 mg/kg sulfinpyrazone. The inhibitory effects of the 35 mg/kg treatment lasted only four hours, whereas the inhibitory effect of the 65 mg/kg treatment lasted at least 12 hours. No sulfinpyrazone was detectable after four hours in any of the treatment groups.

This data further supports the hypothesis that a sulfinpyrazone metabolite may exist in vivo which has an inhibitory effect on platelet aggregation.

Persistence of sulfinpyrazone effects after washing

Cazenave (1977) has demonstrated that when rabbit platelet suspensions are incubated with sulfinpyrazone, there is a reduced adherence to collagen-coated surfaces. However, when platelets are harvested from rabbits treated two hours beforehand with

MAXIMUM
AGGREGATION (%)

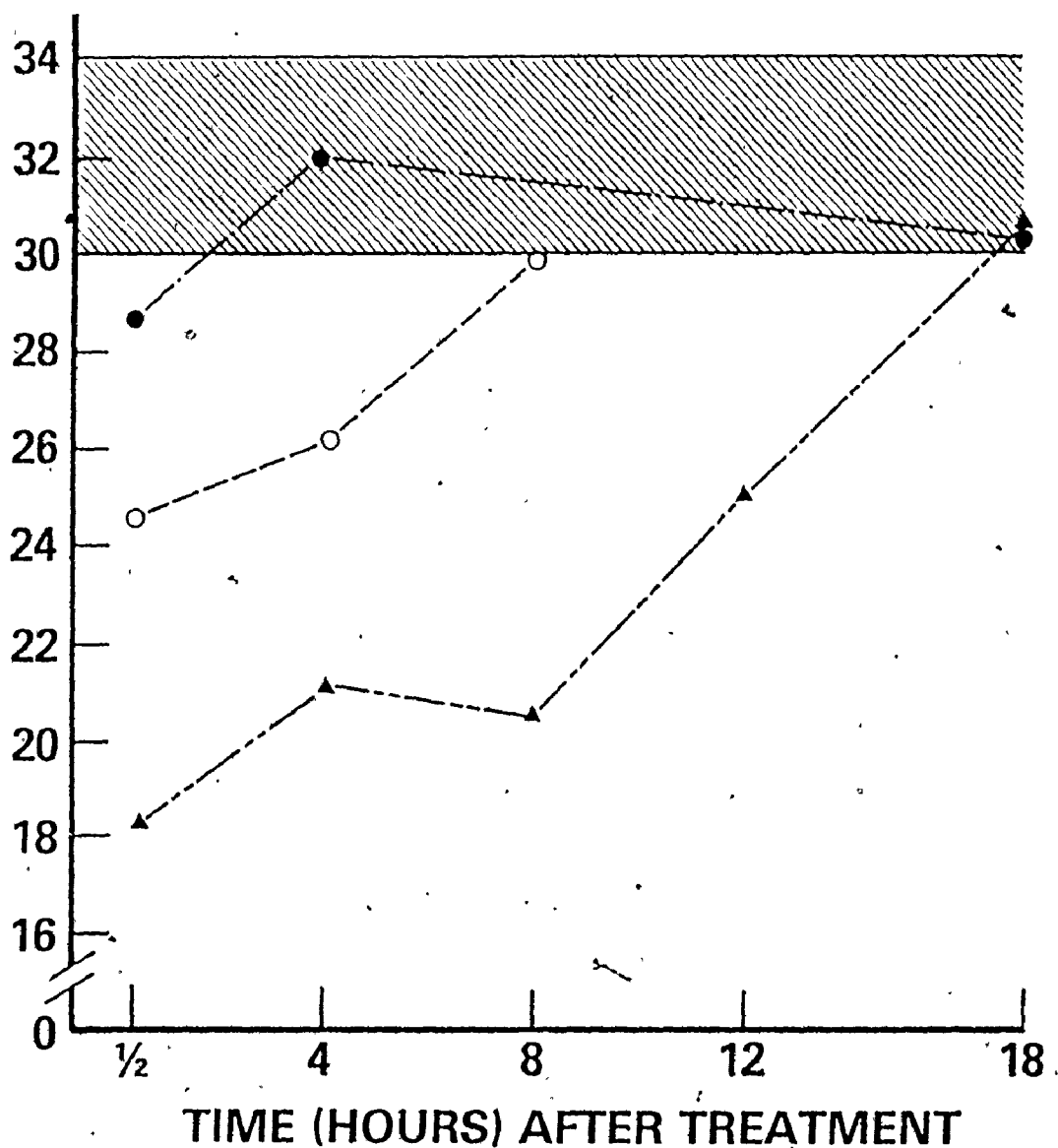


FIGURE 29

PLATELET AGGREGATION, \pm SEM IN RABBITS GIVEN

10 mg/kg SUL (●---●),

35 mg/kg SUL (○---○) OR

65 mg/kg SUL (▲---▲).

