PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Aggregation of Platelets in Damaged Vessels

G. V. R. Born, P. Gorog and M. A. A. Kratzer

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Aggregation of platelets in damaged vessels

By G. V. R. Born, F.R.S., P. Görög and M. A. A. Kratzer

Department of Pharmacology, King's College, Strand, London WC2R 2LS, U.K.

The only certain physiological function of platelets is their aggregation in injured vessel walls as haemostatic plugs. The association of thrombocytopenia with petechial haemorrhages suggests that platelets are somehow required for the functional integrity of small vessels, but no mechanism has yet been established. The pathological aggregation of platelets as thrombi in atherosclerotic arteries is commonly, if not always, initiated by haemorrhage. In artificial vessels, platelets tend to aggregate on the walls wherever blood flow is non-laminar. The mural aggregation of platelets is not prevented by unphysiologically high wall-shear forces. The facts suggest, on the contrary, that the process depends in some way on abnormal haemodynamic conditions.

This contribution is mainly concerned with questions about how haemodynamic conditions in and around vascular leaks affect arriving platelets that aggregate there, and about the chemical agents responsible for making the platelets reactive. The effects of these agents are known mainly from *in vitro* experiments in which aggregation can be quantitatively correlated with biochemical effects by simple and reproducible methods; the relevance to their reactions in haemostasis and thrombosis is uncertain. It is difficult to devise quantitative methods for analysing these processes *in vivo* because of the very low concentrations at which endogenous agents can activate platelets and haemostatic factors in the plasma; the rapidity with which platelets aggregate in a damaged blood vessel; and the complexity and inconstancy of the haemodynamic situation. All these facts must be accounted for in hypotheses of haemostasis. New experimental approaches towards analysing the haemostatic mechanism *in vivo* are described.

Introduction

Platelets aggregate in blood vessels that are damaged by injury, most commonly mechanical, or by disease, most commonly atherosclerosis. Mechanical injury causes aggregates to form both outside and inside any opening through the vessel wall. As these aggregates grow, they diminish and ultimately arrest haemorrhage, at least temporarily; such aggregates are therefore known as haemostatic plugs.

Platelet aggregates that form inside a vessel are thrombi, by definition (French & Macfarlane 1970). Atherosclerotic disease, which is confined to arterial vessels, can apparently induce thrombotic platelet aggregation either when a hardened plaque cracks so that there is haemorrhage into the vessel wall, or when lesions have deformed and constricted the arterial lumen and the flow of of blood is grossly abnormal.

One or other of these conditions initiate coronary and cerebral thrombosis, which manifest themselves clinically as heart attacks and strokes respectively. This paper analyses current evidence connecting haemodynamic effects in diseased arteries with the thrombotic aggregation of platelets in them. In analyses of these processes it is commonly assumed that the experimentally induced aggregation of platelets as haemostatic plugs is a relevant model of the clinical aggregation of platelets as arterial thrombi. The mechanisms may be similar in some respects. However, the haemodynamic conditions are probably very different (figure 1).

Therefore, conclusions based on the haemostatic aggregation of platelets may not necessarily apply to their thrombotic aggregation, even when that is initiated by haemorrhage into the vessel wall; and it is important to beware of oversimplifications in hypotheses put forward to explain these complex processes.

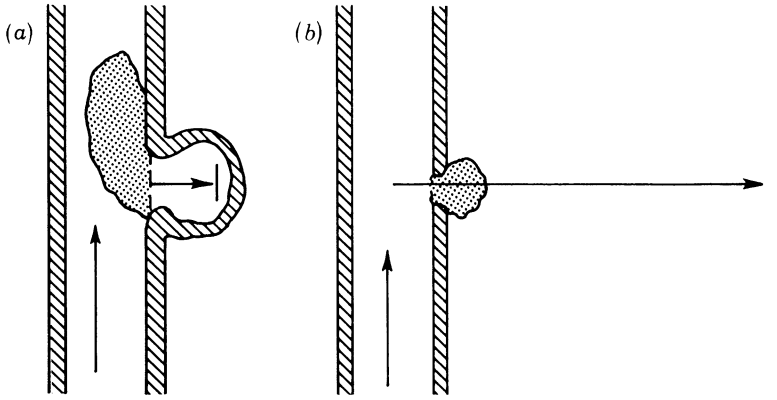

FIGURE 1. Haemodynamic conditions of (*a*) plaque fissure and (*b*) wall injury. In both arteries blood flow is fast. When a plaque cracks, blood makes its way through the fissure into the arterial wall. Flow slows down rapidly so that the extravasated blood becomes a reservoir of thrombogenic agents including presumably ADP, thromboxane A_2 and thrombin in varying proportions. Diffusion of these agents into the arterial lumen can account for the intravascular platelet thrombi that are observed under such conditions.

