J. Physiol. (1968), **195**, *pp.* 715–729
With 2 plates and 6 text-figures
Printed in Great Britain

THE RELEASE OF NUCLEOTIDES, 5-HYDROXYTRYPTAMINE AND ENZYMES FROM HUMAN BLOOD PLATELETS DURING AGGREGATION

By D. C. B. MILLS, I. A. ROBB and G. C. K. ROBERTS

From the Department of Pharmacology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2 and the Electron Microscopy Unit, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C. 2

(*Received* 10 *November* 1967)

SUMMARY

1. Adenosine diphosphate (ADP) and adrenaline caused the aggregation of human platelets suspended in plasma containing citrate anticoagulant and stirred at 37° C. The aggregation occurred in two phases and the second phase was associated with the appearance in the plasma of up to 30 % of the ATP and 55 % of the ADP present in the platelets. The concentration of ADP appearing in the plasma was up to 7 times the concentration added.

2. Radioactivity was released by ADP and by adrenaline from platelets labelled with radioactive 5-hydroxytryptamine; this release was closely correlated with the second phase of aggregation and with the release of nucleotides.

3. Acid phosphatase, β-glucuronidase and adenylate kinase were released to a small extent during second phase aggregation by ADP or adrenaline; thrombin and collagen particles caused significantly greater release of β-glucuronidase than of either acid phosphatase or of adenylate kinase.

4. Morphological changes indicating degranulation of the platelets were observed during the second phase of aggregation produced by adrenaline and by ADP.

5. The second phase of aggregation, degranulation of platelets, and the release of nucleotides, of labelled 5-hydroxytryptamine and of enzymes, were all inhibited by concentrations of amitriptyline which did not inhibit aggregation.

INTRODUCTION

During the clotting of blood, 5-hydroxytryptamine (5-HT) is released into the serum by the action of thrombin on the platelets (Zucker & Borelli, 1955). Thrombin (Käser-Glanzmann & Lüscher, 1962; Grette, 1962), collagen (Hovig, 1963; Spaet & Zucker, 1964) and a number of other high molecular weight materials and particles (see Marcus & Zucker, 1965) produce profound biochemical and morphological changes in the platelets of man and other species. These changes were described for pig platelets treated with thrombin by Grette (1962) who called the process the platelet release reaction. The major features of this reaction are the selective release of large proportions of the adenine nucleotides and 5-HT from the platelets (Grette, 1962; Buckingham & Maynert, 1964) and the disappearance of intracellular organelles (Parmeggiani, 1961; Hovig, 1962; Rodman, Mason & Brinkhous, 1963), together with an increase in the rate of glycolysis (Bettex-Galland & Lüscher, 1960; Karpatkin, 1967). Low concentrations of ADP cause aggregation of platelets (Gaarder, Jonsen, Laland, Hellem & Owren, 1961) and the aggregation produced by thrombin and collagen is due to ADP released from the platelets (Haslam, 1964, 1967).

When platelet aggregation in human citrated plasma is followed turbidimetrically (Born, 1962) the curves obtained with adrenaline (O'Brien, 1963) or with ADP (Macmillan, 1966) can be resolved into two phases. During the second phase platelets release an aggregating agent with the characteristics of ADP (Macmillan, 1966). Imipramine and chlorpromazine inhibit this second phase of aggregation at concentrations which have no effect on the first phase (Mills & Roberts, 1967*a*); they also inhibit aggregation by collagen, and the second phase of aggregation by thrombin (Thomas, 1967). It seems that these substances may act by inhibiting the release of ADP from the platelets.

We have now shown that ADP and adrenaline, like thrombin and collagen, can cause the release of nucleotides and 5-HT from human platelets in citrated plasma and that this release is inhibited by compounds similar to imipramine. A preliminary report of some of these findings has been published (Mills & Roberts, 1967*b*).

METHODS

Reagents. ADP, ATP and collagen and adrenaline acid tartrate (Sigma London, Ltd.) were made up and stored as previously described (Mills & Roberts, 1967*a*, *c*). Firefly lantern extract, *p*-nitrophenyl phosphate and *p*-nitrophenyl glucuronide were also obtained from Sigma London Ltd. Pyruvate kinase, phosphoenolpyruvate and nicotinamide adenine dinucleotide, reduced form (NADH) were from Boehringer Corp. (London) Ltd. and bovine thrombin from Parke, Davis and Co. Detroit, Michigan, U.S.A. Triton X-100 was a gift from

Lenning Chemicals Ltd. London and 5-hydroxytryptamine [3′-^{14}C]creatinine sulphate was obtained from the Radiochemical Centre, Amersham. Amitriptyline was obtained from Roche Products Ltd. Welwyn Garden City, Herts.

Platelet-rich plasma. Human venous blood was collected by free flow into 0·1 vol. of 3·8 % trisodium citrate (giving a final citrate concentration in the plasma of about 31 mM). Platelet-rich plasma was prepared by centrifuging at 320 *g* for 10 min; the platelet count, determined by phase contrast microscopy, was between 1·5 and 6·3 × 10^8/ml. and contamination by red cells was always less than 1·0 %.