**SUL—PPP CONCENTRATIONS IN ALL RABBITS WERE
LESS THAN 0.5 μ g/ml AT 4 HOURS**

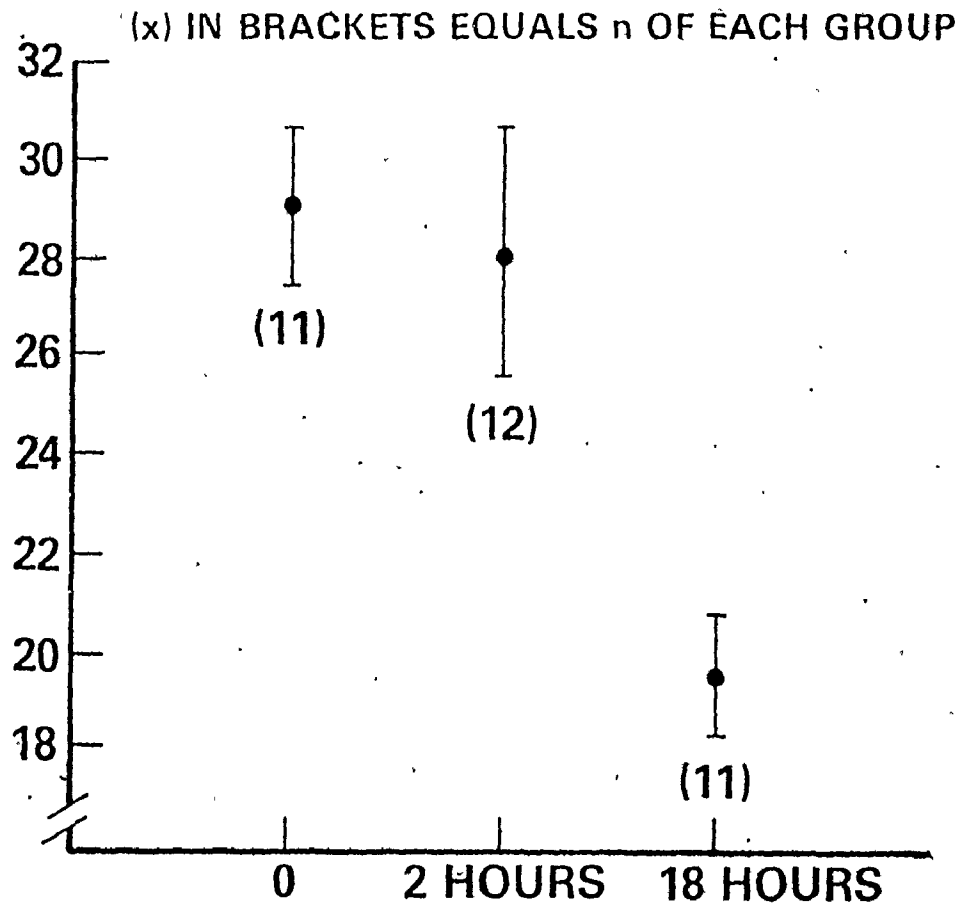
sulfinpyrazone and washed three times, there is no reduction in platelet adherence. These experiments suggest that the effect of sulfinpyrazone on the platelet is reversible.

Experiments were performed to determine whether the effect of the hypothetical metabolite was also reversible. Platelets were collected from three groups of rabbits, washed three times and labelled with ^{51}Cr . One group of rabbits had been treated 18 hours beforehand with 100 mg/kg sulfinpyrazone ($n = 11$), the second group of animals ($n = 12$) had been treated two hours beforehand with 100 mg/kg sulfinpyrazone and the third group of rabbits ($n = 11$) received no sulfinpyrazone. The platelets from these three sets of donors were then injected into three groups of normal recipient rabbits. One hour later, platelet aggregation in vivo was determined. Aggregation of platelets obtained from rabbits which had been treated two hours beforehand was 28% and not significantly different from the control rabbit platelet aggregation of 29%. However, aggregation of platelets obtained from rabbits treated 18 hours beforehand was less than 20% (Figure 30). These results indicate that whereas the sulfinpyrazone effect could be washed off the platelet, the effect of the "metabolite" could not.

Part IV. Summary

Sulfinpyrazone could not be detected in the plasma eight

MAXIMUM AGGREGATION



TIME PLATELETS EXPOSED TO SUL IN VIVO
BEFORE WASHING AND ^{51}Cr -LABELLING

FIGURE 30

COLLAGEN—INDUCED PLATELET AGGREGATION IN VIVO OF PLATELETS OBTAINED FROM

- i) NORMAL RABBITS OR FROM RABBITS GIVEN 100 mg/kg SUL
- ii) 2 HOURS BEFOREHAND OR
- iii) 18 HOURS BEFOREHAND

hours after being injected intravenously. However, in vivo collagen-induced platelet aggregation was inhibited for at least 18 hours. This prolonged inhibition of platelet aggregation appeared to be due to a sulfinpyrazone metabolite. Both the sulfinpyrazone and the metabolite produced an inhibition of platelet prostaglandin synthesis. The effect of sulfinpyrazone appeared to be reversible and could be washed off the platelet surface whereas the effect of its metabolite on the platelet appeared to be irreversible.

SUMMARY

An intra-arterial infusion of collagen into rabbits resulted in a temporary disappearance of a percentage of circulating platelets. This change in platelet population could be detected by both visual and radioactivity counting. The platelet disappearance was due to a direct interaction of the platelet with the collagen and appeared to be independent of the coagulation system. This test was used to study some of the inconsistencies obtained when assessing platelet function suppressing drugs in vivo and ex vivo. It was demonstrated that aspirin and sulfinpyrazone inhibited collagen-induced platelet aggregation in vivo and ex vivo, whereas dipyridamole only inhibited platelet aggregation in vivo. The lack of any dipyridamole inhibitory effect ex vivo was attributed to the anticoagulant used. Thus, when the ex vivo effect of dipyridamole was tested in citrated plasma, no inhibitory effect was shown, but when the dipyridamole effect was tested in heparinized plasma, dipyridamole was shown to effectively inhibit platelet aggregation.

The in vivo collagen/platelet test was also used to study the pharmacodynamics and mechanism of action of some of these drugs, thus allowing a better understanding of how these drugs work in a biological system similar to that in which they would be eventually used.

The prolonged aspirin effect seen in bleeding studies in vivo

and aggregation studies ex vivo does not appear to be related to an acetylation of the megakaryocytes nor an effect of acetylated plasma proteins on newly circulating platelets. Aspirin was shown to inhibit platelet malondialdehyde synthesis and this effect was irreversible. In addition, aspirin was shown to inhibit a second mechanism of the platelet which was reflected by the decreased response to the collagen stimulus independent of the prostaglandin effect. This effect was overcome with time. Furthermore, aspirin was shown to shorten the jugular vein bleeding time. This effect was attributed to an acetylation effect of aspirin on cyclo-oxygenase which resulted in a decreased production of PGI₂.

