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OBSERVATIONS ON THE CHANGE IN SHAPE OF BLOOD PLATELETS BROUGHT ABOUT BY ADENOSINE DIPHOSPHATE

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SUMMARY

1. An optical method used for measuring platelet aggregation was adapted for measurements of the change in shape of platelets that rapidly follows the addition of the aggregating agent adenosine diphosphate (ADP).

2. Measurements were made using dilute suspension of platelets with sufficient EDTA to prevent their aggregation.

3. Measurements of the velocity and magnitude of the optical effects of the shape change were highly reproducible.

4. Volumetric measurements showed that the shape change is not associated with an increase in mean platelet volume.

5. The velocity of the shape change had a temperature coefficient of about 4·5.

6. The velocity and magnitude of the shape change were not affected by pH between 5·8 and 9·2.

7. The dependence of the velocity of the shape change on the ADP concentration was in accordance with Michaelis–Menton kinetics. The K_m was about $7{\cdot}2 \times 10^{-7}$M.

8. The velocity of the shape change was inhibited competitively by ATP, adenosine and 2-chloroadenosine but not by AMP. When the inhibitors were added after the maximum of the shape change they caused a concentration-dependent diminution in the record of the change.

9. The results suggest that the shape change is initiated by reaction of the agonist ADP with specific receptor sites on the platelet membrane which leads to energy-requiring changes in the structures responsible for maintaining the disk shape of normal platelets.

INTRODUCTION

When mammalian blood platelets suspended into plasma at 37° C are observed photometrically (Born, 1962), addition of adenosine diphosphate (ADP), 5-hydroxytryptamine (5-HT), or thrombin causes a rapid increase in the optical density of the plasma which precedes the decrease associated with aggregation of the platelets. The increase in optical density is associated with a change in morphology. The platelets which are normally disks become more spherical and throw out variously shaped projections from their surfaces (Macmillan & Oliver, 1965; O'Brien & Heywood, 1966; McLean & Veloso, 1967). The magnitude of the optical effect associated with the change in shape under otherwise similar conditions varies with the species, increasing in the order man, guinea-pig, and rabbit. The change occurs when the plasma contains ethylenediamine tetraacetate (EDTA) in concentrations sufficient to prevent aggregation completely (Zucker & Zaccardi, 1964); this means that the primary reaction between these agents and the platelet occurs in the absence of ionic calcium in the plasma. Aggregation brought about by some other agents, notably adrenaline and collagen, is apparently not preceded by the change in shape.

Investigation of the change in shape may throw light on several problems. First, like platelet aggregation the reaction is characterized by the specificity with which it is elicited on adding ADP or other aggregating agents, viz. 5-HT and thrombin, the action of which depends on ADP. Secondly, there may be similarities with the mechanisms by which adenine nucleotides bring about mechanical changes elsewhere, for example, in muscle and in mitochondria. Thirdly, this effect of different pharmacological agents is interesting as an example of drug-receptor interactions. For these reasons, the change in shape caused by ADP has been investigated by the photometric method modified to permit accurate measurement of the kinetics.

Evidence obtained with the Coulter Counter has been taken to mean that the change in shape is associated with an increase in mean platelet volume of up to 30% (Bull & Zucker, 1965; Salzmann, Neri, Chambers & Ashford, 1969). The Coulter Counter measures changes in electrical impedance caused by the passage of particles through a small orifice. The change in shape is associated with a shift in the size distribution of the impedance signals which are increased. This question has now been investigated by a different technique which permits the direct measurement of mean platelet volume and of the closeness with which platelets are able to pack together under high centrifugal forces before and after they have undergone the change in shape. With this method it has been established that the change in shape is not accompanied by an increase in the average volume of the platelets.

Some of the findings have been reported to the Physiological Society (Born, 1969).

METHODS

Platelet-rich plasma was prepared from human blood and the concentration of platelets determined as already described (Born & Hume, 1967). To prepare plasma from rabbits and guinea-pigs, the animals were anaesthetized with Nembutal (30 mg/kg body wt.) and bled out through a polyethylene cannula inserted into a femoral artery; small quantities of rabbit blood were obtained from a needle inserted into the marginal vein of the ear. The blood flowed freely into plastic centrifuge tubes in which it was mixed with one tenth volume of 3·8 % trisodium citrate (0·129 M). The tubes were centrifuged at 226 *g* for 10 min at room temperature (20–22 °C). The supernatant platelet-rich plasma was transferred with a plastic Pasteur pipette to plastic measuring cylinders and kept at room temperature while samples were taken for the experimental runs.

The change in shape was investigated with the apparatus originally designed for measuring platelet aggregation (Born, 1962; Mills & Roberts, 1967). A modified EEL Long Cell Absorptiometer (Evans Electroselenium Ltd., Halstead, Essex) contained a 1 ml. sample of platelet-rich plasma in silicone-treated glass tubes (10 × 50 mm) in a jacket through which water was circulated so maintaining constant temperature, usually 37 °C. The plasma was stirred from below by a polyethylene-covered magnetic stirrer which was rotated in the tube at about 1000 rev/min. White light passed through the tube containing the platelet-rich plasma to fall on the photocell of the apparatus. A simple attenuator and back-off circuit was employed to connect the output from the photocell to the pen-recorder (Vitatron UR 400) of 10 mV full-scale sensitivity on which the output was continuously recorded. The changes in light transmission due to the change in shape were small. Therefore, the recorder chart was calibrated so that full-scale deflexion represented only about 10 % change in transmission. The procedure used for measuring the change in shape was modified from that used for measuring aggregation as described under Results.

The high speed of the change in shape made it necessary to add reagents as rapidly as possible. This was done by injecting them in small volumes (20 µl. or less) from a constant-rate spring syringe (CR 700–20 from Hamilton Company Inc., Whittier, California) into the stirred platelet-rich plasma. Injection of a dye such as Evans blue indicated that mixing was complete in less than 0·5 sec.

Reagents. ADP sodium salt (Sigma, London, Ltd.) was neutralized to pH 7·0 with NaOH and stored as 50 mM solution in the deep freeze. For use, dilutions were made as required in Tris-saline (139 mM-NaCl, 15·4 mM-Tris-HCl pH 7·4). Hydroxymethyl [^{14}C]inulin (CAF 400, batch 1) was obtained from the Radiochemical Centre, Amersham; it had a specific activity of 12·6 mc/m-mole. The vial containing 199 µc was dissolved in distilled water to give a solution containing 5 µc/ml.

RESULTS

Description of photometric records of the change in shape

Changes in light transmission through platelet-rich plasma were recorded in such a way that a decrease in transmission produced an upward movement of the recording pen. A typical record of the change in shape is shown in Fig. 1; it was produced by adding ADP (1×10^{-6} M) to a mixture of

0·1 ml. rabbit platelet-rich plasma and 0·9 ml. Tris-buffered saline containing EDTA (final concentration 4×10^{-3} M); the final concentration of platelets was $9{\cdot}8 \times 10^7$/ml. The optical record showed several different parameters which could be measured, as follows:

(1) Without any delay greater than that due to mixing, the light transmission decreased rapidly for 3–4 sec. This *fast phase* was taken to indicate the velocity of the change in shape and was measured by the slope of the trace on a faster chart speed.