Platelet aggregation. Aggregation of platelets was studied photometrically (Born, 1962) in silicone-treated glass tubes at 37° C using a modified apparatus (Mills & Roberts, 1967*a*) with continuous recording of transmission. At the end of each run, 0·1 ml. of 0·1 M ethylenediaminetetra-acetate (EDTA) was added to the sample tube containing 1·0 ml. platelet-rich plasma. The magnetic stirrer was removed and the tube cooled in an ice/methanol bath at −5° C. It was then centrifuged at 650 *g* for 1 min in a bench centrifuge to remove aggregates and most of the platelets. The supernatant was transferred with a silicone-treated Pasteur pipette to a polypropylene centrifuge tube and the remaining platelets were removed by centrifuging at 15,000 *g* for 10 min at 0° C. The supernatant was decanted and samples were taken for various estimations.

Enzyme determinations. Adenylate kinase (EC 2.7.4.3.) was determined in the backward direction by coupling the conversion of ATP and adenosine monophosphate (AMP) to ADP to the oxidation of NADH (Haslam & Mills, 1967). Acid phosphatase (EC 3.1.3.2.) was determined with *p*-nitrophenyl phosphate as substrate (Linhardt & Walter, 1963). β-Glucuronidase (EC 3.2.1.31.) was measured with *p*-nitrophenyl glucuronide as substrate (Szasz, 1967) with 4 hr incubation at 37° C. Samples of 0·1 or 0·2 ml. of plasma were used for enzyme assays, and the content of these enzymes in the platelets was determined on samples of platelet-rich plasma in which the platelets were lysed by the addition of 1 vol. of 1 % Triton X-100. Results are expressed as enzyme units (e.u.) at 37° C using 6·2 × 10^3 for the molar extinction of NADH at 340 mμ and 18·8 × 10^3 for the molar extinction of *p*-nitrophenol at 400 mμ in alkali.

Release of 5-hydroxytryptamine. For determining the release of 5-HT, platelet-rich plasma was incubated with 0·5 μM [3′-^{14}C]5-hydroxytryptamine creatinine sulphate (32 c/mole) for 1 hr at 18–20° C. During this time 90–95 % of the radioactivity was incorporated into the platelets. Radioactivity was measured by counting samples of 0·2 ml. plasma absorbed on a half-circle of glass fibre filter paper (Whatman GFA, Reeve Angel Co. Ltd.) with 15 ml. Diotol scintillation fluid (Herberg, 1960) in a Packard Tricarb scintillation counter. The amounts of radioactivity remaining in the plasma did not change significantly during the course of the experiments.

Release of adenine nucleotides. The firefly luminescence method (Holmsen, Holmsen & Bernhardson, 1966) was used for the determination of ATP and ADP. Samples of 0·2 ml. plasma were added to 0·6 ml. 66 % aqueous ethanol v/v and stored in a deep freeze until assayed. The samples were warmed to room temperature, centrifuged at 650 *g* for 5 min to remove precipitated proteins and portions of the supernatant taken for assay. For the determination of ATP plus ADP, 50 μl. of the supernatant was added to 0·2 ml. of a reaction mixture with the following composition: potassium phosphoenolpyruvate, 0·8 mM; $MgSO_4$, 24 mM; KCl, 78 mM; triethanolamine hydrochloride buffer at pH 7·4, 20 mM; pyruvate kinase, 0·6 e.u./ml. Samples for ATP alone were added to 0·2 ml. of the same reaction mixture which had been inactivated by boiling. After incubation for 5 min at 37° C the reaction was stopped by heating to 100° C for 3 min and the samples transferred with two washes of 1·1 ml. water to glass cuvettes and placed in a cuvette holder adjacent to the photomultiplier of a modified Aminco-Bowman fluorimeter. Firefly lantern extract was prepared by lightly homogenizing the equivalent of 50 mg dried lanterns in 5 ml. water in an all-glass, hand homogenizer and stored in ice. It was found to be relatively stable on storage

for 1–3 days in the deep freeze. Using a 1·0 ml. tuberculin syringe, 0·2 ml. of the suspension was injected with 0·8 ml. air into the cuvette through a small hole in the top of the cuvette holder. The light emitted was recorded during the injection on a 1 mV pen recorder at a chart speed of 10 cm/min and the maximum output used in calculations. Internal standards of ATP and ADP were used to calibrate each batch of lantern extract. For the determination of total platelet ATP and ADP, 0·1 ml. of 0·1 M EDTA and 0·9 ml. of 1% Triton X-100 were added to 1·0 ml. platelet-rich plasma which was allowed to stand at 0° for 30–60 min; this caused lysis of the platelets. Ethanol was then added to a final concentration of 50% and the samples were stored and assayed as for plasma.