The inhibition of platelet function by dipyridamole was associated with the concentration of dipyridamole circulating within the plasma. The concentration of dipyridamole were increased in the presence of salicylate. Thus, it was suggested that the threshold level of dipyridamole needed to inhibit platelet function in vivo could be achieved with lower doses of dipyridamole providing salicylate was present in the system.

Inhibition of platelet function by sulfinpyrazone was shown to be related to an inhibition of platelet malondialdehyde synthesis. This inhibitory effect of sulfinpyrazone was reversible and disappeared when the platelets were washed in Tyrodes solution. Platelet function was also inhibited at a time when the sulfinpyrazone had been cleared from the plasma. This effect was attributed to an undescribed

metabolite of sulfinpyrazone which was formed in vivo and had a potent inhibitory effect on the platelet also associated with an inhibition of prostaglandin synthesis. Whether the site of action of this metabolite was also at the cyclo-oxygenase level or at another position in the pathway, was not ascertained. It is possible that this agent acts elsewhere since the effect of the metabolite was irreversible and could not be washed off the platelet.

DISCUSSION

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CHAPTER IVDISCUSSION

The three platelet function inhibiting drugs which have shown most promise in the management of thrombotic disease are aspirin, sulfinpyrazone and dipyridamole. The effects of these drugs on platelet function have been assessed in vivo, ex vivo and in vitro and the results of these studies have shown a number of inconsistencies. For example, both sulfinpyrazone and dipyridamole have been shown to prevent thrombosis in certain clinical circumstances and to normalize reduced platelet survival (an in vivo test of platelet function) but when given in therapeutic doses, are weak inhibitors of platelet function tested ex vivo. On the other hand, aspirin has no effect on normalizing reduced platelet survival in various clinical thromboembolic states but is a potent inhibitor of platelet function when tested ex vivo. One explanation for these differences is that the inhibitory effects of these drugs on platelets varies with the nature of the stimulus to which the platelet is exposed in vivo and ex vivo. Alternatively, the preparatory procedures necessary to prepare the platelets for study ex vivo may alter the effects of these drugs on platelet function. Furthermore, the use of in vitro and ex vivo platelet function tests, while simple and economical, have a number of limitations which can be overcome by the use of in vivo platelet function tests. For example, with the latter test it is possible

D

to analyze the pharmacokinetics and metabolism of many drugs and study the relationship between drug/plasma concentrations and drug effects. Therefore, this study was undertaken to develop an in vivo test system which could be used 1) to examine reasons for the discrepancy between the drug effects when measured in different test systems, and 2) to study the relationship between the effects of these drugs on platelet function and their circulating plasma levels.

DEVELOPMENT OF THE IN VIVO COLLAGEN-INDUCED PLATELET AGGREGATION

The collagen-induced platelet aggregation test

The infusion of collagen can be used to study the platelet/collagen interaction in vivo and the effects of platelet suppressive drugs on this interaction. To do this effectively, a number of points must be considered. First, it is important not to infuse concentrations of collagen which will impair the pulmonary circulation since this could lead to death or severe stress of the experimental animal (Ni.shizawa et al, 1972). We avoided this problem by infusing the collagen intraarterially. This resulted in the formation of platelet/collagen aggregates which were contained within the arterial and capillary beds. This was confirmed by both direct examination of arterial and venous blood and histological examination of the microcirculation.

Second, the platelet/collagen response could be measured either microscopically or isotopically. We demonstrated that homologous ⁵¹Cr-labelled platelets respond to the collagen infusion in a manner similar to autologous non-labelled platelets. This allowed us to assess the effects of aspirin, sulfinpyrazone and dipyridamole on the platelet/collagen interaction using the isotopic method which avoided the technical difficulties associated with the determination of in vivo platelet aggregation, microscopically.

It was also important to consider the involvement of other factors associated with the activation of the coagulation system which may influence the platelet response. Walsh (1974) has

demonstrated that collagen may activate Factor XII. Thus, it would seem reasonable to expect that the collagen infusion may activate the coagulation system. Therefore, the interaction which we would be studying would include not only a platelet/collagen interaction but also a platelet/thrombin interaction. This possibility appears unlikely since in our experiments the platelet response to collagen in heparinized rabbits was the same as the platelet response in non-heparinized rabbits. The explanation for the lack of any significant thrombin generation may be related to the presence of natural inhibitors of coagulation in the rabbit such as antithrombin III.

However, further studies would have to be done to determine whether or not this is an important control mechanism.

Conclusion

The assessment of platelet function suppressing drugs in vivo can be studied by measuring ^{51}Cr -platelet recovery following an intra-arterial collagen infusion into rabbits. This test system has the advantages that:

- 1) the results of the in vivo test system can be compared directly to the results of an ex vivo test system using the same stimulus which allows a study of the effects of other factors on the platelet/stimulus interaction, and
- 2) ^{51}Cr -platelets behave similarly to the total platelet population and therefore the measurement of their recovery by isotopic methods can be

used to quantify the collagen-induced platelet aggregation response of all of the platelets, determined both accurately and objectively.

Although this test system requires certain technical skills in order to prepare ^{51}Cr -labelled platelet suspensions for re-injection, this limitation is small when compared to its many advantages.

EFFECT OF ASPIRIN, SULFINPYRAZONE AND DIPYRIDAMOLE ON
COLLAGEN-INDUCED PLATELET AGGREGATION IN VIVO AND EX VIVO

1. Differences of effects in vivo and ex vivo

When the effects of aspirin, sulfinpyrazone and dipyridamole were tested on collagen-induced platelet aggregation in vivo and ex vivo, the results were different in vivo and ex vivo. Aspirin and sulfinpyrazone were effective inhibitors of platelet aggregation in both test systems, although considerably more sulfinpyrazone was needed to achieve the maximum inhibitory effect ex vivo. Dipyridamole did not have any effect ex vivo whereas it did inhibit platelet function in vivo. Since the stimulus used in both test systems had been kept consistent, it seemed possible that the inconsistent findings with dipyridamole could be attributed to the procedures associated with the preparation of the samples for ex vivo testing. Therefore, a number of experiments were performed to examine the effects of some of these preparative procedures on the effects of dipyridamole on platelet aggregation ex vivo.