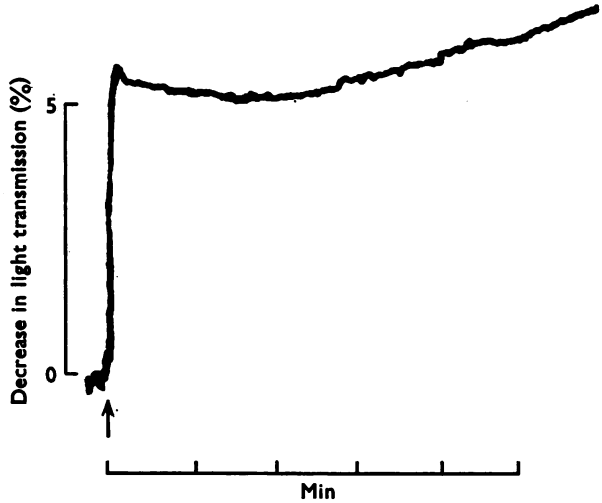

Fig. 1. Trace of the changes in light transmission through a dilute suspension of rabbit platelets after the addition of ADP (1×10^{-6} M) at the arrow. The suspension contained $9{\cdot}8 \times 10^7$ platelets in 0·1 ml. citrate plasma plus 0·9 ml. EDTA-Tris-saline (a mixture containing 0·154 M-NaCl, 0·0154 M-Tris-HCl pH 7·4 and 0·004 M-EDTA).

(2) Next the trace passed through a maximum; this was taken to be proportional to the maximal size of the change in shape and was measured by the distance from the base line.

(3) The light transmission then increased slightly and remained on a *plateau* for a few minutes, which was also measured by its distance from the base line.

(4) Finally, there was a further, slow decrease in light transmission which continued for at least 15 min. This *slow phase* was again measured by the slope of the trace.

The reproducibility of the first three measurements was estimated in an experiment in which the same concentration of ADP (4×10^{-7} M) was added to seven samples of the same rabbit platelet-rich plasma at about 10 min intervals. The results were (mean ± S.E. of mean): velocity 47·1 ±

1·1 cm/min; maximum 32·7 ± 0·5 mm; and plateau 28·0 ± 0·3 mm. This showed that the measurements were highly reproducible.

Magnification of the optical effect

The optical effect of the change in shape as it appears on the usual records of platelet aggregation is too small for accurate measurements. One reason is the rapidity with which aggregation supervenes; in the present experiments, aggregation was prevented by the addition of EDTA.

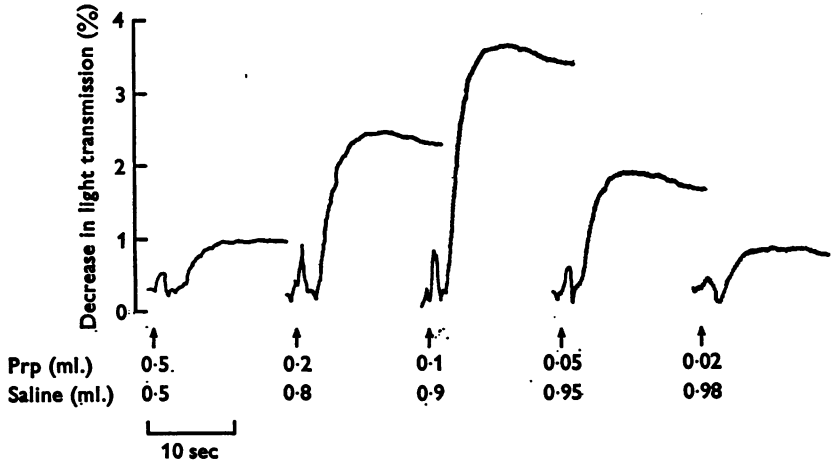

Fig. 2. Effect of diluting platelet-rich plasma (Prp) on the magnitude of the optical effect occurring during the change in shape. Citrated plasma containing $7{\cdot}2 \times 10^8$ platelets/ml. was diluted with EDTA-Tris-saline in the proportions shown. ADP (1×10^{-6} M) was added at the arrows.

Another reason is that aggregation measurements are conveniently made with undiluted plasma in which the platelet concentration is high. It was found that the optical effect of the change in shape could be increased simply by diluting the plasma; this is shown in Fig. 2. When platelet-rich rabbit plasma was diluted with isotonic (0·154 M) saline the optical effect of the shape change caused by ADP increased with the platelet concentration to reach a maximum at about 7×10^7 platelets/ml. and decreased with higher concentrations; with human plasma the maximum was at a platelet concentration of about 5×10^7/ml. (Fig. 3). The effect of diluting with platelet-poor plasma was similar. Therefore, for most measurements platelet-rich plasma was diluted to a standard concentration of 5×10^7 platelets/ml. with saline containing 4×10^{-3} M-EDTA which inhibited aggregation. Between 1×10^{-3} and 1×10^{-2} M-EDTA had no significant effect on the change in shape (Fig. 4).

Comparison of the optical effect of the change in shape caused by ADP with that of swelling caused by hypotonicity

When platelet-rich plasma was diluted in the aggregometer tube by the rapid addition of an equal volume of distilled water, the light transmission decreased in two phases (Fig. 5). The first phase was more rapid than the second into which it merged after 1–2 min. The first phase was much slower than the fast phase of the change in shape caused by ADP (Fig. 5).

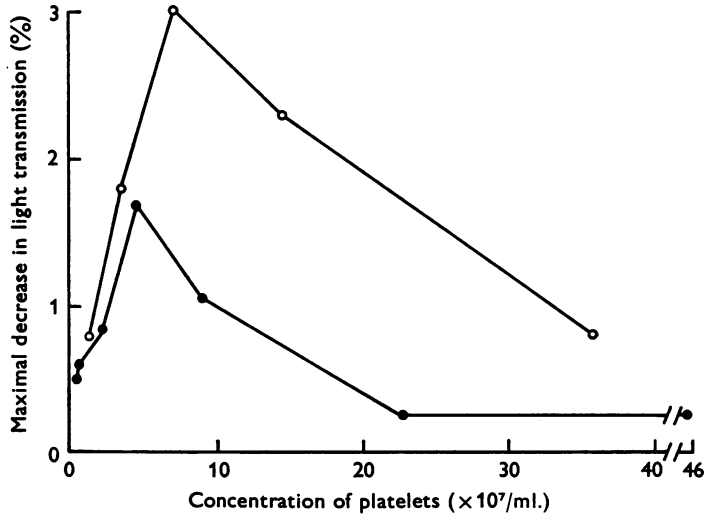

Fig. 3. Maximal decrease in light transmission accompanying the change in shape, as a function of platelet concentration; the latter was varied by diluting citrated rabbit (○—○) or human (●—●) plasma with EDTA-Tris-saline.

