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SOURCE AND CONCENTRATION OF EXTRACELLULAR ADENOSINE TRIPHOSPHATE DURING HAEMOSTASIS IN RATS, RABBITS AND MAN

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SUMMARY

1. A new technique was developed for the measurement of extracellular free ATP in very small samples of whole blood using the luciferin–luciferase enzyme system. The method had a very low background corresponding to approximately 10^{-16} mol ATP.

2. ATP was measured in blood as it emerged during haemostasis following precise puncture of rat and rabbit arteries and after standardized incisions of human skin by the Simplate device.

3. The initial concentration of free ATP in blood emerging 2–4 s after vascular injury was about 2×10^{-7} M in rats and rabbits and about 2×10^{-6} M in humans.

4. The free-ATP concentration increased to 2×10^{-5} M 3–5 min after injury and these increases could be prevented by heparin (20 u./ml).

5. The source of the initial free ATP was identified as damaged cells in the injured vessel wall. Sufficient ADP, both released as such with ATP and generated by enzymic dephosphorylation of ATP, would be present at the site of injury to initiate haemostatic aggregation of platelets.

INTRODUCTION

When a small artery or vein is cut, platelets adhere to and aggregate at the site of vessel injury leading to the rapid build up of a platelet plug, which diminishes and finally arrests the haemorrhage. The rate of accumulation of platelets in the haemostatic plug in the first few seconds is very high (approximately 10^4 platelets/s) and so activation of these platelets must occur within less than 1 s (Born & Richardson, 1980).

Of the several mechanisms proposed to account for such rapid and extensive platelet aggregation, the most common requires that activation be initiated by adhesion of platelets to collagen exposed where damaged vessel walls are denuded of endothelium (e.g. Mustard, Moore, Packham & Kinlough-Rathbone, 1977). Adhering platelets then release other agents, including thromboxane A_2 and ADP, which in turn are responsible for the adhesion of more platelets. This hypothesis does

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not account for the rapidity of haemostatic platelet aggregation (Hugues, 1959; Born & Richardson, 1980) because aggregation of platelets by collagen *in vitro* begins only after a delay or lag period of several seconds (Wilner, Nossel & LeRoy, 1968), even under optimum conditions for rapid reactivity. The reason for this delay is that although the adhesion of platelets to collagen is almost instantaneous, their subsequent aggregation depends on the delayed release of aggregating agents from the platelets themselves.

There is evidence favouring the hypothesis that the nucleotide adenosine 5′-diphosphate (ADP) is the physiological initiator of platelet aggregation. First, it has been calculated that the activation time of blood platelets after addition of ADP is shorter than 0·2 s (Born & Richardson, 1980). Secondly, infusion of different ADP-removing enzyme systems into the blood flowing towards fresh arterial injuries prolongs the bleeding time from them (Zawilska, Born & Begent, 1981) much more than by inhibiting the synthesis of endogenous thromboxane A_2 (Begent & Born, 1983). Furthermore, when ADP is applied ionophoretically to the outside of small blood vessels, platelet thrombi form inside them on normal endothelium with great rapidity (Begent & Born, 1970). If physiological haemostatic plug formation is initiated by ADP it must be present in blood plasma immediately following injury of the vessel. To find out whether this is true, we developed a new technique for measuring minute quantities of ATP in small samples of blood (Kratzer & Born, 1981). Using the firefly enzyme system luciferin–luciferase (Strehler & Trotter, 1952; De Luca & McElroy, 1978) the presence of ADP was inferred from the presence of ATP. Our results indicated that within 2 s after arterial injury the blood plasma contained enough ADP to account for the haemostatic aggregation of platelets, and that the source of these nucleotides was injured cells in the vessel wall.

A preliminary report of these experiments has been published (Born & Kratzer, 1981).