2. Stability of dipyridamole effect

It was possible that the effect of dipyridamole could be lost during the one to two hour interval required for blood processing for ex vivo testing. This possible explanation for the lack of a dipyridamole effect ex vivo was excluded since it was shown in vitro that the inhibitory effect of dipyridamole remained essentially unchanged when the platelets were stored in citrated plasma incubated with dipyridamole.

3. Effect of anticoagulants on the drug effect

Since calcium is a necessary component for both platelet aggregation and deaggregation (Mustard et al, 1975; Detwiler et al, 1977) and plays an important role in the action of dipyridamole on the platelets (Vigdahl et al, 1971), it seemed possible that agents which chelate calcium may also alter the drug effects. The alteration of a drug effect due to the reduction of calcium has also been shown for aspirin. Thus, Gordon and McIntyre (1974) showed that the inhibitory potency of aspirin was much greater in citrated plasma than in heparinized plasma. Therefore, two possible explanations for the inconsistent results found between the effects of these drugs when tested ex vivo and in vivo are that either the chelation of calcium in the citrated samples resulted in an altered drug effect or that citrate has an effect distinct from its chelation effect which may interfere with the drug effect.

The data in this study supports the second explanation. Dipyridamole, although having little or no effect on collagen-induced platelet aggregation in citrated samples ex vivo, was an effective platelet inhibitor when tested in heparinized samples. This effect could have been due to either the presence of citrate or the reduced level of calcium in the plasma. The results of the experiments in which dipyridamole was incubated in platelet suspensions containing various concentrations of citrate, heparin or divalent cations, was consistent with the conclusion that citrate interferes with the

interaction of dipyridamole with the platelet.

4. Limitation of ex vivo testing


The demonstration that citrate significantly alters the effect of dipyridamole on the platelet-collagen interaction emphasizes a limitation of using ex vivo and in vitro test systems. It is possible that other steps in the procedure necessary for preparing platelets for ex vivo testing may also augment or inhibit other drug effects on the platelet. Since citrate has been shown to inhibit the effects of dipyridamole as well as enhance the effects of aspirin (Gordon and MacIntyre, 1974), it is suggested that screening of platelet function suppressing drugs is not always appropriate in samples collected into this anticoagulant. Although heparin did not influence the test system used, it is possible that it could interfere with the measured effectiveness of other platelet suppressive drugs ex vivo. Other investigators have shown that heparin in higher concentrations can both enhance and inhibit platelet aggregation although in this study we have shown that two units/ml heparin has little effect. Possibly, the most suitable test medium with which to test these drugs ex vivo is the platelet suspension in which no anticoagulants are present. Even this system is limited since the preparation of a platelet suspension requires the washing of platelets which may result in the removal of loosely bound drugs from the platelet.

THE ANTI-THROMBOTIC EFFECT OF ASPIRIN

The prolonged effect of aspirin on platelet function

Aspirin results in the prolongation of the bleeding time and has been reported to be associated with increased bleeding in a variety of clinical situations (Gast, 1964; Mielke et al, 1969; Neivert, 1945; Singer, 1945; Wising, 1952). This effect on hemostasis has been attributed to the effect of aspirin on the inhibition of platelet function (Evans et al, 1968; O'Brien, 1968; Zucker and Peterson, 1970). Aspirin has been shown to selectively inhibit prostaglandin production (Smith and Willis, 1971) and, in particular, to acetylate cyclo-oxygenase thus blocking prostaglandin synthesis (Roth et al, 1975; Jafari et al, 1976). This aspirin effect lasts the lifetime of the platelet (O'Brien, 1968; Zucker and Peterson, 1970). However, in spite of this irreversible effect of aspirin on the platelet, only a few studies have demonstrated any beneficial effect of aspirin on thrombosis in man (Fields et al, 1977; Elwood et al, 1974; Canadian Stroke Study, unpublished). It would appear that the optimal dose of aspirin for clinical effectiveness is unknown.

It has been suggested that only small doses of aspirin are required to produce an optimal anti-thrombotic effect. This is based upon studies demonstrating that low concentrations of aspirin maximally inhibit prostaglandin synthesis (Roth and Majerus, 1975; Roth et al, 1975). It is possible, however, that in order to maintain an aspirin effect, particularly on those platelets newly released into the circulation, that larger doses of aspirin may be required to inhibit



prostaglandin synthesis in megakaryocytes. If this were the case, there would be an advantage to using higher doses of aspirin to inhibit platelet function of those platelets associated with the megakaryocyte.

O'Brien (1968) was the first to suggest that the megakaryocyte may be affected by doses of aspirin higher than that needed to inhibit the function of circulating platelets. More recently, Jafari and co-workers (1977) have reported some data which they interpret as indicating that aspirin does inhibit prostaglandin synthesis in both the platelet and megakaryocyte. They showed that platelet prostaglandin E production measured after a single oral ingestion of aspirin, increased at a rate slower than the rate of influx of newly released platelets into the circulation. They concluded that prostaglandin synthesis by megakaryocytes was compromised and hence, prostaglandin production of newly released platelets was reduced. A possible argument against their observations is that new platelets may be sequestered in the spleen for the first two days as has been suggested by Shulman et al (1968). This phenomenon would explain the delay in correction of any aspirin effect.

When we measured the production of MDA by platelets released from megakaryocytes following aspirin treatment, we demonstrated that these platelets produced normal amounts of MDA. There are a number of possible explanations for this observation. First, high doses of aspirin may have no effect on the megakaryocytes. A second, more

likely possibility, is that aspirin in high concentrations acetylates the cyclo-oxygenase of the megakaryocyte, but that this effect is rapidly reversible because the megakaryocyte is able to resynthesize new enzymes. This second explanation, however, is not entirely consistent with the observations of O'Brien and Jafari. In order to produce a prolonged effect of aspirin on the megakaryocyte, which would be reflected by impaired function of young platelets, it would seem necessary to maintain some specific aspirin concentration. However, these investigators have observed a progressive prolongation of the aspirin effect with increasing single doses of aspirin.