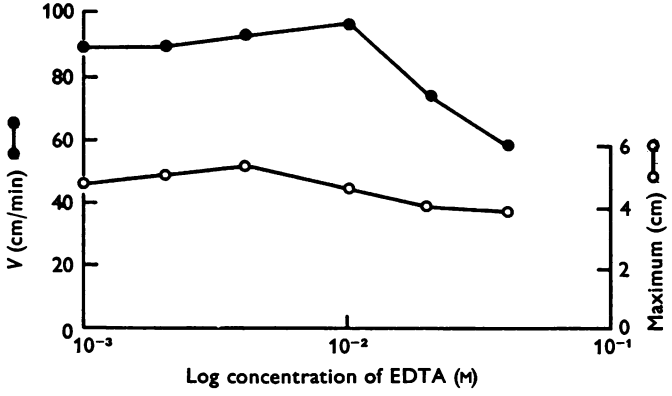

Fig. 4. Effect of EDTA concentration on the fast phase of the change in shape of rabbit platelets caused by ADP (1×10^{-6} M). Changes in both velocity (●—●, cm/min) and the maximum (○—○, cm) are shown.

The velocity of the second phase in the hypotonic plasma was similar to that of the slow phase of the change in shape.

When the slopes of the faster and slower increases in hypotonic plasma were plotted, the distance from their intersection to the base line was similar to the maximum of the change in shape (Fig. 5). Since exposure to hypotonicity could be presumed to cause swelling of the platelets, the optical measurements just described seemed to provide support for the conclusion

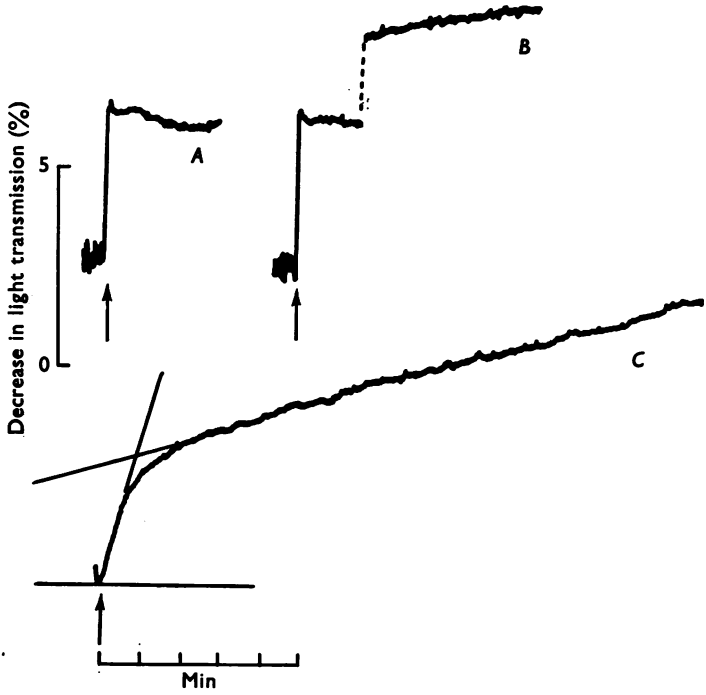

Fig. 5. Comparison of the optical effects during the change in shape and during the swelling of platelets. Citrated rabbit plasma containing $10{\cdot}5 \times 10^8$ platelets/ml. was incubated at 37° C as follows: In *A* and *B*, 0·1 ml. plasma was mixed with 0·9 ml. EDTA-saline, and ADP was added at the arrows to a concentration of 2×10^{-7} M. The break in the curve of *B* was for a period of 10 min. In *C*, at the arrow, 0·1 ml. plasma was mixed with 0·4 ml. EDTA-saline and 0·5 ml. distilled water.

(Bull & Zucker, 1965) that the change in shape is also associated with swelling although at a much faster rate. The conclusion is, however, wrong, as the following experiments show.

Direct measurement of the effect of the change in shape on the volume and packing density of platelets

It has been widely accepted that the change in shape is associated with an increase in mean platelet volume (Bull & Zucker, 1965; Salzmann *et al.*

1969). This conclusion was based on measurements made with the Coulter Counter, the assumption being that the observed changes in impedance are related solely to cell volume. Although this assumption is apparently correct for larger cells, such as erythrocytes, its validity for platelets has not been established. Another method was, therefore, developed for the direct determination of changes in mean platelet volume; this method also measures the closeness with which platelets are able to pack together under the influence of large centrifugal forces before and after undergoing the change in shape.

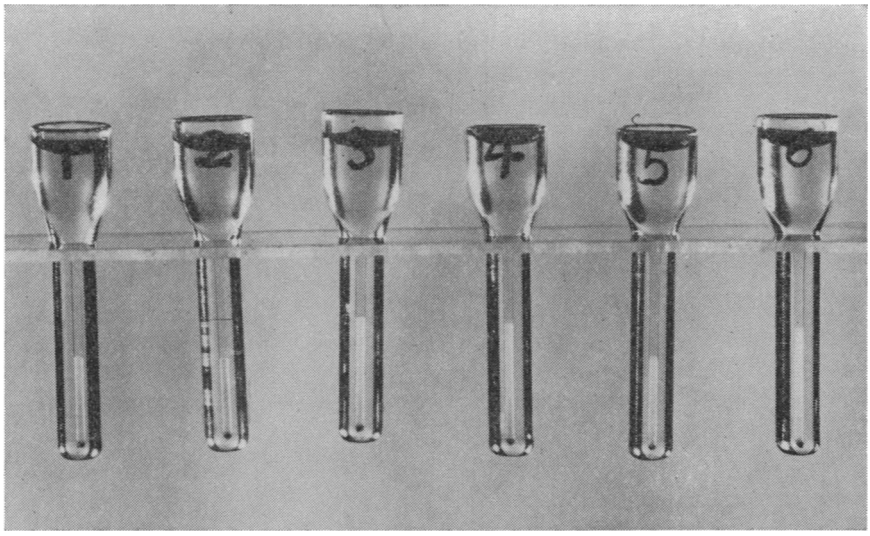

Fig. 6. Thrombocrit tubes containing platelet-rich plasma after centrifugation for 3 min at 10,000 *g*. Clear platelet-free plasma is above the white columns of packed platelets which are clearly demarcated from the short, dark columns of packed red cells in the bottom of the tubes. The tubes shown were from the experiment of Table 1 (*q.v.*).

A new kind of thrombocrit tube was used (Fig. 6). The tube, made of borosilicate glass, was small enough to fit into the metal bucket of the 6-place swing-out head (catalogue No. 69102) of the high-speed attachment to the MSE Major refrigerated centrifuge (Model 17). The reservoir of the tube had a capacity of 0·6 ml., its lower end was shaped like a funnel which merged smoothly into a capillary tube 30 mm long and with an internal diameter of 0·9 mm. Breakage of the tube during centrifuging was prevented by placing a 2 mm thick rubber disk in the bottom of the bucket and filling it with water; when the cap was screwed down the tube was held firmly in place.