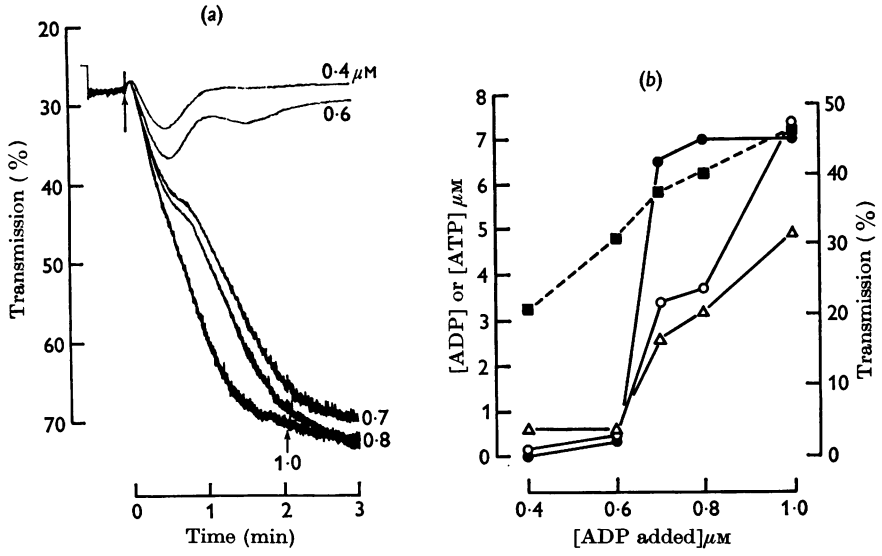
Electron microscopy. Samples for electron microscopy were fixed initially by adding 20% glutaraldehyde to give a concentration of 0·1% in the platelet-rich plasma. Platelets and aggregates were then centrifuged at 900 *g* for 30 sec at room temperature. The plasma was removed with a Pasteur pipette and the sediment gently resuspended in 3% glutaraldehyde in 0·1 M phosphate buffer pH 7·3 and kept at 4° C for 1 hr. The samples were centrifuged, washed 3 times with buffer and finally resuspended in 2% osmium tetroxide in isotonic phosphate buffer pH 7·3 containing glucose (Millonig, 1961) for 1 hr at 4° C. The fixative was removed by centrifuging and the platelets were washed once with water, dehydrated through graded alcohols and embedded in Araldite. Ultra-thin sections (700–900 Å) were examined in a Phillips EM 75 electron microscope.

RESULTS

Text-figure 1*a* demonstrates the abrupt transition from reversible to biphasic aggregation which occurs with critical concentrations of ADP; Text-fig. 1*b* shows the concentrations of ADP and of ATP found in the plasma 3 min after the addition of ADP. The parameters used to describe the first and second phases were V (the initial rate of aggregation) and ΔT (the change in transmission after 3 min; Mills & Roberts, 1967*a*) respectively.

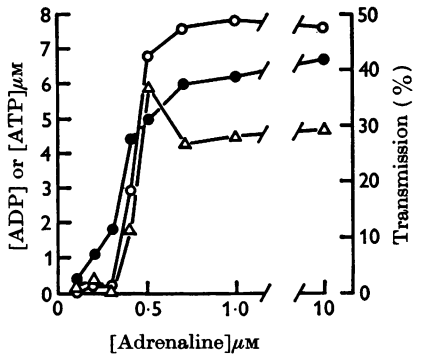
When aggregation was produced by 1·0 μM of added ADP, the concentration of ADP in the plasma after 3 min was 7 μM. Over the range of ADP concentrations added there was a continuous increase in V but a sudden increase in ΔT between 0·6 and 0·7 μM. The appearance of adenine nucleotides in the plasma corresponded closely with the occurrence of the second phase. With increasing concentrations of added ADP (0·8–1·0 μM), the ADP concentration continued to rise in the plasma although ΔT had reached maximum. The appearance of adenine nucleotides in the plasma during the second phase can be attributed to their release from the platelets. Since the concentration of ADP required to produce the second phase of aggregation and the release of nucleotide varied from one donor to another over the range 0·5–5 μM, it was not possible to treat results from different donors together; the figures given below are the results of representative individual experiments.

Release of ATP and ADP from platelets was also observed during the second phase of aggregation produced by adrenaline. In the experiment of Text-fig. 2, concentrations of adrenaline up to 0·3 μM produced first phase



Text-fig. 1(*a*). Superimposed traces of aggregation curves obtained with a range of ADP concentrations added to citrated human platelet-rich plasma containing $4{\cdot}91 \times 10^8$ platelets/ml. stirred at 37° C in the aggregation apparatus. ADP was added at the arrow to give the final concentrations (μM) shown beside each trace.

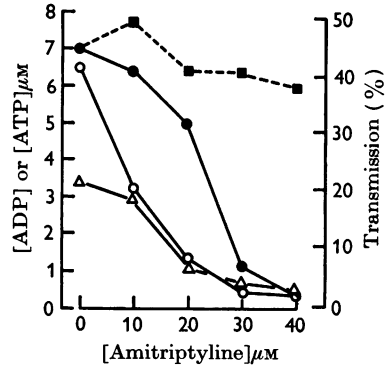
(*b*) Measurements of the first and second phases of aggregation *V*, (■ - - - ■) and ΔT (●——●) made on the traces shown in (*a*) are plotted in units of % transmission against the right-hand axis. Concentrations of ATP (△——△) and ADP (○——○) found in the plasma 3 min after the addition of ADP are plotted against the left-hand axis in n-moles/ml. plasma.



Text-fig. 2. The aggregation produced by a range of concentrations of adrenaline measured by the change in % transmission 3 min after adrenaline (ΔT, ●——●) is plotted against the right-hand axis in units of % transmission. Concentrations of ATP (△——△) and ADP (○——○) found in the plasma 3 min after adrenaline are plotted against the left-hand axis in n-moles/ml. plasma.

aggregation only and no nucleotides were found in the plasma. At 0·4–0·5 μM adrenaline the appearance of the second phase was accompanied by the release of ATP and ADP; at higher concentrations of adrenaline the amount of ATP found in the plasma fell to 4·3 μM while the ADP concentration continued to rise to a maximum of 7·7 μM at 1 μM adrenaline. During the second phase up to 30% of the platelet ATP and 55% of the ADP were released; the ATP/ADP ratio in the plasma was between 0·5 and 1·0.

Text-figure 3 shows that the release of nucleotides from platelets during the second phase is inhibited by amitriptyline which, like imipramine, inhibits the second phase of aggregation by ADP (Mills & Roberts, 1967*a*).