A third possible explanation for the prolonged effect on the platelet is that aspirin has at least two effects on the platelet, low doses of aspirin blocking prostaglandin production and higher doses exerting additional effects. This explanation is supported by our observations that higher doses of aspirin are required to maximally inhibit collagen-induced platelet aggregation than those doses required to inhibit platelet MDA production. Other investigators have also demonstrated that aspirin may alter platelet function in at least two ways. Loew and Vinazzer (1976) found that 4 to 10 times the dose needed to inhibit platelet aggregation in vitro was required to inhibit platelet release of factors 3 and 4. Whether these dose-related effects seen by these investigators and the dose-related effect seen by us represents similar effects have not been determined. However, both sets of data support the conclusion that platelet

prostaglandin production is inhibited by low doses of aspirin and that other platelet functions are inhibited by higher doses of aspirin.

Another remote possibility is that acetylated plasma proteins coming in contact with newly released platelets alter their function. This possibility seems unlikely since aspirin-treated platelet poor plasma does not alter collagen-induced platelet aggregation or platelet MDA production in normal rabbits.

These experiments raise the possibility that aspirin in doses that block prostaglandin synthesis may not necessarily produce optimal antithrombotic effects and that it may be necessary to inhibit the platelet function, which is independent of prostaglandin synthesis, in order to achieve such an antithrombotic effect.

Aspirin effect on the vessel wall

Platelets do not adhere to intact vascular endothelium. The mechanism for this protection has not been well understood but a number of hypotheses have been offered over the years. Recently, a number of investigators have demonstrated that the vessel wall itself produces a number of prostaglandins (Blumberg et al, 1977; Jones, 1977; Moncada et al, 1977; Needleman et al, 1976). Some of these cause vessel wall contraction and enhance platelet aggregation while others cause vessel wall relaxation and inhibit platelet aggregation (Gerrard et al, 1977; Hadhazy et al, 1976; Moncada, 1977). In

particular, it has been shown that prostacyclin or PGI₂, which is produced primarily by the endothelium (Herman et al, 1977) is a potent inhibitor of platelet aggregation (Moncada et al, 1976, 1977; Needleman et al, 1976a, 1977). When the vessel wall is damaged, platelets adhere to the underlying surface. It is hypothesized that the extent of this platelet adherence to the damaged vessel is controlled by the relative concentration of PGI₂ produced by the damaged endothelial cells and, possibly by the underlying cells (Herman et al, 1977; Marcus, 1977). Since aspirin is a potent inhibitor of both platelet prostaglandin synthesis and prostaglandin synthesis of other tissues (Flower, 1974), it is conceivable, therefore, that any antithrombotic effect of aspirin achieved with the inhibition of platelet prostaglandin production may be abolished if the synthesis of PGI₂ is inhibited. Preliminary studies by Cazenave (unpublished) has shown in vitro that more platelets adhered to damaged aorta which was pretreated with aspirin than did platelets to non aspirin-treated damaged aorta. Thus, it is possible that whereas inhibition of prostaglandin synthesis in the platelet results in an antithrombotic effect, inhibition of prostaglandin synthesis in the vessel wall may result in an increased thrombotic effect.

In all clinical studies in which aspirin has had any anti-thrombotic effect (Canadian Stroke Study, unpublished; Field et al, 1977; Harris et al, 1974; Zechert, 1973), the dose of aspirin was

in excess of the amount required to inhibit platelet prostaglandin synthesis and may have been sufficient to mediate a second effect on the platelet. It is unlikely, however, that these daily doses (1200 - 2500 mg) were sufficient to inhibit PGI₂ formation by the vessel wall since it has been shown that 20-400 times the amount of aspirin required to acetylate platelet cyclo-oxygenase and therefore maximally inhibit platelet prostaglandin, is required to inhibit prostaglandin synthesis in cells of the vessel wall (Baenziger et al, 1977; Burch et al, 1977; Patrono et al, 1976; Weksler et al, 1977). This would suggest that the dose of aspirin required to inhibit PGI₂ in vivo would be in great excess to that dose of aspirin used as an antithrombotic agent clinically and, therefore, that the possibility that the drug could induce thrombosis at these doses would be unlikely.

In this thesis, an in vivo animal model was used to investigate the effects of aspirin on the vessel wall and the duration of this effect. The experimental model used was the jugular bleeding time performed in thrombocytopenic animals. The results demonstrated that aspirin, in very high concentrations, given systemically, instilled locally or applied topically, results in a decrease in the bleeding time in these thrombocytopenic animals. This effect was independent of the influence of aspirin on platelets and was not due to a pH or osmotic effect on the vessel wall. It was hypothesized that the aspirin effect on the vessel wall was due to an acetylation of

cyclo-oxygenase in the cells of the vessel wall and subsequent inhibition of PGI_2 synthesis. This hypothesis is supported by the observations that indomethacin treatment also produces a shortening of bleeding time in thrombocytopenic animals and that the flow rate of fluid perfused through the bleeding time puncture wound in the jugular vein is reduced after high dose aspirin treatment (Blajchman et al, 1977), indicating that the size of the hole is smaller in these animals. This suggests that PGI_2 causes a relaxation of the vessel from its state of contraction which is initiated by the injury stimulus and that aspirin abolishes the effect of PGI_2 .

These data indicate that considerably higher doses of aspirin are required to inhibit prostaglandin synthesis of the vessel wall than those required to inhibit prostaglandin synthesis in platelets and that the aspirin effect on the vessel wall is reversible and of a short duration. Thus, the data obtained here supports other findings that, given in the doses used clinically, aspirin is unlikely to enhance thrombosis since it is unlikely to influence prostacyclin synthesis by the vessel wall and, even if it did, that its effect would be relatively short-lived.