In view of the great effect of temperature on the change in shape (see below), the platelet-rich plasma was kept at 37° C throughout. The photometric records of a typical experiment are shown in Fig. 7. For each run, a 1 ml. sample was pipetted into the aggregometer tube and stirred for 2 min with 25 μl. hydroxymethyl [^{14}C]inulin (5 μc/ml.); then 40 μl. 0·1 M-EDTA was added. In control runs after stirring for a further 1 min, 0·6 ml. was transferred into a thrombocrit tube; in experimental runs, after stirring for a further 0·5 min, the change in shape was induced by the

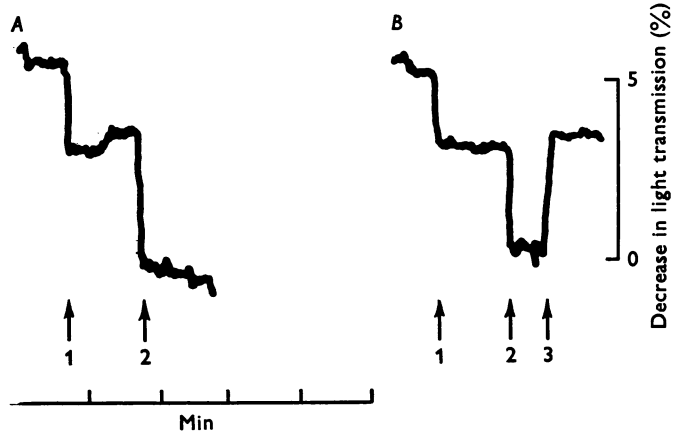

Fig. 7. Changes in light transmission caused by adding to 1 ml. citrated rabbit plasma containing $8{\cdot}0 \times 10^8$ platelets/ml. successively 25 μl. radioactive inulin (at first arrow) and 40 μl. 0·1 M-EDTA solution (at second arrow) in control run (*A*) followed, in test run (*B*) by 10 μl. ADP solution (at third arrow). In this experiment, mean platelet volume and trapped plasma volume were then determined: see text.

addition of 10 μl. 1×10^{-3} M-ADP. After another 0·5 min, 0·6 ml. was transferred into a thrombocrit tube. The tubes were centrifuged at 37° C for 3 min at 10,000 *g*.

After centrifugation, the bottom of the capillary tube contained a small sediment of erythrocytes, usually less than 1 mm in length. Above and sharply demarcated from it was a greyish-white column (Fig. 6) consisting of packed platelets. The lengths of the red and white columns were measured to the nearest 0·5 mm; the measurements were facilitated, as in a clinical thermometer, by the magnifying effect of the thick-walled capillary tube. From the radius of the capillary (0·45 mm.) and the length of the white column, its volume was calculated in μl.

The volume of trapped plasma in this column was determined as follows. First, 0·3 ml. of the platelet-free supernatant plasma was transferred to a scintillation counting vial. Then the reservoir of the thrombocrit tube

and its capillary down to just (about 0·5 mm) above the top of the white column were washed out four times with saline through a syringe and needle, to which was attached a plastic tube (Portex PP 25) fine enough to pass down the capillary. In this way all radioactivity was removed from above the packed platelet column. Then the column was quantitatively transferred into a counting vial with a 0·3 ml. water, using the same syringe and tube. The radioactivities of the supernatant plasma and of the packed platelet column were determined by scintillation counting in a Packard Model B. On the evidence (Born & Gillson, 1959; Born & Bricknell, 1959) that radioactive inulin neither penetrates into platelets nor is adsorbed onto their surfaces, the volume of plasma in the packed platelet column was calculated from the radioactivities. No correction was made for sedimented red cells because the extracellular space between them is known to be very small, 2–5 % of the packed cell volume (Born, Day & Stockbridge, 1967).

The protocol (Table 1) of a typical experiment, that of Fig. 6, shows that triplicate determinations agreed closely. Four experiments of this kind gave similar results (Table 2); they showed that: (1) the change in shape caused by ADP was not associated with an increase in mean platelet volume; the mean volume of the platelets was 6·6 μ^3 in the absence and 6·8 μ^3 in the presence of ADP; (2) on the other hand, the change in shape was associated with a large increase, i.e. 24–61 %, in the volume of plasma trapped in the packed platelet column.

To find out whether this effect was due specifically to ADP, it was replaced in two more experiments by guanosine diphosphate (GDP); this substance affected neither the mean platelet volume nor the trapped plasma volume. To confirm that the method was capable of demonstrating increases in mean platelet volume, a 1 ml. sample of a platelet-rich plasma was diluted with 0·6 ml. isotonic (0·154 M) saline, and another with 0·6 ml. distilled water to make the plasma 0·118 M with respect to NaCl; this did not cause lysis of the platelets and produced the optical effect shown in Fig. 8. In the hypotonic plasma the mean platelet volume increased by 37 % (the plasma volume between the packed platelets also increased by 44 %).

Therefore, in the absence of any demonstrable change in platelet volume, it must be concluded that the optical effect is caused solely by changes in shape.

Effect of temperature

The velocity of the rapid reaction of rabbit platelets to ADP increased rapidly with temperature between 25 and 37° C (Fig. 9). The temperature coefficients in two experiments were 4·4 and 4·5. At 40° C, the velocity was less than at 37° C. In one of these experiments, the effects of

TABLE 1. Measurements made to determine the effect of ADP (10 μM) in the change in shape on mean platelet volume and on the volume of plasma between platelets sedimented under 10,000 g for 3 min

An unanaesthetized rabbit was bled from an ear vein into 0·1 vol. 3·8 % trisodium citrate. The blood was centrifuged at 22° C and 250 g for 20 min. The supernatant plasma contained $7{\cdot}96 \times 10^8$ platelets/ml. Each thrombocrit tube was filled with 0·6 ml. of this plasma taken from the aggregometer tube, as described in the text.

Thrombocrit contents tube no.	Length of packed column (mm)		Volume of white column (μl.)	Radioactivity of cell-free supernatant plasma		Radio-activity of packed cell column (c.p.m.)	Volume of plasma in packed cell column (μl.)	Corrected packed cell volume (μl.)	Corrected packed cell volume of 10^8 platelets (μl.)	Volume of plasma between 10^8 platelets packed in column (μl.)
	Red	White		(c.p.m./ 300 μl.)	(c.p.m./ μl.)					
1 Control, i.e. plasma without ADP	1·0	8·0	5·1	57,256	191	331	1·73	3·4	0·72	0·37
2 Control	1·0	8·0	5·1	55,328	184	332	1·80	3·3	0·70	0·38
3 Control	1·0	7·5	4·8	56,140	187	314	1·68	3·1	0·66	0·36
4 Plasma with ADP	1·0	10·0	6·4	55,290	184	524	2·85	3·5	0·75	0·60
5 Plasma with ADP	1·0	9·0	5·7	52,712	176	412	2·35	3·3	0·70	0·50
6 Plasma with ADP	1·0	9·5	6·0	54,822	183	473	2·58	3·4	0·72	0·55

TABLE 2. Mean platelet volume, and plasma volume between platelets sedimented under 10,000g for 3 min, after the change in shape produced by ADP (10 μM)