METHODS

Drugs and reagents

ATP, ADP, neutralized charcoal, and luciferin–luciferase reagent in glycine buffer and human albumin (Article LO633) were purchased from Sigma, London. Injectable sodium heparin solution (1000 u./ml) was obtained from Weddel Pharmaceuticals, London. Solutions of luciferin–luciferase reagent (10 mg) were prepared daily by addition of water (250 μl) that had been purified by filtration through charcoal and contained luciferin (5×10^{-4} M), luciferase (0·09 mg/ml) $MgSO_4$ ($1{\cdot}9 \times 10^{-2}$ M) and EDTA (4×10^{-3} M) as well as glycine and human albumin.

Animal experiments

Male Wistar rats (200–300 g) were anaesthetized with sodium pentobarbitone (60 mg/kg body weight I.P.). Both carotid arteries were exposed. The clotted blood from the field of operation was removed with absorbent tissues and the area superfused with physiological saline for 5 min. A small Perspex plate, 5 mm thick with a conical hole (i.d. increasing from 1 to 5 mm) was placed on each artery to form a small reservoir. The vessel was then punctured with a 100 μm steel needle through the hole. Blood samples (1 μl) were taken from the reservoir at increasing times for determination of free ATP.

Male New Zealand white rabbits were anaesthetized with sodium pentobarbitone (30 mg/kg body weight I.V.). To induce deep anaesthesia, the animal was also given a mixture of N_2O/O_2 (2:1) with about 1·5% v/v halothane. After removing hair from the ear with an electric razor the skin was covered with silicone grease (Dow Corning, Midland, U.S.A.). The main ear artery was punctured through the skin with the tip of a drawn-out glass pipette (tip diameter 100 μm) which was then

withdrawn and samples of emerging blood (1 μl) were taken for free-ATP measurements. In some experiments, the ear was ligated at its base. A polyethylene tube (o.d. 0·8 mm) was inserted into the central artery and the vessel perfused at 4·5 ml/min at 37 °C with physiological saline to which heparin (5 u./ml) and bovine serum albumin (1 %) were added. Perfusion pressure was monitored with a Grass Polygraph 79C recorder. 5 min after the start of the perfusion, when the pressure had stabilized at about 90 mmHg, the artery was punctured as described above 2 cm beyond the site of cannulation and 1 μl samples of the emerging saline perfusate were collected for ATP determination.

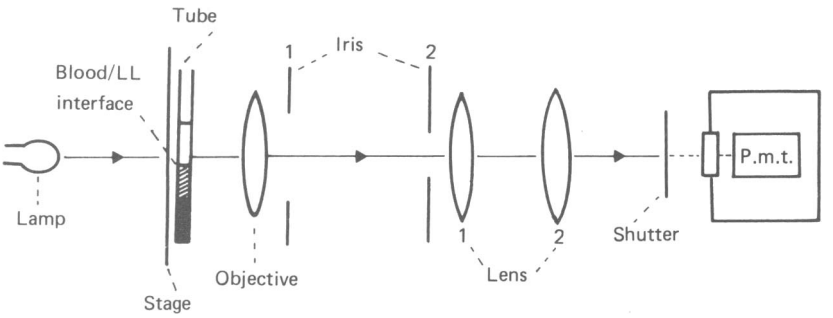

Fig. 1. Schematic diagram of the experimental arrangement to measure free ATP in whole blood samples. Blood was drawn into a polyethylene tube together with luciferin–luciferase (LL) solution. Free ATP in the blood plasma diffused into the enzyme solution and produced light just above its interface with blood. The emitted light was captured by an objective and imaged on to iris 2. Lens 1 and 2 imaged the iris on to the photocathode of the photomultiplier (P.m.t.). The lamp was used to control the measuring area of the polyethylene tube by observing its image on iris 2.

Human experiments

In eight apparently healthy male volunteers, who claimed not to have taken any drugs for 2 weeks, an incision was made with the Simplate bleeding time device (General Diagnostics, Los Angeles, U.S.A.) on the skin of the forearm, and 1 μl samples were collected from the emerging blood for determination of free ATP.