DIPYRIDAMOLE EFFECT ON PLATELETS

Effect of dipyridamole on the platelet ex vivo

Dipyridamole has little effect on platelet function ex vivo after the administration of "therapeutic" doses of dipyridamole (Harker and Slichter, 1970; Warlow et al, 1974) although in high concentrations dipyridamole inhibits platelet function in vitro (Emmons et al, 1965; Mills and Smith, 1971; Philp et al, 1973) and, when given in high doses, inhibits experimentally-induced platelet thrombosis in animals (Cucuianui et al, 1971; Mayer and Hammond, 1973). Two studies performed in man have demonstrated that dipyridamole is a potentially effective antithrombotic agent in man. Thus, Sullivan and co-workers found that in 100 patients with cardiac valve replacements given 400 mg dipyridamole daily, in combination with warfarin, thromboembolic events were reduced in the 18 months following treatment as compared to patients given warfarin alone (Sullivan et al, 1969). In addition, Harker and Slichter (1970, 1972) found that the reduced platelet survival seen in patients with prosthetic heart valves was normalized after dipyridamole therapy.

The present studies indicate that the lack of a dipyridamole effect when measured ex vivo may be due in part to an ex vivo artifact. Thus, it was demonstrated that sodium citrate significantly interferes with the effect of dipyridamole on the platelet. In all the studies previously referred to in which no dipyridamole effect was demonstrated, citrate was used as the anticoagulant. Therefore, it

is possible that the lack of effect of dipyridamole seen in those studies is due to a citrate effect. A recent study by Rajah and associates has shown that dipyridamole inhibited platelet aggregation ex vivo in citrated plasma obtained from patients with prosthetic heart valves when therapy was maintained for a minimum of nine days, suggesting the possibility that with higher drug levels accumulated over time, the citrate effect may be overcome (Rajah et al, 1977).

The effect of aspirin on the dipyridamole plasma level

Harker and Slichter (1970, 1972) have shown that a low dose of dipyridamole, in combination with 1 gm aspirin, given daily, was as effective as a higher dose of dipyridamole in normalizing the reduced platelet survival seen in patients with prosthetic heart valves whereas when the low dose of dipyridamole or aspirin were given separately, they had no effect. The effect of the combination treatment has been attributed to a "synergism" of the two drugs on platelet function. However, this effect can be explained by an alternative mechanism. If the effect of dipyridamole on platelet function is related to concentration in the plasma, it is possible that an alteration in the pharmacokinetics of dipyridamole by aspirin may result in an altered effect of dipyridamole.

The dipyridamole effect on in vivo collagen-induced platelet aggregation has been shown by us to be related to the concentration of dipyridamole within the plasma. This is supported by the data

of Rajah, et al (1977). These investigators have also shown that the inhibitory effect of dipyridamole on ex vivo collagen-induced platelet aggregation was related to the dipyridamole plasma concentration.

Thus, it was predicted that any compound which altered the distribution or blocked the clearance of dipyridamole could cause either an increase in the dipyridamole effect or a prolongation of that effect. We found that the concentration of dipyridamole at any given interval after dipyridamole treatment was higher when the dipyridamole treatment was preceded by aspirin or salicylate, and as a consequence, the effect of dipyridamole on in vivo platelet aggregation was prolonged.

The mechanism of this effect, however, has not been elucidated.

There appear to be a number of possibilities:

- 1) salicylate saturates the glucuronide pathway and reduces the clearance of dipyridamole from the plasma compartment;
- 2) salicylate alters the hepatic blood flow so that the dipyridamole distribution is altered in the plasma compartment; or
- 3) salicylate saturates the tissue and plasma protein sites to which dipyridamole is normally bound and alters the distribution and/or clearance of dipyridamole.

Evidence that the glucuronide pathway is saturated by aspirin has been demonstrated by Cohen, (1976) and Levey and Tsuchiza (1972). These investigators demonstrated that the $T_{1/2}$ of circulating aspirin and salicylate was increased to 20 hours when these compounds were given in high doses as compared to a $T_{1/2}$ of three to six hours

when given in the usual therapeutic dose. This effect on the $T_{1/2}$ was attributed to the saturation of the glucuronide pathway. Since dipyridamole is cleared primarily through the same pathway (Zak et al, 1963), it is possible that the clearance of dipyridamole is blocked by the saturation of the pathway by salicylate.

There is no evidence to support the second hypothesis. In addition, it has been reported that salicylate in very high doses increases biliary outflow (Nickerson, 1970), which if correct, would be expected to increase clearance of dipyridamole.

The most likely mechanism to explain the effect of salicylate on the pharmacokinetics of dipyridamole is that salicylate saturates the tissue and/or plasma proteins to which dipyridamole is normally bound. Aspirin and salicylate have been shown to saturate plasma proteins and to bind competitively with other drugs, displacing some of them from the protein so that the pharmacokinetics of the second drug are altered (Davidson and Smith, 1961; Yu et al, 1963). Since dipyridamole binds to both plasma proteins and tissues (Kubler et al, 1969; Niewiarowski et al, 1975), it is possible that when aspirin or salicylate are given in high enough doses before the dipyridamole treatment, the sites to which dipyridamole bind, are saturated. This would result in a redistribution of dipyridamole within the plasma and tissue compartments. In addition, the transportation of dipyridamole by plasma proteins to the glucuronide pathway would be altered, and thus, clearance would also be

decreased.

Until protein binding studies are performed, no definite conclusions on the mechanism of the interaction between salicylate and dipyridamole can be made.

If a similar phenomenon did occur in man, these results may have an important implication since they would support the use of a lower dose of dipyridamole in combination with salicylate for the treatment of certain thromboembolic conditions.