No. of expt.	Mean platelet volume (μ^3)	Corrected packed volume of 10^8 platelets (μl.)				Volume of plasma between 10^8 platelets sedimented under 10,000 g for 3 min (μl.)				
		Without ADP	Mean	With ADP	Mean	Ratio, with ADP / without ADP	Without ADP	Mean	With ADP	Mean	Ratio, with ADP / without ADP
1	4·1	0·42 0·39	0·41	0·38 0·45	0·41	1·00	0·44 0·58	0·50	0·78 0·81	0·83	1·61 Note (1)
2	5·1	0·53 0·49	0·51	0·51 0·54	0·52	1·02	0·64 0·53	0·58	0·95 0·99	0·95	1·60 Note (1)
	6·4	0·65	0·64	0·64	0·64	1·00	0·57	0·57	0·91	0·91	1·59
3	6·9	0·72 0·70 0·66	0·69	0·74 0·70 0·72	0·72	1·04	0·37 0·38 0·36	0·37	0·61 0·50 0·55	0·55	1·50
4	6·5	0·64 0·64 0·67	0·65	0·68 0·70	0·69	1·06	0·35 0·37 0·34	0·35	0·43 0·43	0·43	1·24

Note (1). In these experiments, glutaraldehyde (0·025 M) was present during the centrifugations; the results were not used in the calculation of mean platelet volume or of mean intercellular plasma volume.

temperature on the velocity of *swelling* of platelets in hypotonic plasma was also determined. For this purpose, platelet-rich plasma was mixed in the reaction tube with an equal volume of distilled water at different temperatures which were measured with a thermocouple. The velocity of swelling increased with temperature between 18 and 37° C, but very slightly, the temperature coefficient being only about 1·5; above 37° C

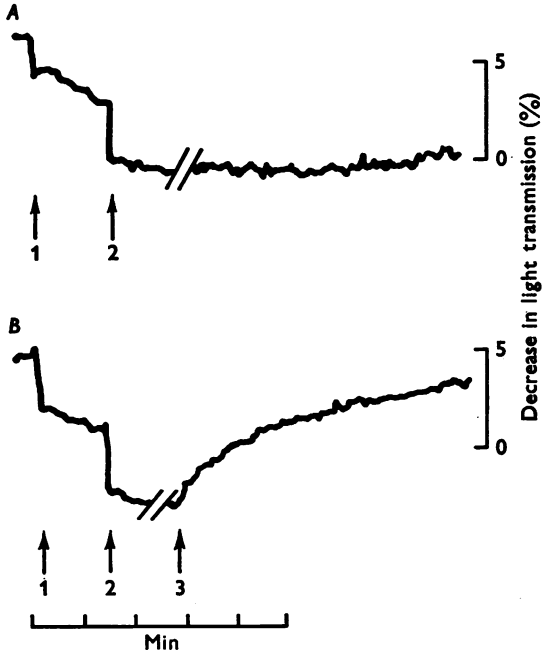

Fig. 8. Changes in light transmission caused by adding to 1 ml. platelet-rich plasma as in Fig. 7 successively 25 μl. radioactive inulin (at first arrow) and 40 μl. 0·1 M-EDTA (at second arrow) followed in control run (*A*) by 0·3 ml. 0·54 M-NaCl and in test run (*B*) by 0·3 ml. distilled water (at third arrow). The breaks in the traces indicate that these dilutions caused large increases in light transmission which are irrelevant and therefore not shown in the Figure. These samples were then used for determining mean platelet volume and trapped plasma volume: see text.

the rate, if anything, decreased slightly (Fig. 9). That the platelets swelled in this hypotonic plasma was confirmed microscopically; the platelet count was constant showing that the platelets were not lysed.

In all subsequent experiments on the change in shape, the measurements were made at 37° C.

Effect of pH

In a series of runs, each sample of rabbit platelet-rich plasma was diluted in the aggregometer tube with EDTA-saline containing 0·0154 M-

Tris-HCl pH 7·4 to a volume of 1 ml. containing 5×10^7 platelets. The pH was decreased by the addition of 1 N-HCl or increased by the addition of 1 N-NaOH; the added volumes were so small (at most 60 μl.) that the light transmission was hardly altered. After stirring for 15 sec, ADP was added to induce the change in shape. As soon as the maximum was passed, the pH of the platelet suspension was measured with a glass electrode.

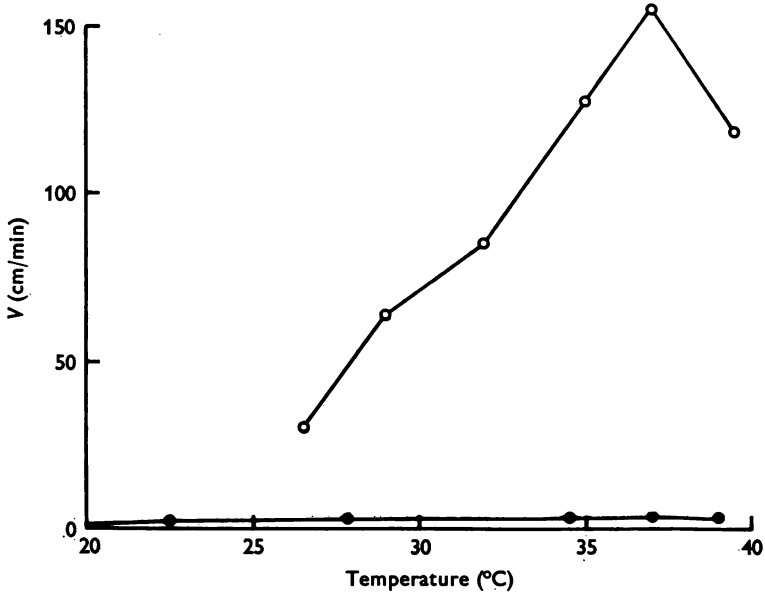

Fig. 9. Effect of temperature on the velocity (V) of the change in shape produced by ADP at 2×10^{-7} M (○—○) and on the velocity of platelet swelling in hypotonic plasma corresponding to about 0·08 M-NaCl (●—●).

Neither the velocity nor the maximum of the change in shape were affected by increasing the pH from 5·8 to 9·2. Below pH 5·8 and above pH 9·2 both velocity and maximum decreased.

Effect of ADP concentration

The fast phase, i.e. velocity, of the change in shape increased with increasing concentrations of ADP in the manner shown in Fig. 10*a*; the reciprocal of the velocity plotted against the reciprocal of the concentration gave a straight line (Fig. 10*b*) from which an apparent K_m for ADP was calculated of about 7·2 ($\pm$ 1·1) $\times 10^{-7}$ M, corresponding to an affinity constant ($1/K_m$) of about $1{\cdot}4 \times 10^6$. In another, similar experiment this value was $2{\cdot}2 \times 10^6$ so that the mean affinity constant was $1{\cdot}8 \times 10^6$.

The maximum and the plateau of the optical effect also increased with ADP concentration, but the latter did not affect the velocity of the slow phase.