Erythrocyte suspension

To produce erythrocyte suspensions free of platelets, heparinized whole blood (50 ml) was centrifuged at 1000 *g* for 10 min. After removing the buffy coat, the supernatant plasma was transferred to polyethylene tubes (Eppendorf 3810) and centrifuged at 8000 *g* for 5 min to produce platelet-poor plasma. The packed red cells were resuspended in that plasma to a final haematocrit of 44 %. Preliminary experiments showed that enough platelets remained to interfere with ATP determinations; ADP (5×10^{-5} M) was added to the suspension to induce the release reaction in remaining platelets. The suspension was incubated at 37 °C for 1 h to allow free ATP to be broken down by dephosphorylating enzymes. When the concentration of free ATP was below 10^{-8} M, calibration experiments were begun.

Measurement of ATP

Luciferin–luciferase reagent (1 μl) was drawn into a polyethylene tube (i.d. 0·5 mm) fitted to an Oxford Sampler 2 μl micropipette (BCL, London). Holding the pipette vertically, the 1 μl sample of blood or physiological saline was drawn into the tube, care being taken to avoid the formation of air bubbles. The open end of the tube was plugged with Sealing Wax D553 (Radiometer, Copenhagen), removed from the pipette and mounted vertically on the stage of the light-measuring device (Fig. 1). The luminescence arising from diffusion of free ATP into the reagent (corresponding to a volume of 0·22 μl) just above its interface with the blood sample was focused by a microscopic objective (10 ×, aperture 0·4) on to iris 2 (i.d. 2 cm). Lens 1 (*f* 50 cm) and lens 2 (*f* 16 cm) were used to focus the diffraction plane of the microscopic objective (iris 1) on to the photocathode of a 6256B

photomultiplier (EMI, Hayes, Middlesex), contained in a TE-241 RF PTM housing (Products for Research, Danvers, U.S.A.) and run at 1400 V with a 479 N stabilized power supply (Brandenburg, Thornton Heath, Surrey). Single photoelectrons were integrated with a Reigate series GSA-10, RTM-10 counter (Panax, UK) and the output was displayed on an SR 6255 chart recorder (Watanabe, Japan). By enclosing the entire apparatus in a black light-tight box in a darkroom and by cooling the photomultiplier to −75 °C with frozen CO_2 it was possible to reduce the background to 0·8 counts/s.