THE EFFECT OF SULFINPYRAZONE ON PLATELET FUNCTION

The effect of sulfinpyrazone on platelet function

Sulfinpyrazone has been shown to be effective in prolonging reduced platelet survival seen in a number of thrombotic conditions (Blakely, 1977; Weily and Genton, 1970; Smythe et al, 1965; Steele et al, 1973), and in preventing thrombosis of arterial venous shunts in patients undergoing chronic hemodialysis (Kaegi et al, 1974). The effect of this drug in those conditions has been attributed to the inhibition of platelet function (Packham et al, 1967; Zucker and Peterson, 1970). Recently, Ali and McDonald have shown that the in vitro inhibitory effects of sulfinpyrazone on collagen-induced platelet aggregation and release are associated with an inhibition of platelet prostaglandin production (Ali and McDonald, 1977).

In the present study, the relationship of the inhibition of platelet prostaglandin production ex vivo and inhibition of platelet function in vivo, and the relation of these effects to the concentration of sulfinpyrazone in the plasma, were investigated. It was demonstrated that there was a progressive increase in inhibition of platelet aggregation at a time when there was no detectable sulfinpyrazone in the plasma. A similar pattern of inhibition of in vivo collagen-induced platelet aggregation in guinea pigs following sulfinpyrazone treatment has also been observed in another laboratory (White, personal communication).

Two possible explanations were suggested to explain the effect. First, it was possible that the prolonged effect on platelet function

seen long after the drug was cleared from the plasma was due to a non-specific effect of the initially high concentration of sulfinpyrazone achieved with the bolus injection; and, second, a metabolite of sulfinpyrazone was formed which was responsible for the continuation of the inhibition of platelet function after the sulfinpyrazone had been cleared from the circulation. The first possibility has been excluded because it was shown that when the same dose of sulfinpyrazone was given as an infusion instead of a bolus in order to overcome any possible toxic effects arising from the initially high plasma concentrations, the inhibitory effect at 18 hours still persisted. Furthermore, platelet poor plasma prepared from rabbits that had been treated with sulfinpyrazone 18 hours beforehand, was able to inhibit malondialdehyde production of normal platelets. Thus, this provides direct evidence that there is something in the plasma, presumably a sulfinpyrazone metabolite, which inhibits platelet function. The identification of the metabolite, however, has not yet been achieved.

Aldridge and Johnson, and Dieterle and his associates, have shown that 85-95% of all ingested or injected sulfinpyrazone can be accounted for in the urine within the first 48 hours after treatment (Aldridge and Johnson, 1972; Dieterle et al, 1975). At least one-half of the sulfinpyrazone is eliminated as sulfinpyrazone and the rest as its metabolites, para-hydroxy sulfinpyrazone, β -glucuronide sulfinpyrazone and a third undefined metabolite. It is possible that the unaccounted for 5-15% residual can influence

platelet function and is responsible for exerting the prolonged effect seen in our experiments. Thus far, we have been unable to characterize the compound but have been able to demonstrate that whatever exists at 18 hours is firmly bound to the platelet and cannot be washed off. This is distinctly different from sulfinpyrazone itself which is readily removed from the platelet with washing, and has a reversible effect as has been demonstrated in this study and elsewhere (CazanaVe et al, 1977a; Davis et al, 1975). The differences in the effects of the two sulfinpyrazone compounds suggest that they may act at different sites in the prostaglandin pathway, or that the metabolite blocks platelet release by some other mechanism. Although both aspirin and sulfinpyrazone appear to act on cyclo-oxygenase, and so inhibit prostaglandin formation (Roth and Majerus, 1974; Ali and MacDonald, 1977), only sulfinpyrazone is effective in normalizing platelet survival. It is entirely possible, therefore, that the effect of sulfinpyrazone in rabbits is contributed to by the metabolite, which accumulates over time and acts by other mechanisms. This would also explain some of the observations in the study of Mustard et al (1967) in which they showed that the effect of sulfinpyrazone on platelet survival in rabbits required a treatment of at least 15 days. One might argue that this time span was required to accumulate sufficient metabolite to have an effect. It is important to determine whether this metabolite is formed in man and these studies are presently being carried out.

SUMMARY

Summary

This study was undertaken in order to investigate some of the factors which could account for the discrepancies found when the effects of aspirin, dipyridamole and sulfinpyrazone on platelet function are assessed ex vivo and in vivo. An in vivo test system was developed which made it possible to examine the relationship between the plasma drug levels of these three agents and their effects on platelet function in vivo. This study confirmed other observations that all three drugs inhibit platelet function in vivo and provided new information which could prove useful to their use as antithrombotic agents.

The studies demonstrated that:

1. Autologous platelets disappear temporarily from the circulation following an intra-arterial infusion of (acid soluble) collagen. This disappearance of circulating platelets can be detected by visual counts and expressed as percent drop. The percentage drop in circulating platelets has been described as maximum platelet aggregation.
2. When rabbits which previously had been injected with homologous ⁵¹Cr-labelled platelets, are infused intra-arterially with collagen, the labelled platelets also disappear temporarily from the circulation. The pattern of this disappearance as determined by radioactivity is similar to the pattern of disappearance of

the autologous platelets when determined visually.

3. Maximum platelet aggregation of both autologous and homologous platelets is inhibited in the presence of aspirin, sulfinpyrazone or dipyridamole.
4. Maximum platelet aggregation is not altered when the partial thromboplastin time exceeds 150 seconds and therefore, it is concluded that the platelet response is due to a direct interaction between the platelet and the polymerizing collagen.
5. The measurement of maximum platelet aggregation of homologous ⁵¹Cr platelets determined by radioactivity following an intra-arterial collagen infusion into rabbits appears to be a valid and reliable in vivo test system with which to test potentially anti-platelet agents.
6. Citrate blocks the effect of dipyridamole on platelet aggregation. This effect is not due to the chelation of calcium because when calcium is reduced in the platelet environment and citrate is not present, the dipyridamole inhibitory effects are enhanced.
7. Dipyridamole, which had not been shown to be an effective inhibitor of platelet aggregation in citrated plasma after a single bolus treatment, does inhibit platelet aggregation when tested in heparinized plasma.
8. The assessment of platelet suppressant drugs ex vivo is

limited. Sodium citrate itself has been shown to alter both platelet function and the effect of a drug on platelet function.