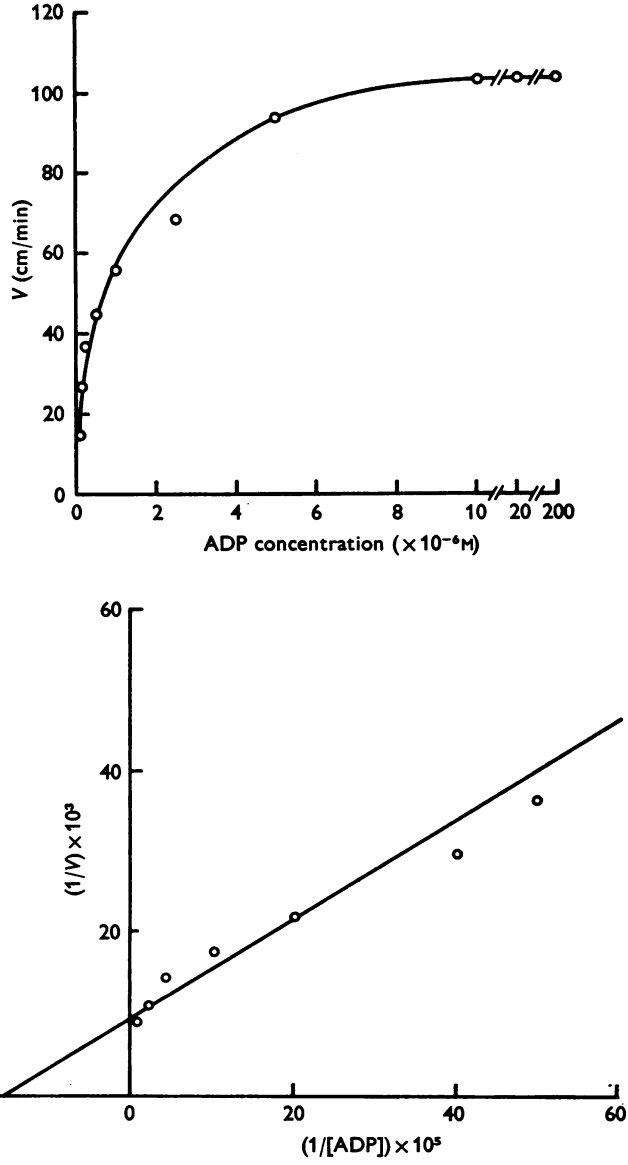

Fig. 10*a*. Effect of ADP concentration of velocity (v) of the change in shape of rabbit platelets at a concentration of 5×10^7/ml. diluted plasma. *b*. Double reciprocal plot of the results shown in Fig. 10*a*. The line was calculated by the method of Wilkinson (1961).

Inhibition by ATP

Platelet aggregation by ADP is inhibited by ATP (Born, 1962). It is difficult to elucidate the mechanism of this inhibition because in platelet-rich plasma the antagonist, ATP, is rapidly dephosphorylated to the agonist ADP. This complication did not arise in investigating the effect of ATP on the change in shape because of its rapidity and because the presence of EDTA prevented the enzymic break-down ATP to ADP.

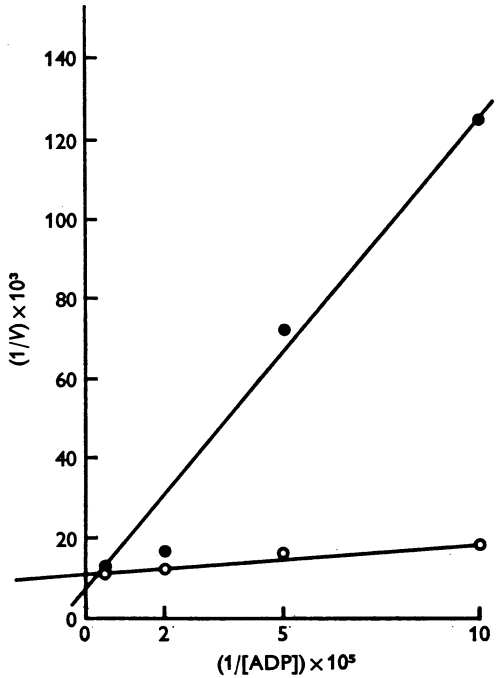

Fig. 11. Inhibition by ATP of the velocity (V) of the change in shape produced by ADP, shown as a double reciprocal plot of $1/V$ against 1/ADP concentration: without ATP (○—○) and with 5×10^{-5} M-ATP (●—●).

A sample of ATP was used which contained no ADP demonstrable by paper chromatography. This sample diminished the velocity of the change in shape induced by ADP. Quantitative investigation of the inhibition was made difficult by the progressive decline in responsiveness of platelets in plasma kept at room temperature, by the steepness of the dose–response curve to ADP and by the variability in the ATP concentration required to demonstrate inhibition with different plasmas. Nevertheless, double-reciprocal plots of the best experiments suggested that the inhibition of the reaction velocity by ATP was competitive (Fig. 11). When ATP was

added immediately after the maximum, the height of the plateau was diminished in proportion to the ATP concentration (Fig. 12).

Inhibition by adenosine and 2-chloroadenosine

Platelet aggregation by ADP is inhibited by adenosine and some of its analogues, notably 2-chloroadenosine (Born & Cross, 1963; Born, 1964; Born, Haslam, Goldman & Lowe, 1964; Maguire & Michal, 1968). The fast phase, maximum, and plateau of the change in shape were affected by

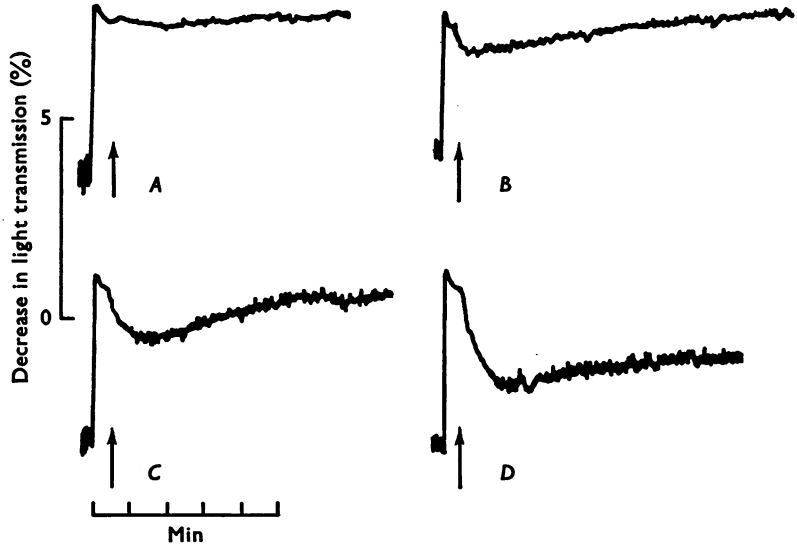

Fig. 12. Reversal by ATP of the change in shape produced by ADP at 1×10^{-6} M in rabbit platelets at a concentration of 5×10^{6} ml. diluted plasma. At the arrows, ATP was added as follows: none in *A*; 1×10^{-5} M in *B*; 5×10^{-5} M in *C*; and 5×10^{-4} M in *D*.
The original records showed dilution artifacts which were identical in control runs when saline was added and after the addition of the same volumes of ATP solution; these artefacts have been omitted from the tracings shown here.

these substances, the inhibition increasing with concentration up to at least 10^{-4} M except the fast phase which was maximally inhibited to about 75% at about 5×10^{-5} M (Fig. 13). The inhibition of aggregation by adenosine or 2-chloroadenosine increases with the time interval between addition of the inhibitor and the subsequent addition of ADP (Born, 1964). The effect of these substances on the velocity of the shape change did not increase with time and was apparently also competitive (Fig. 14). When added just after the maximum, adenosine or 2-chloroadenosine at 10^{-4} M also caused a decrease in the plateau. For the same decrease, the rate at