By contrast, when injury opens an artery the outflow of blood through the wall continues without the formation of such a reservoir. Thrombogenic agents appearing in the extravasated blood induce extravascular platelet aggregation, as observed under these conditions.

CLINICAL AND PATHOLOGICAL FEATURES OF ARTERIAL THROMBOSIS

Any hypothesis for thrombotic platelet aggregation must be able to account for the following facts. 1. Thrombi do not form in normal arteries. 2. Thrombi form in atherosclerotic arteries. 3. Arterial thrombi consist initially of aggregated platelets. 4. Atherosclerosis increases slowly, whereas thrombosis occurs rapidly and is individually unpredictable; therefore, atherosclerotic arteries must be subject to sudden, unpredictable events capable of initiating platelet aggregation. 5. Most occlusive thrombi are associated with fissures in underlying atheromatous plaques.

The erythrocyte-haemodynamics hypothesis (Born 1979*a*) proposes that the sudden unpredictable event that starts arterial (typically coronary) thrombosis is plaque fissure; haemorrhage through the fissure is associated with increased haemodynamic stress causing ADP (and other adenine nucleotides) to appear in the plasma; this ADP is principally responsible for activating platelets and their aggregation as mural thrombi.

Much progress has been made with support for this hypothesis (Born *et al.* 1976; Born & Wehmeier 1979).

Evidence includes the following. 1. In atherosclerotic arteries platelet thrombi form only when blood flow is sufficiently abnormal, i.e. as a result of haemorrhage into a fissure, or in tortuous and/or stenotic regions. 2. In artificial blood vessels mural thrombi of platelets grow where, and only where, flow is non-laminar. 3. In artificial vessels the formation of platelet thrombi in non-laminar flow depends on the presence of red cells. 4. Adhesion and aggregation of platelets on artificial surfaces increase with red cell concentration and are abolished by ADP-removing enzymes. 5. The haemodynamic stress associated with experimental haemorrhage is insufficient in duration and magnitude to activate platelets directly, but sufficient in both to induce release of red cell ADP (Born & Wehmeier 1979).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The thrombogenic adhesion of platelets to vessel walls therefore depends indirectly on the haemodynamic properties of the blood as it flows through arteries constricted and/or fissured by atherosclerosis.

Gross and histological appearances of arterial thrombi establish that their central mass consists mainly of aggregated platelets. What, therefore, is the mechanism responsible for rapid and extensive platelet aggregation in an artery as an apparently random event in time? Close serial sectioning of obstructed coronary arteries established some time ago that the platelet thrombus responsible is usually, if not invariably, associated with recent haemorrhage into an underlying atherosclerotic plaque (Friedman & Byers 1965; Constantinides 1966; Davies & Thomas, this symposium). The haemorrhages occur through fissures or fractures in the plaque, and it is a reasonable assumption that the sudden appearance of such a fissure or fracture is the random, individually unpredictable event affecting coronary arteries that has to be assumed to account for the clinical onset of acute myocardial infarction (Born 1979*a*). Why such a defect should develop at a particular moment is uncertain. Perhaps it is analogous to the sudden appearance of fine cracks in the wings of jet aircraft which is ascribed to the cumulative effects of variable stresses on metal known as metal fatigue (Gordon 1978; Frost *et al.* 1974). The chance event of plaque fissure can in principle be prevented only by preventing atherosclerosis, which is, as we know, still very problematical. Fortunately, the subsequent thrombotic process due to platelet aggregation is now understood to the extent that it may become preventable by drugs before long.