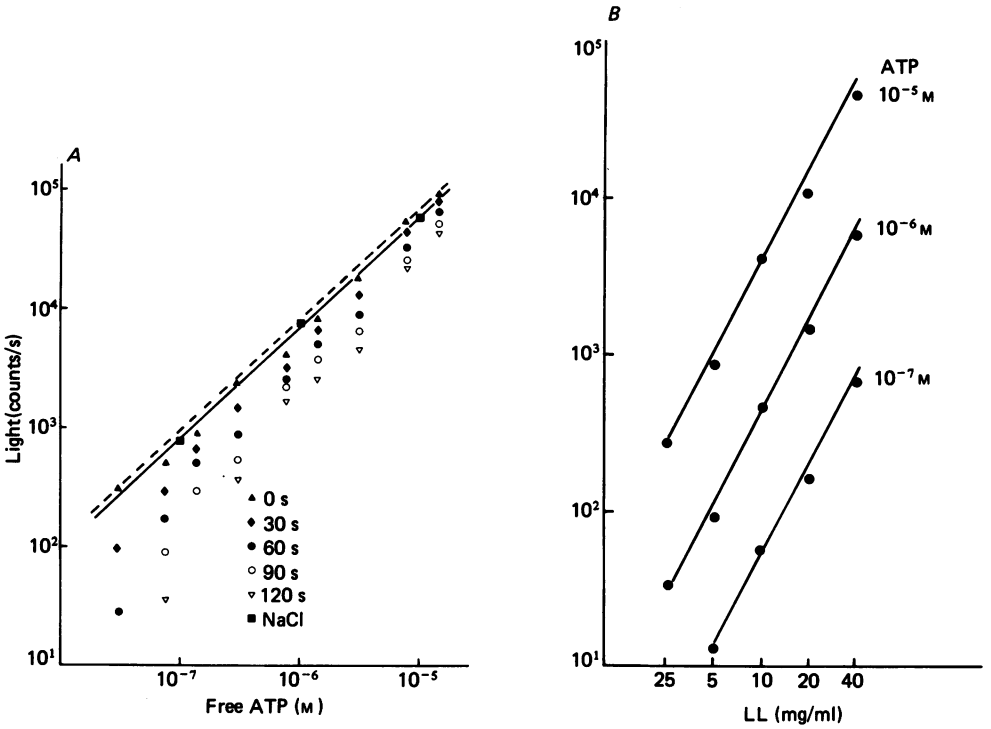

Fig. 2. *A*, light output of a platelet-free blood sample of rabbit *vs.* free-ATP concentration. Samples were taken for measurement 30, 60, 90 and 120 s after injection of ATP into the platelet-free blood, and light output extrapolated to zero time. ATP was also injected into physiological saline and the luminescence was compared with the blood samples. There was no significant difference between the light output of saline (NaCl) (continuous line) and the blood samples extrapolated to zero time (dashed line). The saline results were multiplied by 1·82 to correct for the absence of erythrocytes. *B*, light output of the measuring tube *vs.* luciferin–luciferase (LL) concentration for three different ATP concentrations (as indicated) in physiological saline containing 5 mM-$MgSO_4$. Measurement lines exhibit a slope of 2 on this double log graph.

RESULTS

Measurement of ATP in physiological saline

The light output as a function of luciferin–luciferase concentration was determined for three ATP concentrations (10^{-5}, 10^{-6} and 10^{-7} M) in physiological saline. The log *vs.* log plots were linear for each ATP concentration with slopes of 2, indicating that light output increased with the square of the luciferin–luciferase concentration (Fig. 2*B*). Maximal sensitivity was attained with luciferin–luciferase as supplied

at a concentration of 40 mg/ml; higher concentrations induced haemolysis and were therefore avoided. The background counts (< 1 count/s) corresponded to less than 10^{-16} mol ATP, i.e. less than the amount of ATP contained in one human erythrocyte.

Measurements of free ATP in suspensions of erythrocytes

Attempts to obtain calibration curves for luminescence as a function of free-ATP concentration in whole blood were frustrated by variable amounts of ATP released from platelets following the addition of ATP to the blood. Therefore, calibration curves were made with suspensions of erythrocytes. Following the addition of ATP, luminescence decreased rapidly with time, presumably due to dephosphorylation of ATP by plasma enzymes. Therefore, ATP solutions (5 μl) were added to erythrocyte suspensions (200 μl) and luminescence was determined in samples taken after 30, 60, 90 and 120 s and extrapolated back to zero time. Light emission was proportional to free ATP, and a log *vs.* log plot for zero-time values was linear for ATP concentration from 10^{-8} M to 2×10^{-5} M as shown in Fig. 2*A* for rabbit blood. The zero-time calibration curve for saline was almost identical with that for erythrocyte suspensions, showing that the presence of erythrocytes did not interfere with the light measurements. The calibrations were closely similar also for rat and human red cell suspensions (results not shown). Before all experiments the instrument was calibrated with the saline calibration curve.

Possible sources of error in measuring technique

There was always an unavoidable delay of 20–30 s between taking a blood sample and determining its free ATP. Therefore, we had to investigate the possibility that during that delay, the free ATP concentration either decreased by the action of dephosphorylating enzymes or increased by leakage of ATP from cells damaged by the sampling procedure or by release from activated platelets.

When ATP (final concentration $1{\cdot}3 \times 10^{-6}$ M) was added to a suspension of rat erythrocytes in platelet-free plasma and luminescence measured in samples taken at time intervals up to 2 min, light emission decayed with a half-time of approximately 20 s (Fig. 3). However, the decay of light emission was much slower in the first of these samples, the half-time being approximately 210 s (Fig. 3); decay was probably inhibited by EDTA present in the luciferin–luciferase reagent as supplied. As ATP was routinely measured within 20 s of sampling, the decay of light emission within this time was less than 10 % and could be disregarded.