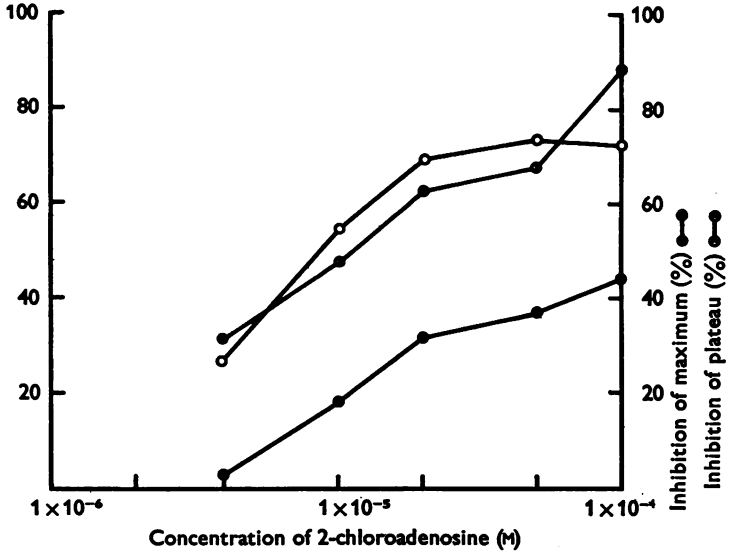

Fig. 13. Inhibition (per cent) by 2-chloroadenosine of the velocity (○—○), the maximum (●—●), and the plateau (◑—◑), of the change in shape produced by ADP at 4×10^{-7} M in rabbit platelets at a concentration of $7{\cdot}8 \times 10^{7}$/ml. in plasma diluted with EDTA-saline.

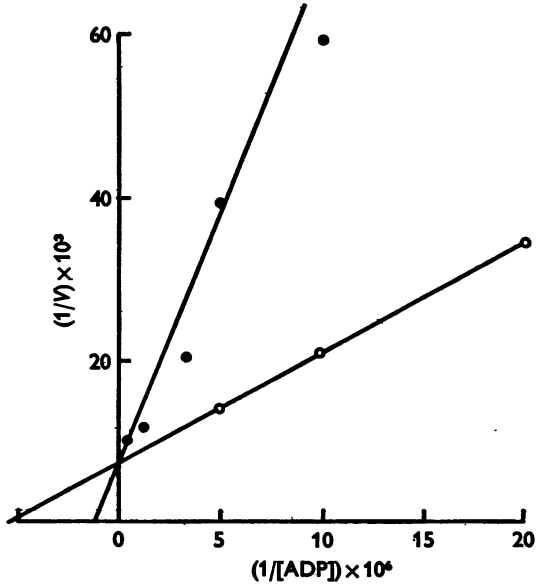

Fig. 14. Inhibition by 2-chloroadenosine of velocity (V) of change in shape produced by ADP, shown as double reciprocal plots of $1/V$ against 1/ADP concentration. The concentration (M) of 2-chloroadenosine was zero (○—○) and 1×10^{-5} (●—●).

which the new plateau was approached was faster with ATP than with adenosine or 2-chloroadenosine.

The velocity of the change in shape caused by ADP was not reduced by adenosine monophosphate (AMP) at 2×10^{-4} M, by guanosine or inosine triphosphates at 1×10^{-4} M, or by inorganic pyrophosphate at 1×10^{-3} M. The compound 5′-azido 5′-deoxyadenosine, which has a greater vasodilator action than adenosine (Jahn, 1965) was also inactive at 2×10^{-4} M.

DISCUSSION

The first thing that happens when platelets are exposed *in vitro* to certain aggregating agents including ADP is a change in shape from smooth disks to more spherical forms with variously shaped protrusions. This has been established with both the light and the electron microscope (Tocantins, 1948; Zucker & Borelli, 1954; Setna & Rosenthal, 1958; Macmillan & Oliver, 1965; White & Krivit, 1967). The visual evidence of a change in shape has been correlated with two indirect measurements. One is an increase in optical density of platelet-rich plasma that occurs as soon as one of the aggregating agents is added (Macmillan & Oliver, 1965; O'Brien & Heywood, 1966); the other is a shift in the size distribution as measured with the Coulter Counter (Bull & Zucker, 1965; Manucci & Sharp, 1967).

The results in this paper confirm that the change in shape is associated with changes in light transmission, which can be made to provide measures of both velocity and magnitude. However, a new technique for the direct measurement of packed platelet volume has shown conclusively that the change in shape is not accompanied by an increase in the mean volume of the platelets. That the technique was capable of demonstrating such an increase was established by measuring the swelling of platelets in hypotonic plasma. It seems that changes in volume cannot always be distinguished from changes in morphology of cells as small as platelets and that, therefore, the Coulter Counter cannot be relied on to establish changes in volume.

Although the mean volume of the platelets remained the same, the shape change *was* associated with a considerable increase in the volume of plasma trapped between the platelets when they were packed together by centrifugation at 10,000 *g* for 3 min. This indicates that the average distance between packed platelets is increased by the newly formed protrusions and that these are remarkably rigid. The rigidity may be due to microfibrils which can be seen in some electron micrographs of the protrusions (Silver, 1965). The microtubules which encircle the platelets beneath the outer membrane and are supposed to provide a cytoskeleton apparently disappear during the change in shape (Behnke, 1965; Sixma &

Molenaar, 1966); possibly they provide the elements of the microfibrils in the protrusions.

The volume of trapped plasma was similar to that found in earlier experiments under rather different conditions (Born & Bricknell, 1959). This suggests that this is the minimum space between platelets packed together as closely as possible by high centrifugal forces. This space is, however, much greater than the space between platelets in the second phase of aggregation (Born & Hume, 1967) when almost the whole of their surfaces are in the closest possible apposition. The increased close packing presumably implies that the rigidity of the protrusions disappears and that the platelets become more deformable by forces acting from outside.

The optical method for following the change in shape has so far been used wholly empirically. Nothing is known about the relation between number, sizes, and properties of the protrusions on the platelets and the observed decrease in light transmission. It is reasonable to assume that the rate of this decrease measures the velocity at which the protrusions are formed and that the maximum of the decrease provides a measure of their magnitude. The results of measurements based on these assumptions have provided useful information about the nature of the change in shape.

The high velocity of the change in shape induced by ADP supports the suggestion (Born, 1964) of a trigger mechanism in which ADP acts like a drug such as acetylcholine in causing contraction of smooth muscle. This implies that the platelet surface has specific receptors for ADP with which it interacts without necessarily being bound to them for more than a very short time indeed. The assumption that the reaction is analogous to such a drug-receptor mechanism is supported by other observations, viz. (1) the maximum and plateau in the light transmission records and (2) the antagonism by ATP.