How does haemorrhage into a ruptured plaque start off platelet thrombogenesis? This can be regarded as part of the general question of how platelets are caused to aggregate through haemorrhage, most effectively from arteries. An explanation commonly put forward is that the process is initiated by platelets adhering to collagen exposed where damaged vessel walls are denuded of endothelium (Mustard *et al.* 1977; Packham & Mustard 1977). Adhering platelets then release other agents, including thromboxane A_2 and ADP, which in turn are responsible for the adhesion of more platelets as growing aggregates. This explanation is unlikely to be correct for the following reasons. First, haemostatic and thrombotic aggregates of platelets grow without delay and very rapidly (Hugues 1959). When an arteriole 200 μm in diameter is cut into laterally, the rate of accession of platelets to the haemostatic plug is of the order 10^4/s in the first seconds (Born & Richardson 1980). In contrast, the aggregation of platelets by collagen begins, even under optimal conditions for rapid reactivity, only after a delay or lag period of several seconds (Wilner *et al.* 1968). Secondly, platelets tend to aggregate as mural thrombi when anticoagulated blood flows through plastic vessels (Didisheim *et al.* 1972), for example in artificial organs such as oxygenators or dialysers (Richardson *et al.* 1976), which contain no collagen nor anything else capable of activating platelets similarly. This implies that there are conditions under which platelets are activated in the blood by something other than collagen or other constituents of the walls of living vessels. The plaque on which a thrombus grows has usually narrowed the arterial lumen. At constant blood pressure the flow of blood is faster through the constriction than elsewhere in the artery. Therefore, high flow and wall shear rates are no hindrance to the aggregation of platelets as thrombi (Born 1977). Indeed, the question arises of whether the activation of platelets, which precedes their aggregation, *depends* in some way on abnormal haemodynamic conditions.

Measurements of the haemodynamic forces required to activate platelets directly (Hellums & Brown 1977) indicate that the blood flow over atherosclerotic lesions *in vivo* is unable to do so (Colantuoni *et al.* 1977). The activation therefore must be *indirect*. Now, it has been known

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

for many years that platelets can be activated by at least one agent, namely ADP, derived from the red cells, which outnumber and surround the platelets in the blood (Gaarder *et al.* 1961).

Clear evidence of increased platelet adhesiveness brought about by the operation of flow-mechanical factors on erythrocytes was provided by experiments in which blood was made to flow through branching channels in extracorporeal shunts (Rowntree & Shionya 1927; Mustard *et al.* 1962). Deposits of platelets formed consistently on the shoulders of a bifurcation in the flow chamber but nowhere else in the channels. In such divergent flow situations there is boundary-layer separation, which is accompanied by flow delays or stasis (Fox & Hugh 1966). This might by itself be expected to increase the probability of platelet aggregation. However, when the chambers were perfused not with blood but with platelet-rich plasma, no deposit was formed, showing that red cells were also essential.

The dependence of the deposition of platelets from flowing blood on the presence of red cells could be caused by physical or chemical mechanisms or, of course, by both acting synergistically. A *physical mechanism* would depend essentially on an increase in the *diffusivity* of platelets caused by the flow behaviour of the erythrocytes. Indeed, the diffusivity of platelets in flowing blood has been estimated to be two orders of magnitude greater than that calculated for platelets diffusing in plasma (Turitto *et al.* 1972; Turitto & Baumgartner 1975). This is consistent with the enhanced radial fluctuations of erythrocytes or of latex microspheres (2 μm in diameter) in flowing suspensions of red cell ghosts (Goldsmith 1972). High platelet diffusivity is required also to explain the growth of mural thrombi. This must depend on successful platelet–platelet collisions, the rate of which between platelets following streamlines near the walls would hardly be sufficient to account for the rapidity of growth observed *in vivo* (Begent & Born 1970; Richardson 1973; Born & Richardson 1980).

Is ADP involved in arterial thrombogenesis?

There is increasing evidence for a *chemical mechanism* in the increased adhesiveness of platelets in the presence of red cells, i.e. through their ADP. The concentrations of ADP required for activating platelets are small, probably less than 10^{-8} M (Frojmovic 1978) and ADP is also rapidly dephosphorylated in blood (Haslam & Mills 1967), so that its direct demonstration there under conditions relevant to thrombogenesis is difficult.