Text-fig. 3. Effect of range of concentrations of amitriptyline added 0·5 min before ADP (1 μM) to platelet-rich plasma containing 4·91 × 10^8 platelets/ml. First phase (*V*, ■----■) and second phase (ΔT, ●——●) are plotted in units of % transmission against the right hand axis. Concentrations of ATP (△——△) and ADP (○——○) found in the plasma 3 min after the addition of ADP are plotted against the left-hand axis in n-moles/ml. plasma.

The plasma sample used in this experiment showed two phase aggregation with 1·0 μM added ADP, and pretreatment with 10–40 μM amitriptyline reduced both the extent of second phase aggregation and the amount of nucleotide released. At 10 μM, amitriptyline had little effect on aggregation although the ADP release was reduced by one half.

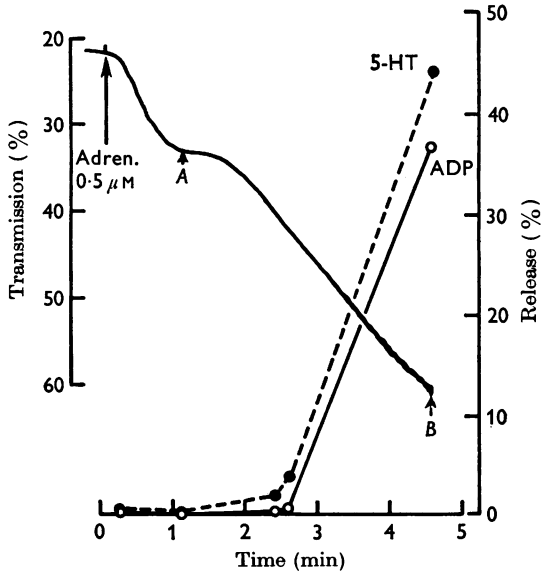
As ADP can cause nucleotide release, we examined the possibility that it can also cause the ultrastructural changes which accompany the release reaction. Plate 1 shows electron micrographs of aggregates fixed 3 min after adding ADP to platelet-rich plasma at a concentration which gave two-phase aggregation. In Pl. 1*a* an aggregate is shown in which most of the platelets appear to have lost their granules although the outlines of individual platelets can still be seen. Platelets at the edge of the aggregate have undergone the greatest changes. Large pseudopods can also be seen

which are entirely free of granules, but are continuous with areas containing granules deeper in the aggregate. When the same concentration of ADP was added after pretreatment with 20 μM amitriptyline, the second phase of aggregation was partly inhibited and the platelets were less densely packed and retained their granules (Pl. 1*b*). With 40 μM amitriptyline, most of the aggregates had dispersed after 3 min and the electron microscopic appearance of the individual platelets was essentially normal. Adrenaline caused ultrastructural changes similar to those caused by ADP; the changes become visible only during the second phase of aggregation (Pl. 2).

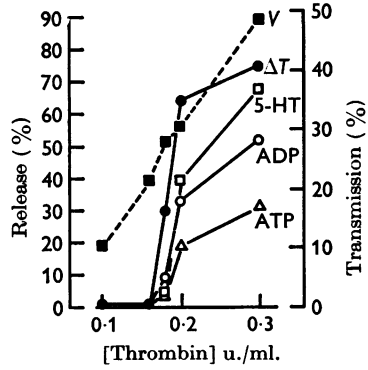
These observations suggested that the release of 5-HT associated with degranulation by thrombin (Grette, 1962), and by collagen (Spaet & Zucker, 1964) might be brought about by adrenaline and by ADP. We therefore followed the release of 5-HT by measuring the appearance of radioactivity in the plasma during the aggregation of platelets which had previously been allowed to take up radioactive 5-HT. Table 1 shows the results of an experiment in which thrombin, ADP and adrenaline are compared. With concentrations chosen to give near-maximal second phase aggregation, all three agents released substantial quantities of 5-HT although ADP caused less release than either adrenaline or thrombin. Release by all three agents was inhibited by 30 μM amitriptyline. The release of 5-HT by thrombin and adrenaline correlated well with the release of nucleotides. In this experiment the amount of ADP needed to provoke second-phase aggregation was so high that no attempt was made to measure released nucleotides.

The association of the release by adrenaline of ADP and 5-HT with the second phase of aggregation is demonstrated by the time course experiment shown in Text-fig. 4. Little or no release of 5-HT or ADP occurred until the second phase was well under way. In a parallel experiment samples were fixed for electron microscopy at various points along the aggregation curve. Electron micrographs of aggregates fixed during the first (*A*) and second (*B*) phases are shown in Pl. 2 and corresponding points on the aggregation curve are marked in Text-fig. 4. The platelets in first phase aggregates were loosely packed and showed no degranulation, whereas during the second phase the platelets became densely packed and contained few granules.