In further experiments, we measured the decrease of light output from whole blood with platelets present. The ear artery of a rabbit was punctured, a sample from the first drop of emerging blood was drawn into the measuring tube and light measurement was started after 20 s. Light emission decreased with a half-time of 110 s ($n = 3$) but after 5 min it increased rapidly to well above the initial values. The experiment was repeated after injecting the rabbit intravenously with heparin to produce an initial concentration of about 20 u./ml blood. In this experiment, light emission decreased with a half-time of 95 s ($n = 3$) but there was no subsequent increase. This suggested that the increase in light emission observed in the absence of heparin represented release of ATP from platelets which had been activated by thrombin. This late increase in light emission was observed only with rabbit and not with rat or human

blood. We concluded that accurate determinations of free-ATP concentration during haemostasis could be made when light emission was measured within 20 s after taking the blood sample.

Another question concerned the background level of ATP in whole blood as determined by our technique. It was clearly essential to establish conditions for sampling blood containing as little free ATP as possible. Rabbit blood containing

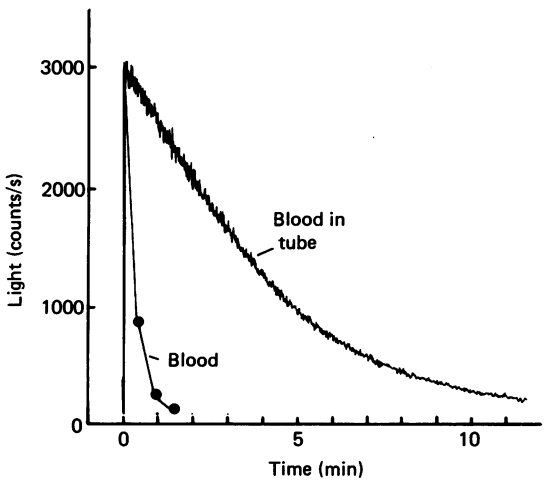

Fig. 3. Decay of light of platelet-free blood from rat. ATP was injected into a sample (200 μl) at a final concentration of $1{\cdot}3 \times 10^{-6}$ M. A sample (1 μl) was drawn into the tube together with luciferin–luciferase (1 μl) and the tube inserted into the measuring stage immediately. Light emission was quantified *vs.* time (curve 'blood in tube'). Samples were also taken at different times after injection of ATP and the *initial* light output was determined (curve 'blood'). The vessel wall was punctured 20 s before zero time.

20 u. heparin/ml was carefully dispensed into Eppendorf centrifuge tubes without shaking or stirring. After 1 h the free-ATP concentration was $2{\cdot}6 \pm 0{\cdot}4 \times 10^{-8}$ M (mean $\pm$ S.E. of the mean; $n = 6$). When the experiment was repeated with an erythrocyte suspension or with platelet-rich plasma, free ATP was $2 \pm 0{\cdot}5 \times 10^{-9}$ M ($n = 6$) and $7 \pm 1{\cdot}4 \times 10^{-10}$ M ($n = 4$) respectively. It was possible to measure ATP at concentrations in the order of 10^{-10} M in blood collected from cannulated rat carotid arteries, giving an absolute background of 10^{-16} mol ATP in 1 μl blood. This concentration was a thousand times lower than that measured during the haemostatic process.

Free-ATP concentration during haemostasis

The first drop of blood collected 2–4 s after puncturing a rat carotid artery or a rabbit ear artery contained free ATP at concentrations of around 2×10^{-7} M (Table 1). After cutting human skin with the Simplate bleeding time device, the free-ATP concentration was about $1{\cdot}8 \times 10^{-6}$ M, i.e. about ten times higher. In all three species, free-ATP concentrations increased to approximately 2×10^{-5} M after 3–6 min, coinciding with blood clotting. The increases could be prevented during haemostasis in rabbit and rat if heparin (20 u./ml blood) was injected intravenously just before

the experiments began. Fig. 4*B* shows typical examples of the changes in free ATP concentration with time during bleeding from rabbit ear artery and from human skin. Some time courses were biphasic with the free-ATP concentration decreasing to a minimum about 1 min after injury and increasing greatly thereafter. This secondary

TABLE 1. Free ATP in blood after injuring rabbit ear artery, rat carotid artery and human skin

	ATP concentration 2 s after injury ($M \times 10^{-7}$)	Maximal ATP concentration ($M \times 10^{-5}$)	ATP concentration after 5 min with 20 u. heparin/ml ($M \times 10^{-7}$)
Rabbit ear artery	2·4±0·7 (29)	2 (3)	0·5 (3)
Rat carotid artery	2±1·4 (5)	1±0·5 (5)	0·18 (3)
Human skin	18±4 (7)	1·4±0·1 (7)	

All values are expressed as mean±S.E. of the mean, with the number of observations, *n*, in parentheses.