It has recently become possible to demonstrate the appearance of free ADP in blood *directly* in concentrations sufficient to activate platelets (Schmid-Schönbein *et al.* 1979). In specially designed apparatus, whole blood or resuspended red cells are exposed to controlled, different shear stresses for known time periods. The apparatus is designed to cover the range of these variables presumed to be relevant to the situations *in vivo*. The experiments show that ADP appears in the plasma in concentrations required for platelet activation (0.1–1.0 μM) but in direct proportion to free haemoglobin, indicating that platelet activation can result from small degrees of haemolysis due to haemodynamic stresses such as occur during haemorrhage, whether external or through a plaque fissure. It is not yet certain whether the appearance of free ADP is rapid enough to account for aggregation *in vivo*. This process appears to be faster than the release of ADP from the platelets themselves or of thromboxane A_2 produced by them which, in any case, induces aggregation via ADP (personal communications from B. Samuelsson and A. Marcus).

When blood vessels are injured so that they bleed, circulating platelets adhere to the damaged

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

vessel wall and aggregate within the first seconds. The mechanism of the initial platelet aggregation remains uncertain. To investigate the initiation stage of haemostasis, the carotid arteries of rats were punctured with a 100 μm needle and free ATP, as an indicator of ADP, was measured in the emerging blood (Kratzer & Born 1981). This was brought into contact with luciferin–luciferase in a polyethylene tube, internal diameter 0.8 mm. The light produced at the blood–enzyme interface was measured with a sensitive photon-counting device which gave background counts of 1 photoelectron per second and could detect less than 10^{-8} M ATP in 2 μl blood.

When an artery was injured, the emerging blood contained about 10^{-7} M ATP in a first peak after about 2 s. After about 1 min the ATP concentration rose to a second peak of about 5×10^{-6} M. This was decreased by heparin or by chlorpromazine. The source of ATP accounting for the first peak remains uncertain; possibly this ATP is released from red cells undergoing high shear stress from the haemodynamic effects of haemorrhage. The observations suggest that the second peak represents ATP released from platelets.

The release reaction of platelets has been assumed to subserve a positive feedback mechanism responsible for their aggregation in haemostasis and thrombosis (Born 1965). This assumption is based mainly on *in vitro* experiments. Considerable uncertainty remains about the contribution of the release reaction to the initiation of haemostasis *in vivo*. The rapidity of the process and the presence of other tissues makes it impossible to follow the reaction quantitatively *in vivo* by methods that permit this *in vitro*. We have therefore applied quantitative electron microscopy to find out how quickly the concentration of dense bodies decreases in platelets during their haemostatic aggregation (Görög & Born 1981).

In mice, platelets were enriched in dense bodies by pretreatment with 5-hydroxytryptamine. Mesenteric arteries were incised with a sharp blade. Bleeding was stopped by a micromanipulator-operated device about 15 and 60 s after the cut. The cut segments were immediately fixed *in situ* with glutaraldehyde and postfixed. Serial sections were made for electron microscopy. Platelets isolated from peripheral blood of the same animal were prepared similarly. Electron micrographs were projected on to a television screen and numbers of dense bodies and total platelet areas were determined by an image analysing computer. After 15 s there were no significant differences in numbers of dense bodies in platelets from different parts of the haemostatic plugs ($8.31 \pm 0.57/100$ μm² (mean ± s.e.m.)) and in platelets from the blood $8{\cdot}93 \pm 0{\cdot}38$). On the other hand, after 60 s the parts furthest from the cut contained fewer dense bodies than the nearer parts and the overall dense body number (5.86 ± 0.05) was significantly smaller ($p < 0.001$) than that of platelets from the blood (14.45 ± 0.09). The results suggest that haemostatic aggregation of platelets does not initially depend on their release reaction. Furthermore, as the action of aspirin on platelets is inhibition of the release reaction these results also explain, in part at least, the comparative ineffectiveness of aspirin in clinical trials of the prevention of reinfarction.

Our observations suggest a new approach to the prophylaxis of arterial, e.g. coronary, thromboses (Born 1979*b*). This approach would require the demonstration that their incidence is diminished by drugs that, in clinically acceptable blood concentrations, do not inhibit platelet function directly but which inhibit the release of activating agent, presumably ADP, from red cells during rheological stresses such as occur in potentially thrombogenic arteries. Such a demonstration may then also explain the effect of dipyridamole or sulfinpyrazone in preventing increased utilization of circulating platelets under potentially thrombogenic conditions. This

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

cannot easily be accounted for by any direct action on platelets by either drug at its clinically effective concentration. Perhaps these drugs act mainly on the red cells to diminish their activating effect on platelets.