When thrombin is added to platelet-rich plasma, the second phase of aggregation appears at a critical concentration below which aggregation is reversible (Thomas, 1967). The concentration of thrombin required to produce second phase varies from one donor to another so that in these respects thrombin is similar to ADP. An experiment in which the release of nucleotides and of 5-HT was measured 2 min after the addition of thrombin



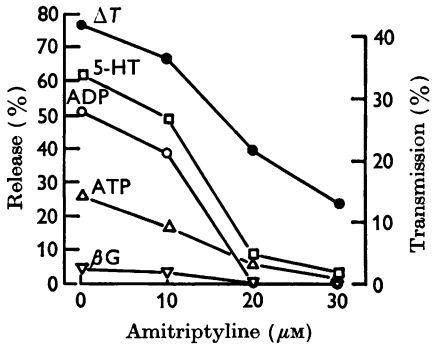
Text-fig. 4. Samples of platelet-rich plasma were stirred in the aggregation apparatus for various times after the addition of 0·5 μM adrenaline. The % release of radioactive 5-HT (●----●) and of ADP (○——○) is plotted against the right-hand axis and a composite aggregation curve is drawn against the % transmission scale (left-hand axis). The platelet-rich plasma contained $6{\cdot}23 \times 10^8$ platelets and 6×10^6 red cells/ml. and 3·02 n-moles ADP/10^8 platelets. The arrows *A* and *B* indicate points on the aggregation curve corresponding to the samples taken in another experiment for electron microscopy (Pl. 2).



Text-fig. 5. First phase (*V*, ■----■) and second phase (ΔT, ●——●) of aggregation produced by adding thrombin to platelet-rich plasma, are plotted against the right-hand axis in units of % transmission. Percentage release of ADP (○——○), ATP (△——△) and of radioactive 5-HT (□——□) are plotted against the left-hand axis. The platelets contained 3·2 n-moles ADP and 4·9 n-moles ATP per 10^8 cells, and there were $4{\cdot}86 \times 10^8$ platelets/ml. plasma.

is shown in Text-fig. 5. Whereas the first phase (V) increased linearly with thrombin concentration, the second phase (ΔT, measured 2 min after the addition of thrombin) appeared suddenly at 0·16–0·20 u./ml., and was accompanied by release of 5-HT and nucleotides. No release was detected with concentrations of thrombin which produced only reversible aggregation.

Amitriptyline inhibited both the second phase of aggregation and the release of nucleotides and 5-HT caused by ADP, adrenaline and thrombin (Text-fig. 2, Table 1). The results given in Text-fig. 6 show that in the concentration range 10–30 μM, amitriptyline causes an increasing and parallel inhibition of the release of ADP and 5-HT produced by 0·5 μM adrenaline. The release of ATP is also inhibited but in a manner which does not follow so closely the release of 5-HT.



Text-fig. 6. Inhibition by amitriptyline of aggregation and release caused by adrenaline (10 μM). Aggregation measured by ΔT (●——●) 3 min after adrenaline is plotted against the right-hand axis in units of % transmission. Percentage release of ADP (○——○), ATP (△——△), radioactive 5-HT (□——□) and of β-glucuronidase (βG, ▽——▽) are plotted against the left-hand axis. The platelet-rich plasma was the same sample as that used in Fig. 5, and the platelets contained 0·00036 e.u. β-glucuronidase per 10^8 cells.

Acid phosphatase is released from platelets by thrombin (Zucker & Borelli, 1958) and the release of lysosomal enzymes exemplified by acid phosphatase has been put forward as a general mechanism for the platelet release reaction (Kowalski, Kopeć, Węgrzynowicz, Hurwic & Budzyński, 1966). Adenylate kinase is released from human platelets during aggregation by ADP (Haslam & Mills, 1967). We have measured the release from platelets during aggregation of β-glucuronidase, a lysosomal enzyme (Gianetto & De Duve, 1955); acid phosphatase, which has been found in both lysosomal and non-lysosomal granules in platelets (Siegel & Lüscher, 1967); and adenylate kinase. The latter, a low molecular weight enzyme present in soluble form in cytoplasm, was chosen as a control for platelet

damage since its activity in platelets is high compared with that in plasma (Haslam & Mills, 1967). Table 2 shows the release of nucleotides and of enzymes produced by ADP, thrombin and collagen. Whereas ADP caused

TABLE 1. Release of adenine nucleotides and of [^{14}C]5-HT from platelets during aggregation

Samples of 1·0 ml. human citrated platelet-rich plasma previously incubated with radioactive 5-HT were incubated with stirring for 3 min at 37° C after the addition of the aggregating agent. Amitriptyline, when included, was added as a 10 mM solution in 0·154 M-NaCl, 0·5 min before the aggregating agent. At the end of the incubation the platelets were removed by centrifuging and samples of the plasma taken for measurement of radioactivity and nucleotide content. The platelets ($4{\cdot}86 \times 10^8$/ml. plasma) contained 4·9 n-moles ATP and 3·2 n-moles ADP/10^8 cells.

Aggregating agent and concentration	Amitriptyline conc. (μM)	V (% T/min)	ΔT (% T)	Release (%) 5-HT	ADP	ATP
ADP, 5 μM	0	36	11	1·4	—	—
ADP, 10 μM	0	44	36	26·3	—	—
ADP, 10 μM	30	46·5	19	7·8	—	—
Thrombin, 0·16 u./ml.	0	23	0·6	1·3	0·6	0·4
Thrombin, 0·30 u./ml.	0	50·5	43	68·2	52	31·4
Thrombin, 0·30 u./ml.	30	51	41·5	56·3	35	17·5
Adrenaline, 10 μM	0	14	44	62	51	26·3
Adrenaline, 10 μM	30	14	14	3·6	0	1·7

TABLE 2. Release of nucleotides and of enzymes during platelet aggregation

Samples of 1·0 ml. citrated human platelet-rich plasma were stirred at 37° C in the aggregation apparatus. Three minutes after the addition of the aggregating agent the platelets were removed by centrifuging and measurements made of acid phosphatase (AP), β-glucuronidase (βG) and adenylate kinase (AK) in the supernatant plasma. Ethanol extracts of the plasma were assayed for ADP and ATP. There were $4{\cdot}31 \times 10^8$ platelets/ml. platelet-rich plasma and the platelets contained (per 10^8 cells) 0·017 e.u. AP, 0·00054 e.u. βG, 0·078 e.u. AK, 5·2 n-moles ATP and 3·7 n-moles ADP.