9. Acetylated plasma has no effect on collagen-induced platelet aggregation in vivo nor platelet malondialdehyde production ex vivo.
10. Malondialdehyde production of new platelets released from the bone marrow is not compromised by the previous exposure of the megakaryocytes to high concentrations of aspirin nor the presence of acetylated plasma proteins.
11. Low concentration of aspirin maximally inhibits platelet malondialdehyde production ex vivo. This effect is permanent and lasts the lifetime of the platelet.
12. The effect of aspirin on platelet malondialdehyde production can be dissociated from its effect on collagen-induced platelet aggregation. Therefore, it is concluded that aspirin affects platelet function by at least two mechanisms, one independent of the prostaglandin pathway.
13. Aspirin shortens the bleeding time of punctured jugular veins after either systemic or local administration of the drug. This aspirin effect on the vessel wall is independent of its effect on the platelet because the bleeding time is shortened in both normal and thrombocytopenic animals. The aspirin effect on the vessel wall is not a non-specific effect due to non-physiological pH nor increased osmolarity of the vessel

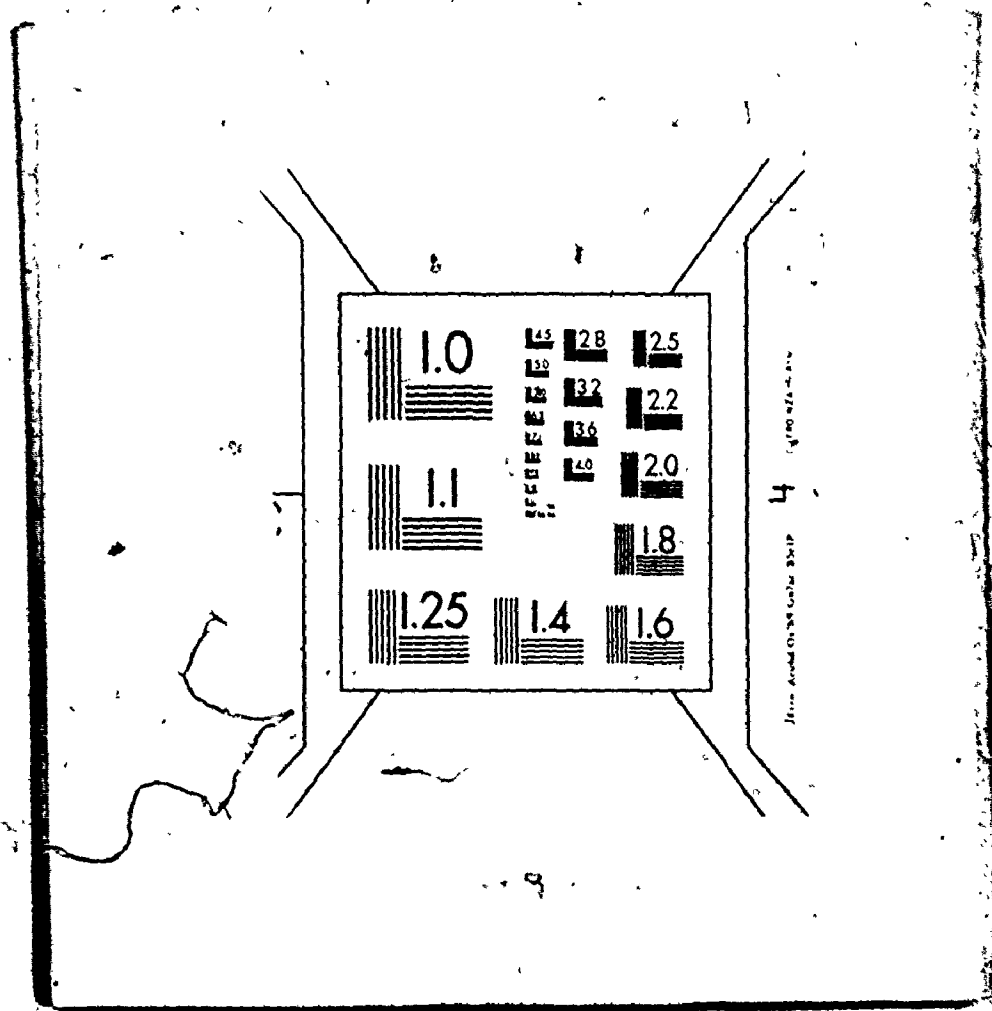
wall environment. The aspirin effect on the vessel wall is attributed to an acetylation of cyclo-oxygenase and subsequent inhibition of vessel wall prostaglandin I_2 because both aspirin and a similar acting agent, indomethacin, have the same shortening effect on the bleeding time. The vessel wall can overcome the aspirin effect which lasts for only about an hour probably because the nucleated cells of the vessel wall can re-synthesize and replace depleted products.

14. The inhibitory effect of dipyridamole on maximum platelet aggregation in vivo is dose-dependent and reversible. The maximum effect lasts for about one hour and is related to the level of dipyridamole circulating in the plasma. The level of dipyridamole circulating in the plasma is increased in the presence of aspirin and sodium salicylate.
15. Sulfinpyrazone inhibits maximum platelet aggregation in a dose-dependent fashion and the effect is related to the level of sulfinpyrazone circulating in the plasma. The effect is mediated in part by a blockage of platelet malondialdehyde production. The sulfinpyrazone effect is reversible and can be washed off the platelet.
16. The inhibition of platelet aggregation observed long after sulfinpyrazone is cleared from the plasma is related to the initial dose of sulfinpyrazone administered but is not related to the presence of sulfinpyrazone within the plasma. This

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effect is attributed to the formation of an active metabolite which is present in the plasma at 18 hours in sufficient concentration to block platelet malondialdehyde production of both autologous and homologous platelets.

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