Some time ago one of us (G.V.R.B.) had the idea that drugs capable of counteracting haemolysis (Seeman 1972) might diminish this activating effect of red cells on platelets and so inhibit their aggregation as thrombi. Experimental evidence for this idea came with the demonstration (Born *et al.* 1976) that chlorpromazine added to anticoagulated human blood, in concentrations that *in vitro* diminish hypotonic haemolysis but have no direct effect on platelet aggregation (Mills & Roberts 1967), prolong the bleeding time from small holes in artificial vessels where extravasation is terminated, as *in vivo*, by aggregated platelets. More recent experiments (Born & Wehmeier 1979) support the conclusion that this effect is accounted for by the antihaemolytic action of chlorpromazine. This has led to the suggestion (Born *et al.* 1976, 1979*a*, *b*) that other drugs possessing this effect of chlorpromazine may diminish the incidence of arterial, particularly of coronary, thrombosis when it is induced by conditions of abnormal stress on the red cells, such as through haemorrhage into atheromatous lesions.

As already proposed (Born 1979*b*), evidence for or against this proposition could perhaps be obtained by comparing the incidence of acute coronary occlusions in populations on long-term treatment with chlorpromazine (or other drugs acting in this respect like chlorpromazine) with the incidence in control populations not on such drugs. The only conceivably relevant evidence of which we have been made aware up to now (through the courtesy of Dr J. A. Baldwin, Director of the Oxford University Unit of Clinical Epidemiology) is an investigation of mortality in Norwegian psychiatric hospitals during the period 1950–62 (Odegard 1967). This concluded that mortality from circulatory disease, predominantly 'coronary disease' and 'infarction', was higher in the mental hospital population than in the general population, although the excess was not as much as that from most other causes. Within the patient population, the excess mortality from coronary disease and infarction was less for schizophrenics than for all other psychoses. Furthermore, the excess mortality from circulatory disease diminished strikingly after 1957, particularly when compared with the period 1926–41, because the mortality did not rise to the same extent in the hospital as in the general population.

These conclusions, if confirmed, are of course open to different interpretations. Epidemiological considerations apart, chlorpromazine has many effects in the body, and also a large number of metabolites. Furthermore, the concentration of chlorpromazine in patients' plasma (Mackay *et al.* 1974) is one to two orders of magnitude lower than that required to prolong the *ex vivo* bleeding time described below. Therefore, if patients' blood were used for determining this bleeding time, a prolongation might be expected only if the drug or a similarly active metabolite were concentrated in red cell membranes. On the other hand, it has been observed that single clinical doses (5–20 mg) of chlorpromazine injected intramuscularly into apparently healthy volunteers cause the Ivy bleeding time to be significantly prolonged (Zahavi & Schwartz 1978).

Our experimental observations with chlorpromazine make it attractive to suggest that the general introduction of this drug for the control of schizophrenic in-patients from about 1955 onwards accounts for their relative protection against cardiac mortality at a time when it was increasing rapidly in Norway and elsewhere, including Britain. It would be interesting to learn of other information that may support or, just as important, invalidate this line of thought. It may be worth while to investigate appropriate populations from this point of view.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Are prostaglandins involved in arterial thrombogenesis?

A different explanation of arterial thrombosis has been widely canvassed (Gryglewski *et al.* 1976). This may be referred to conveniently as the prostacyclin–thromboxane hypothesis, and it proposes that: (1) whether or not thrombosis occurs in arteries depends on a local balance between prostacyclin (PGI_2), which inhibits platelets, and thromboxane A_2 (TXA_2), which aggregates platelets; (2) in normal arteries, thrombosis is prevented by prostacyclin in the blood where its sources are lungs and endothelium; (3) part of the 'endothelial' prostacyclin originates in endoperoxides transferred from adherent platelets; (4) the walls of atherosclerotic arteries synthesize less prostacyclin, and those of artificial blood vessels none at all. Platelets' endoperoxides are therefore utilized for thromboxane A_2 production, which is responsible for activating the platelets and their aggregation as thrombi.