Aggregating agent and concentration		ΔT (% T)	Release (%) ADP	ATP	AP	βG	AK
ADP	0·2 μM	0	1	0	0	0	0
	0·5	10	6	2	1·5	0	0
	1·0	26	31	5	1·5	0	0·8
	2·0	39	28	20	0·8	0·7	1·0
	5·0	35	44	24	1·2	0	1·4
Thrombin	0·2 u./ml.	0	0	5	0·3	0	0·1
	0·25	17	12	0	0·3	1·8	1·0
	0·3	33	35	23	0·7	2·2	2·4
	0·4	36	41	20	2·2	5·6	2·9
	1·0*	44	57	16	2·3	11·6	4·1
Collagen suspension	5 μl.	2	0	8	0·3	1·2	0
	7	4	2	4	0·5	3·9	0·2
	10	19	12	13	0·9	1·0	0·5
	15	26	21	15	1·1	4·9	1·2
	20	31	31	20	1·6	9·1	1·3

* Clotting occurred in this sample but not in any of the others.

the release of between 30 and 60 % of platelet ADP, the maximum release of acid phosphatase and β-glucuronidase was less than 3 % and never significantly higher than that of adenylate kinase. Although it was inhibited by amitriptyline, this small release was probably due to platelet damage. Adrenaline caused a similar pattern of release (Text-fig. 6); thrombin and collagen, on the other hand, caused the release of significantly greater amounts of β-glucuronidase than of either acid phosphatase or adenylate kinase.

DISCUSSION

When human platelets in citrated plasma at 37° C aggregate after the addition of ADP, a second wave of aggregation can be demonstrated during which a material is released into the plasma which has the platelet aggregating characteristics of ADP (Macmillan, 1966). We have found that ADP causes the release of ADP, ATP and 5-HT during the second phase of aggregation and that this release is accompanied by ultrastructural changes in the platelets similar to those which occur during the release reaction provoked by thrombin and collagen (Grette, 1962; Spaet & Zucker, 1964). The aggregates consist of densely packed platelets most of which appear to have lost their granules. Hovig (1962) and Rodman *et al.* (1963) failed to show degranulation or nucleotide release during aggregation by ADP, using rabbit platelets and human washed platelets respectively. This supports our observation that a second phase cannot be demonstrated with either washed platelets or those of rats and rabbits. ADP can also cause an increase in the availability of the phospholipid procoagulant material platelet factor 3 (Hardisty & Hutton, 1966; Pizzuto, Giorgio & Didisheim, 1966). Activation of platelet factor 3, and release of 5-HT and of an aggregating agent by ADP have also been observed by Zucker & Peterson (1967). It is justifiable, therefore, to add ADP to the list of agents which can initiate the platelet release reaction.

Certain drugs including imipramine and amitriptyline inhibit the second phase of aggregation at concentrations below those that interfere with aggregation by ADP (Mills & Roberts, 1967*a*). Amitriptyline inhibits the release reaction caused by ADP, adrenaline, thrombin and collagen. The release of ADP, ATP and 5-HT and the loss of granules can all be prevented by pretreatment with 30–40 μM amitriptyline. Inhibition of release can be demonstrated at lower concentrations which have little effect on the optical changes associated with second phase. The way in which amitriptyline acts may be related to the ability of compounds of its type to stabilize biological membranes (for refs. see Mills & Roberts, 1967*a*).

During the release reaction, up to 55 % of the total platelet ADP and 30 % of the ATP appear in the plasma in which the ATP/ADP ratio is

0·5–1·0, compared with 1·5–2·5 in whole platelets. These ratios agree with those found by Ireland (1967) after treating washed platelets with thrombin. The nucleotides released by thrombin or collagen from platelets which have incorporated radioactive adenosine or inorganic phosphate have a lower specific radioactivity and a lower ATP/ADP ratio than the total platelet nucleotides (Holmsen, 1965; Ireland, 1966, 1967). This suggests that the released nucleotides come from a metabolically inert pool. The metabolically active pool is probably in the cytoplasm and mitochondria. It is reasonable to suggest, therefore, that the inert pool is located in and released directly from one or more of the different types of platelet granule. These include lyosomes (Marcus, Zucker-Franklin, Safier & Ullman, 1966), α-granules (Siegel & Lüscher, 1967) and 5-HT storage granules (Tranzer, DaPrada & Pletscher, 1966; Solatunturi & Paasonen, 1966); the latter are rare in human platelets. When 5-HT is released by thrombin it is not metabolized by platelet monoamine oxidase and so presumably also passes directly from its storage granules into the plasma (DaPrada, Bartholini & Pletscher, 1965).