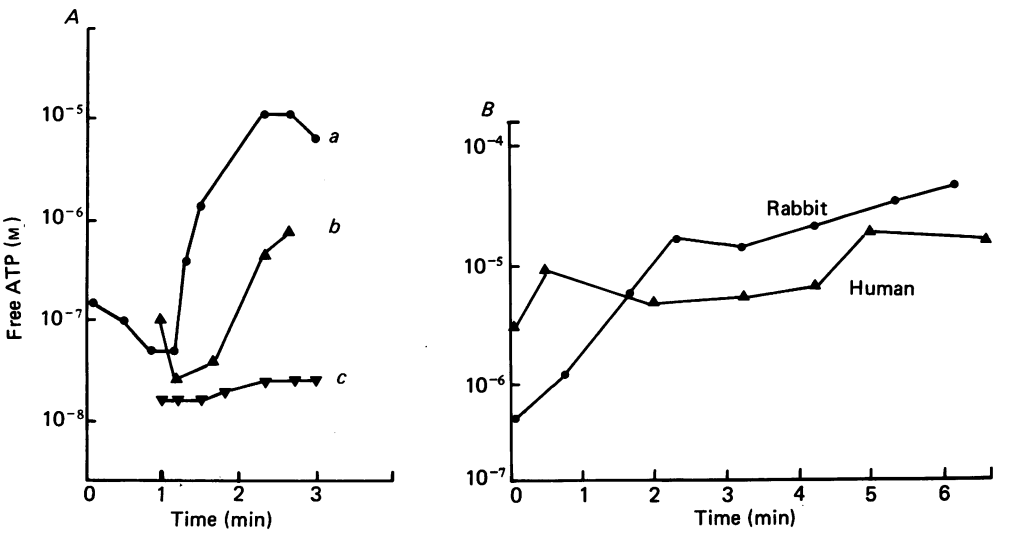

Fig. 4. *A*, free-ATP concentration against time in blood after puncturing rat carotid arteries with a 100 μm steel needle. The rats received no heparin (*a*), 5 u. heparin/ml (*b*), or 10 u. heparin/ml (*c*). *B*, typical examples of free-ATP concentration *vs.* time after puncturing rabbit ear artery with a glass capillary (●) and after injuring human skin (Simplate bleeding time device) (▲). In both experiments, blood was not removed from the injury so that bleeding continued into the blood which had already emerged.

increase in ATP concentration during bleeding from the rat carotid artery was diminished by heparin at 5 and almost abolished at 10 u./ml blood (Fig. 4*A*, *b* and *c*).

Analysis of the cellular source of the initial free ATP

Potential sources of the initial free ATP in blood following injury were the vessel wall, erythrocytes and/or platelets. To determine the source or sources, the following

experiments were done. Rabbits were given 1000 u. heparin I.V. and the shaved surface of an ear was covered with silicone grease and positioned at an angle of 45° to the vertical. An ear artery was punctured through the skin with a glass capillary which confined the injury to the vessel and overlying skin. Drops of blood were made to roll down the hydrophobic silicone-coated skin; this made it possible to collect any particular droplet without mixing it with blood which had emerged before. This permitted determination of time courses of free ATP concentrations (Fig. 5). Even