In view of the extraordinary inhibitory potency of prostacyclin, this hypothesis appears attractive, but it is not entirely satisfactory because it does not explain:

(1) how platelets can produce endoperoxides for utilization by endothelium without the simultaneous formation of thromboxane A_2 by the platelets, which should aggregate them;

(2) that the haemostatic aggregation of platelets does occur under conditions in which it might be expected to be prevented by endothelial prostacyclin (Dejana *et al.* 1980).

(3) why platelet thrombi do not form in artificial blood vessels, i.e. in the absence of prostacyclin, except under particular haemodynamic conditions and in the presence of red cells;

(4) why arterial thrombosis does not occur continuously on all atherosclerotic lesions; and

(5) that the essential clinical characteristics of arterial thrombosis include an unpredictable and sudden onset, commonly in patients with either long-standing or minimal atherosclerotic lesions.

Evidence that the biosynthesis of prostaglandins is initiated by mechanical deformations of cell membranes (Piper & Vane 1971) does suggest a mechanism by which prostaglandins could be brought into thrombogenesis through haemodynamic effects. Platelets themselves produce the aggregating agent thromboxane A_2 (Hamberg *et al.* 1975; Svensson *et al.* 1976). The first step in its formation is the release of arachidonic acid from phospholipids in the cell membrane, catalysed by the enzyme phospholipase A_2 which is normally inactive; how the enzyme is activated physiologically is not known. Perhaps activation is initiated by small distortions of the outer membrane of platelets when they pass through regions in which the haemodynamic forces are greater than in the normal circulation, as during haemorrhage into a ruptured atherosclerotic plaque. This fluid-mechanical activation of platelets would not involve red cells, which apparently do not contain a thromboxane-forming system.

Is collagen involved in arterial thrombogenesis?

There has been ample confirmation of the observation (Didisheim *et al.* 1972) that 'thrombosis induced mechanically in a teflon shunt appears indistinguishable from thrombosis induced by mechanical or electrical injury in living vessels, despite the absence of endothelium, collagen and muscle from the teflon tubing'.

These observations do not support the assertion that haemostatic plugs or thrombi are initiated through the collision of platelets with exposed collagen. On the other hand, it has recently been shown that a nonapeptide sequence of collagen is a specific binding site for

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

platelets (Legrand *et al.* 1980). If this binding depends on multiple interactions (Santoro & Cummingham 1979), these could also induce distortions in the platelet membrane sufficient to activate phospholipase A_2 and thereby the prostaglandin cascade, resulting in the release reaction as observed. This therefore represents yet another way in which prostaglandins could participate in thrombogenesis.

Our knowledge of arterial thrombogenesis is still sufficiently fragmentary that the most reasonable way to conclude is with two quotations that counsel caution: 'Un point de vue unique est toujours faux' (Paul Valéry); and 'The belief in a single truth and in being the possessor thereof is the root cause of all evil in the world' (Max Born).

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Note added in proof (19 *May* 1981). Further evidence has now been obtained that ADP is involved in activating platelets *in vivo* (Krystyna Zawilska, G. V. R. Born & Nicola A. Begent, to be published). Novel techniques were developed for the reproducible determination of bleeding times from small arteries of rats and rabbits in the territory supplied by the superior mesenteric artery. One of its main branches was cannulated and infusions were made into the mesenteric circulation of two ADP-removing enzyme systems: either creatine phosphate (CP) with creatine phosphokinase (CPK) or phosphoenolpyruvate (PEP) with pyruvate kinase (PK). In both species these infusions increased the bleeding time significantly, suggesting that the increases were caused by decreases of free ADP in the blood. To confirm this conclusion, the same as well as other animals were infused with either substrate (CP or PEP) or enzyme (CPK or PK) alone. In neither species was the bleeding time prolonged by infusing enzyme alone. On the other hand, the bleeding times were significantly increased in rats and rabbits infused with substrate (CP or PEP) alone, suggesting that the ADP-removing systems were completed by endogenous enzymes. The plasma of both rats and rabbits were found to obtain CPK at concentrations higher than those in human plasma. In all experiments the arterial blood pressure, the blood platelet concentration and the haematocrit decreased moderately. The influence of these factors on bleeding time values was taken into account by appropriate controls.

These observations, which establish that infusion of different ADP-removing enzyme systems into arterial blood flowing towards fresh arterial injuries greatly increases the bleeding time from them, supports the conclusion that the activation of platelets for primary haemostasis depends on ADP.

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