Our experiments show a close correlation between the release of adenine nucleotides and that of 5-HT, but little correlation between the release of nucleotides and that of either acid phosphatase or β-glucuronidase. Neither ADP nor adrenaline released more acid phosphatase or β-glucuronidase than adenylate kinase. It is possible that the small amount of these enzymes which were released (less than 4% of the total) originated from a few disrupted platelets rather than from a specific release mechanism as appears to exist for nucleotides and 5-HT. Significantly greater release of β-glucuronidase (up to 10% of the total) occurred with thrombin and collagen. This suggests that these agents, as well as initiating the release reaction, may provoke other changes including lysosomal activation (Kowalski *et al.* 1966). In support of this idea, Thomas (1967) has obtained indirect evidence that thrombin and collagen but not ADP cause the release from platelets of catecholamines which potentiate the aggregating action of ADP released at the same time. Low concentrations of thrombin cause reversible aggregation which is not inhibited by imipramine (Mills & Roberts, 1967*a*). Thomas (1967) found that slightly higher concentrations of thrombin can produce two phase aggregation, the second phase of which is inhibited by imipramine. We have now shown that this second phase is associated with the release of nucleotides and 5-HT. The possibility cannot be excluded that small amounts of ADP are also released during the first phase of thrombin aggregation as the measurements were made 2 or 3 min after the addition of thrombin, by which time aggregation had reversed spontaneously.

Adrenaline causes two-phase aggregation in some samples of plasma but

not in others (O'Brien, 1963). The first phase is unusual as the aggregates remain very loose and the platelets do not swell as they do on contact with ADP (Bull & Zucker, 1965). During the second phase the aggregates become densely packed, degranulation occurs and ATP, ADP and 5-HT are released into the plasma. This release is inhibited by amitriptyline at concentrations which do not affect the first phase. The failure of the platelets to swell and the comparative irreversibility of the first phase suggest that it may not involve ADP but be due to a direct action of adrenaline.

The relevance of the platelet release reaction to the formation of haemostatic plugs and thrombi *in vivo* has not been established. However, as pointed out by Born (1965), the rapid transition from the initial adhesion of a few platelets at a site of endothelial damage to the formation of a plug of platelets large and solid enough to form a mechanical obstruction to the blood flow requires some type of chain reaction. The initial stimulus to adhesion has to be passed on to platelets which have not themselves come in direct contact with the damaged tissue. The release reaction may underlie this chain reaction. Platelets exposed to 1·0 μM ADP can release enough ADP to give a concentration of 7 μM in the plasma, affording a 'multiplication factor' of 7; after potentiation by adrenaline this 'multiplication factor' is higher still (Mills & Roberts, 1967*c*). This may be enough to counteract the rapid removal of ADP by dilution in the flowing blood and by enzymic attack (Mills, 1966). Such a process will stop when the supply of fresh platelets is cut off as the aggregate stops the blood flow. The increased density of packing of the platelets during second phase aggregation (Born & Hume, 1967) may contribute to the solidity of the platelet plug which may be stabilized further by the release of clotting factors and the formation of fibrin. If this occurs *in vivo* the release reaction will be of fundamental importance in haemostasis and in thrombosis.

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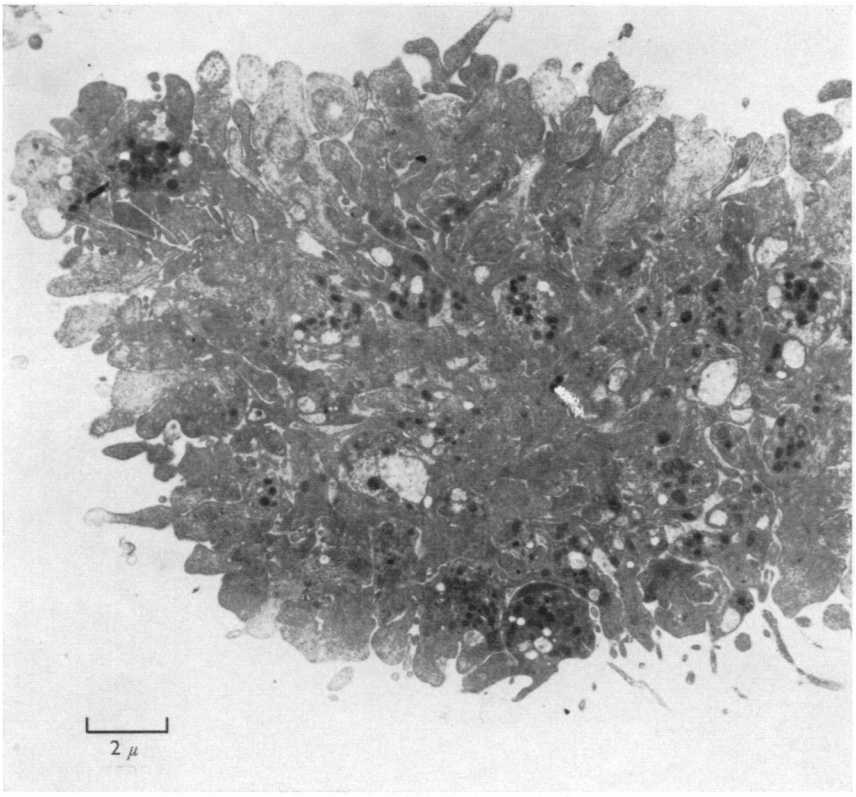