The maximum and the subsequent plateau in the light transmission curves call to mind smooth muscle contractions caused by drugs in which the tracings pass through a maximum and then 'fade' to a plateau. This has been explained in the rate theory of drug action (Paton, 1961) by assuming that both association and dissociation between drug and receptor occur and that stimulant action is proportional to the rate of association. Receptor occupation interferes with the stimulant action by diminishing the pool of free receptors; this would account for 'fade'. Such an interpretation could be applied to the action of ADP in producing the change in shape. It would explain why several attempts to demonstrate bound ADP have failed.

The last phase of the change in shape produces a slow decrease in light transmission which continues for at least 15 min. The rate is similar to the

slow decrease in light transmission seen when platelets are suspended in a hypotonic medium which just fails to lyse them. This similarity suggests that the slow phase of the change in shape is associated with a gradual structural degradation such as would be expected to occur in hypotonic media, possibly accompanied by an increase in volume; this has still to be investigated.

The antagonism by ATP could be accounted for by competition between ATP and ADP at the receptor. AMP, another substance structurally close to ADP, had no antagonistic action. This difference between AMP and ATP can be explained if it is assumed that the primary reaction of ADP is with a protein similar to the postulated mechano-protein present in the outer membrane of mitochondria. The structure of isolated mitochondria is altered in opposite directions by ADP and ATP whereas AMP has no effect (Lehninger, 1964). Indeed, there is much evidence that all contractile protein systems, including those of muscle, react specifically with ADP and ATP but not with AMP or other nucleotides (see e.g. Davies, 1963; Szent-Györgyi, 1968; Pringle, 1968).

This leaves the problem of the mechanism by which adenosine and 2-chloroadenosine inhibit the change in shape produced by ADP. The inhibition did not increase with time and was apparently competitive. The proposition that the inhibition of platelet aggregation by adenosine (Born & Cross, 1963) is quantatively related to its phosphorylation (Rosenberg & Holmsen, 1968) was made untenable by the demonstration (Born & Mills, 1969) that when adenosine uptake by platelets was almost totally prevented its inhibitory effect on aggregation did not diminish but was, if anything, potentiated. On the other hand, the suggestion that adenosine and 2-chloroadenosine simply compete with ADP for its receptor site (Born, 1964) does not appear very likely either, in view of the ineffectiveness of AMP which is structurally closer to ADP.

The change in shape, like aggregation, is inhibited by prostaglandin E_1 (unpublished results). This substance increases the adenyl cyclase activity of human platelets (Wolfe & Shulman, 1969; Zieve & Greenough, 1969). Adenosine greatly increases the concentration of cyclic 3′,5′-AMP in guinea-pig brain (Sattin & Rall, 1970). Should adenosine, like prostaglandin E_1 (Robinson, Arnold & Hartmann, 1969), be found to increase the cyclic 3′,5′-AMP content of platelets, the possibility should be considered that their inhibitory action on the change in shape is connected with this biochemical effect. In the brain preparation 3′5′-cyclic AMP is increased also by ATP, ADP, 3′-AMP and 5′-AMP. Of these, only ATP inhibits the change in shape of platelets whereas ADP, of course, promotes it. Unlike adenosine, the adenine nucleotides cannot penetrate into cells through intact membranes; their site of action must be at the outside of the cell

membrane unless it is assumed that the nucleotides are dephosphorylated to adenosine. Similarities in the antagonistic actions of adenosine and ATP on the shape change of platelets might suggest that their mode of action ATP and its occurrence in the presence of EDTA suggest that ATP acts as such and not after dephosphorylation. Therefore, it may be concluded that, whatever its mode of action, ATP acts on the outside of the platelet membrane.

A particularly interesting observation was the remarkable dependence of the change in shape on temperature. The velocity of the change in shape had a temperature coefficient of about 4·6 between 27 and 37° C. This may be compared with the temperature coefficients of the velocity of muscular contractions which are between 1·5 and 1·7 for both the cat and the sloth (Gordon & Phillips, 1953; Enger & Bullock, 1965). It is not yet possible to give a precise meaning to this high temperature coefficient but it suggests that the change in shape involves one or more reactions with large energies of activation.

The ease with which the change in shape is induced by the slightest abnormalities in the platelet's environment or metabolism suggests, furthermore, that the flat disc shape of the normal platelet, like the biconcave shape of the normal erythrocyte, is a thermodynamically improbable state maintained by the continuous expenditure of energy, and that the change in shape brings about a more probable state. This would suggest that the change in shape may also involve an increase in entropy such as might be associated with the depolymerization of a highly organized protein structure in the microtubules.

The observations can be accounted for by the following hypothesis. The outer membrane of the platelet contains adenine nucleotides, mostly as ATP which is part of a metabolic pool (Holmsen, 1969) maintained by energy metabolism. The change in shape is initiated by a decrease in the ratio of ATP/ADP in the membrane. This decrease can be caused by an increase in ADP added to the medium or released into it from other cells. Alternatively, ATP may be decreased and ADP simultaneously increased in the membrane under conditions in which the resynthesis of ATP is slowed by interference with energy metabolism or when ATP utilization is accelerated in reactions, e.g. kinases, which occur in the membrane.

The hypothesis proposes, therefore, that a decrease in the ATP/ADP ratio in the platelet membrane diminishes the effectiveness of membrane ATP in maintaining the normal shape of the platelet. If this shape is maintained by the bundle of microtubules acting as a cytoskeleton, it is necessary to suggest ways in which a change in the adenine nucleotides in the outer membrane could lead to alterations in the microtubules which are at some distance and clearly separated from it (Behnke, 1967).

Transmission of the effect from the outer membrane to the microtubular system must be very rapid. By analogy with muscle, it seems likely that the transmitter is calcium which is released from the membrane when the ATP/ADP ratio falls.

The filaments seen in platelet protrusions could contain elements from depolymerized microtubules. If these elements were actin-like, the rigidity of the protrusions could be analogous to that of muscles in rigor which contain actin-bound ADP but little or no ATP.

In mitochondria, where the experimental results suggest an analogous molecular situation (Voth & Schafer, 1968), the evidence is against the involvement of a cation-activated membrane ATPase in the primary change. With platelets, the change in shape occurs in the presence of high concentrations of EDTA so that it is independent of ionic calcium or magnesium in the medium. Furthermore, the velocity of the change in shape produced by ADP, unlike that produced by 5-HT (G. V. R. Born, to be published), does not depend on the concentrations of external sodium. This suggests that ATPases affected by cations in the medium are not involved in the change in shape reaction. However, an ATP-utilizing system could, of course, make use of intracellular calcium or magnesium.

The last point to discuss is the functional significance of the change in shape of platelets. Many other types of cell extrude processes such as microvilli or pseudopodia. The function of some of these processes is clear, e.g. pseudopodial extrusions determine motility in amoebae and phagocytosis in granulocytes. The function of microvilli extruded by other cells has still to be established but it is reasonable to suggest that they facilitate contacts which have to be made with other cells (Bangham, 1964).

The only certain physiological function of platelets is to ensure normal haemostasis by adhesion and aggregation in injured blood vessels and by accelerating clotting. This function depends on the platelet's ability to make rapid contact with vascular basement membranes, with exposed collagen fibres, and with other platelets. The making of these contacts is clearly facilitated by the change in shape in which platelets throw out extensions with great rapidity.

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