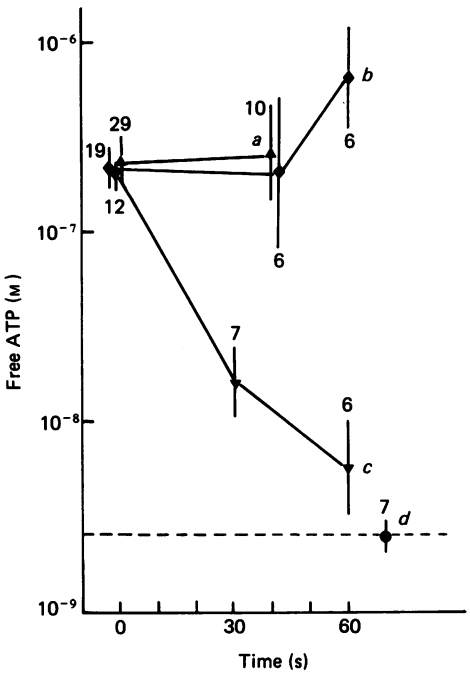

Fig. 5. Free-ATP concentration against time in the emerging blood after puncturing rabbit ear arteries with a 100 μm glass needle. All animals received heparin (20 u./ml) just before the start of the experiment. *a*, controls; *b*, animals treated with indomethacin I.P. 1 mg/kg, 1 h before the start of the experiment; *c*, free ATP in 'saline bleeding'. After perfusion of rabbit ear arteries with physiological saline, the arteries were injured as above. ATP concentration in the outflowing saline was multiplied by 1·82 to correct for the absence of erythrocytes; *d*, free ATP of a saline drop which was in contact with the uninjured ear surface for 30 s. Numbers indicate the number of experiments; vertical bars indicate S.E. of the mean.

under these conditions of minimal injury the first drop of blood collected within 4 s contained $2{\cdot}4 \times 10^{-7}$ M-free ATP and a second sample taken after 40 s contained almost the same concentration (Fig. 5*a*). The rate of loss of blood was about 20 ± 5 μl/min ($n = 10$). When the animal was injected with indomethacin (1 mg/kg body weight) before injury the free-ATP concentration was not changed significantly, while the blood flow increased to 55 ± 30 μl/min ($n = 6$) and the bleeding time also increased (Fig. 5*b*).

Then an ear artery was cannulated at its origin and perfused with heparinized saline

containing albumin. 5 min after the start of the perfusion, the vessel was punctured 2–4 cm distal to the cannulation and the ATP concentration determined in the saline emerging from the wound. Statistical analysis indicated a log normal distribution of the measured values which were therefore submitted to the transformation $x = \log x$ (Gaddum, 1945). ATP values in the extravasated saline were multiplied by 1·82 to correct for the absence of erythrocytes for which the haematocrit volume was 44%. The initial concentration of ATP in extravasated saline was not significantly different from the corresponding values in extravasated blood; but they differed in decreasing rapidly with time (Fig. 5*c*). In little more than 1 min the luminescence due to ATP had decreased to background luminescence, determined by applying physiological saline (40 μl) to the surface of the siliconized ear and making measurements with it 30 s later (Fig. 5*d*).

DISCUSSION

In three species: humans, rabbits and rats, the blood emerging from vessels after injury contained considerable concentrations of free ATP, and so did the fluid emerging from vessels that were perfused with saline. When the results obtained with extravasated normal blood, heparinized blood and heparinized saline were compared, three different origins of ATP could be distinguished.

(1) Because the free ATP in the first drop of blood emerging from injured rabbit ear arteries was not significantly different from that in the first drop of fluid emerging from a similar artery perfused with saline, the source of the ATP could be only damaged cells in the vessel walls. This is consistent with the observation that the greater cell damage caused in humans by the template bleeding technique was associated with a free-ATP concentration an order of magnitude greater than that produced by the more localized puncture of the rabbit ear artery. Much, if not most, of the ATP would come from injured endothelial and smooth muscle cells which contain high concentrations, i.e. 5×10^{-3}–10^{-2} M (Nees & Gerlach, 1983).

(2) The time course of free ATP in the extravasated saline (Fig. 5*c*) showed the concentration as decreasing rapidly, suggesting that nucleotides were washed out from the injured vessel wall cells. In contrast, in extravasated blood during normal haemostasis, the level of ATP (Fig. 5*a*) was nearly constant up to the cessation of bleeding. Therefore, there must have been release of additional ATP, presumably from blood cells.