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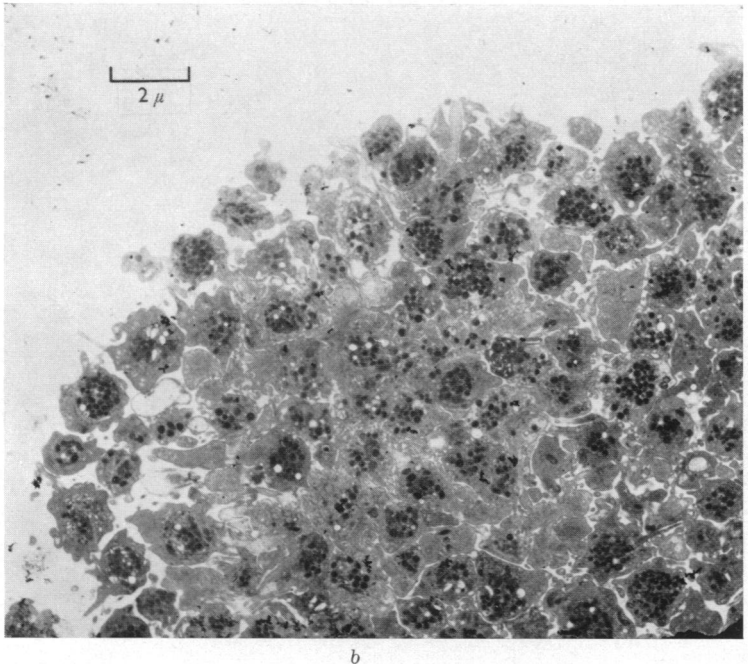

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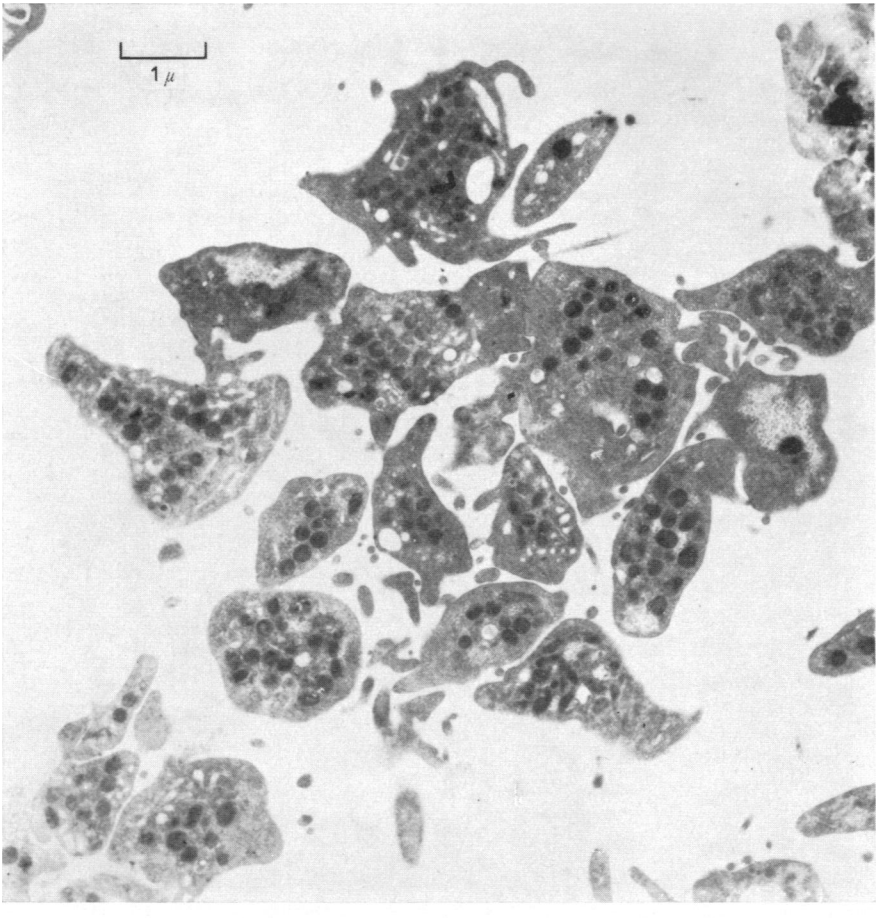

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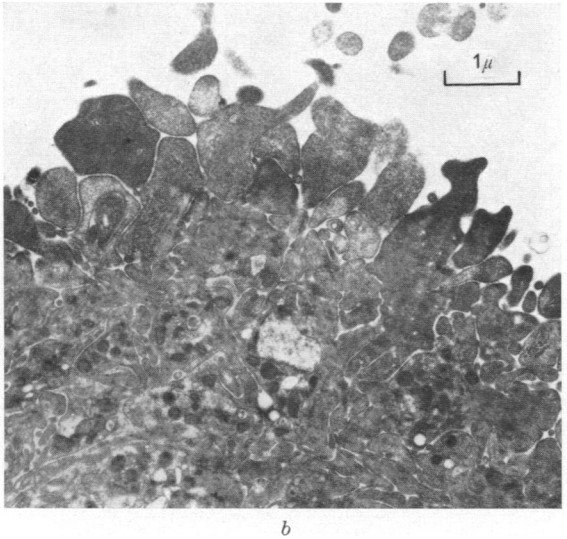

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs of platelet aggregates fixed 3 min after the addition of ADP (5 μM) to platelet-rich plasma. (*a*) untreated and (*b*) after pretreatment with amitriptyline (20 μM) 0·5 min before ADP. In (*a*) the platelets are tightly packed together and few granules are visible. In (*b*) the platelets are more loosely packed and the platelet granules are present in large numbers.

PLATE 2

Electron micrographs of platelet aggregates formed after the addition of adrenaline (7 μM) to platelet-rich plasma, (*a*) fixed 70 sec after adrenaline, at a point on the aggregation curve shown as *A* in Fig. 4, (*b*) fixed 320 sec after adrenaline, at a point corresponding to *B* in Fig. 4. In (*a*) the platelets are very loosely aggregated and appear to have retained their disc shape. In (*b*) the aggregates are densely packed and the platelets have lost most of their granules.