The origin of this ATP might be the blood platelets which adhere at the site of injury and release their dense-body contents into the passing blood stream. A free-ATP concentration of 10^{-7} M could be produced if only about 1% of platelets flowing through a punctured vessel wall were to adhere as a haemostatic plug, with release of the ATP and ADP from their storage granules. The time course of this release during haemostasis *in vivo* has been indicated by a quantitative electron microscopic method (Görög & Born, 1981). After injury of small arteries in the mouse mesentery, there was no significant decrease of dense bodies in the platelet plug after 15 s; but after 60 s the concentration of dense bodies in these platelets had decreased by about 60% compared with platelets in the circulating blood. Thus, under these conditions the release reaction occurred after 15–60 s. This is consistent with our observation of a release of ATP at $t = 40$ s (Fig. 5*b*). ATP levels at this time were not changed

significantly by indomethacin, which would seem to be inconsistent with this ATP coming from platelets because indomethacin inhibits the release reaction. However, inhibition by indomethacin can be overcome *in vitro* by sufficiently high concentrations of ADP. Secretion of nucleotides from platelets can be induced by collagen and thrombin when cyclooxygenase is blocked. Under the conditions of our experiment where large amounts of heparin were present, the contribution of thrombin to ATP release would be expected to be minimal. This result therefore indicates a role for collagen at a later stage of haemostasis.

(3) Free ATP appeared between 3 and 7 min after vascular injury. At this time the concentration of free ATP in the extravasating blood rose to about 2×10^{-5} M, which was much higher than before. The increase occurred in the pool of blood remaining at the injury site, it was followed by clotting and could be prevented by high concentrations of heparin (20 u./ml). This suggests that the release of ATP is induced by thrombin, presumably from platelets. This conclusion is supported by experiments in which the release of ATP from platelets after addition of thrombin to human blood was determined *in vitro* with the luciferin–luciferase technique (Ingerman, Smith & Silver, 1979). The maximal ATP concentration was $6{\cdot}5 \times 10^{-6}$ M which, when corrected for total erythrocyte volume, made the plasma concentration $1{\cdot}2 \times 10^{-5}$ M, in agreement with our *in vivo* value for human subjects which was $1{\cdot}4 \times 10^{-5}$ M.

We have determined only the concentration of free ATP in extravasating blood. Haemostatic platelet aggregation is initiated not by ATP but by ADP (Zawilska *et al.* 1981; Begent & Born, 1983) so that its concentration in the local blood plasma is the important determinant. There are two reasons for inferring the simultaneous presence of free ADP under the conditions in which we have demonstrated free ATP. First, free ATP is cellular in origin: as all viable cells also contain ADP, release of ATP is presumably accompanied by release of ADP. Nees & Gerlach (1983) measured the concentrations of adenine nucleotide in coronary endothelial cells, which are one of the sources of free nucleotides after an artery has been injured. The ratio ADP/ATP is 0·2 and the absolute ADP concentration is in the order of magnitude of 10^{-3} M. This concentration is a thousand times higher than that required for maximal activation of human blood platelets. Adenine nucleotide concentration was determined in several other tissues with the corresponding ADP/ATP ratios, which range from 0·2 to 0·65 (Gerlach, Bader & Schwoerer, 1961; Deuticke & Gerlach, 1966). If cells of these tissues are damaged, ADP is set free in high concentrations.

Secondly, ATP free in the blood is rapidly dephosphorylated to ADP by ectoenzymes present on the surface of endothelial cells, as well as on other vessel wall cells, which become exposed to the flowing blood by vascular injury. The ecto-ATPase of cultured pig aortic endothelial cells is approximately twice as active as the corresponding ADPase, so that contact of ATP with the endothelial surface causes accumulation of ADP. The ecto-ATPase activity of endothelial and smooth muscle cells is remarkably high (Pearson, Carleton & Gordon, 1980; Cusack, Pearson & Gordon, 1983) and ATP near the cell surface will be dephosphorylated to ADP in a very short time.

Our results and these considerations therefore make it probable that ADP will be

present at the site of vascular injury at around 10^{-7} M, which is enough to activate platelets.

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