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QUANTIFICATION OF THE MORPHOLOGICAL REACTION OF PLATELETS TO AGGREGATING AGENTS AND OF ITS REVERSAL BY AGGREGATION INHIBITORS

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SUMMARY

1. In rabbit citrated platelet-rich plasma, the changes in shape of the platelets produced by adenosine diphosphate (ADP) or 5-hydroxytryptamine (5-HT) were observed by photometric and volumetric techniques and by measurements of platelet images on electron micrographs either directly or with an image analysing computer. This permitted the indirect manifestations of the shape changes to be correlated with the morphological features responsible for them, i.e. transformation of disks to more spherical forms and extrusion of blebs and spikes.

2. Following the addition of ADP, an initial brief peak in the light scattering records was associated with marked but transient irregularities in the surface of the platelets. These effects were absent when the other shape changes were produced by 5-HT.

3. When the optical manifestations of the shape changes induced by ADP were reversed by the addition of antagonists adenosine triphosphate, 2-chloroadenosine or prostaglandin E_1, the morphological changes were reversed by a diminution in the number of spikes and the conversion of spherical platelet bodies to a more discoid form.

4. The volume of extracellular plasma trapped between platelets sedimented by centrifugation was proportional to the number of spikes which they extruded. Under all conditions, the volume of the platelets themselves remained remarkably constant at approximately 5×10^{-9} μl./platelet.

5. Addition of a calcium chelating agent alone produced a rapid persistent alteration in the optical properties of platelet-rich plasma. The magnitude of this alteration was proportional to chelator concentration but greater in some plasmas than in others. Similar optical effects were produced when the calcium concentration

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of platelet-rich plasma was increased by adding calcium. The optical effects produced by calcium or its chelators were unusual in that the changes in transmitted and horizontally scattered light were in opposite directions (transmitted light decreased, scattered light increased) whereas, in all other circumstances so far examined, these changes were in the same direction. The chelators caused the formation of spikes on the platelets without appreciably altering their disk shape, which may explain the unusual nature of the optical effects.

INTRODUCTION

When fresh platelet-rich plasma is observed photometrically at 37 °C (Born, 1962; Michal & Born, 1971), addition of ADP causes a decrease in light transmission which precedes the increase associated with platelet aggregation. The decrease in light transmission is associated with a striking change in platelet morphology. The platelets which are normally disk-shaped become more spherical and extrude variously shaped blebs and spikes (MacMillan & Oliver, 1965; O'Brien & Wodehouse, 1968; White, 1974). Another manifestation of the morphological changes is a decrease in the amount of light scattered in the plane perpendicular to the axis of stirring (Michal & Born, 1971). Unlike aggregation, the shape change does not require Ca ions in the medium (Zucker & Zaccardi, 1964; McLean & Veloso, 1967). Therefore, in the presence of a calcium chelating agent aggregation is prevented so that the optical manifestations of the shape change can be examined by themselves.

Optical and other methods have established the following about the shape change of human platelets in response to ADP (Born, 1970; Michal & Born, 1971). There is no change in mean platelet volume. The velocity is high with a half-time at 37 °C of about 2–3 sec, and with a remarkably high temperature coefficient of about 4·5. Neither velocity nor extent of the shape change is affected by pH between 5·8 and 9·2. The velocity depends on the ADP concentration in accordance with Michaelis–Menten kinetics, with an apparent K_m of about 7×10^{-7} M. The velocity of the shape change is diminished by inhibitors of aggregation, including ATP, adenosine, and 2-chloroadenosine, but not by AMP; only with ATP is inhibition clearly competitive (Macfarlane & Mills, 1975). The similarities of the specificities of ADP in inducing, and of adenosine and ATP in inhibiting, shape change and aggregation suggested that inhibition of the shape change prevents the subsequent reaction of the platelets including aggregation and the release reaction. When inhibitors are added after the shape they cause a concentration-dependent reversal of its optical effect.

These observations suggested that the shape change is initiated by a reaction of ADP with specific receptor sites on the platelet membrane postulated earlier to account for aggregation by ADP (Born, 1965, 1967, 1970), and that this reaction is the trigger responsible for initiating the subsequent events leading to aggregation of the platelets. Much work is being done to find means of inhibiting platelet aggregation under conditions in which it may lead to thrombosis (Mills, Smith & Born, 1970; Mustard & Packham, 1973; Sherry, 1973; McNicol, Mitchell, Reuter & van de Loo, 1974; Hirsch, Gent & Genton, 1975; Genton, Gent, Hirsch & Harker, 1975). We have therefore undertaken a further investigation of the shape change and its reversal in which the optical effects have been correlated quantitatively with the

electron microscopic appearance of platelet bodies and their extrusions as well as with thrombocrit measurements of their volumes. Some of the results have been briefly reported (Born, Foulks, Michal & Sharp, 1972*a*).

METHODS

Platelet-rich plasma was prepared from the blood of unanaesthetized restrained rabbits by cutting a marginal ear vein with a scalpel and allowing the blood to drip into a plastic centrifuge tube containing 0·1 volume of 3·8% (w/v) sodium citrate. After mixing, the blood was centrifuged at 200 *g* for 15 min at room temperature of about 20 °C. The supernatant plasma usually contained 5–12 × 10^8 platelets/ml. This platelet-rich plasma was taken off with a Pasteur pipette and kept in a plastic container at room temperature during the experiment.

For *optical measurements* the platelet-rich plasma was diluted with saline to make the concentration of platelets 3 × 10^8/ml. One ml. samples were stirred magnetically in an aggregometer (Michal & Born, 1971) at 37 °C. Aggregating and inhibiting agents were added very rapidly in small volumes (20 μl. or less) from a constant-rate spring-loaded syringe. Changes in transmitted and scattered light associated with changes in platelet shape were measured as already described (Born, 1970; Michal & Born, 1971). Scattered light was measured in the horizontal plane at right angles to the incident light which was polarized in the vertical plane. Upward deflexions in the recordings of both transmitted and scattered light indicate decreases in the output of the photosensitive cells.

Mean platelet volume was determined with specially designed thrombocrit tubes (Born, 1970), using undiluted platelet-rich plasma. Human serum albumin labelled with ^{125}I was added to the plasma before centrifugation and the radioactivity of the platelet pellet was determined for calculating the volume of extracellular plasma trapped with the packed platelets (Born, 1970).

For *quantitative electron microscopy*, the sequence of morphological changes of the platelets was quantified as follows. A 0·8 ml. sample of platelet-rich plasma was stirred in an aggregometer tube for 2 min to bring it to 37 °C. For controls, 0·8 ml. ice-cold 2·5% glutaraldehyde in 0·067 or 0·1 M-cacodylate buffer at pH 7·4 was added to fix the platelets (Sheppard, 1972). After stirring for 0·5 min the contents of the tube were poured into another tube containing 9 vol. of cold glutaraldehyde buffer. After mixing, the tube was left at 0 °C for about 30 min to complete fixation. The fixed platelets were sedimented by centrifugation at 1500 *g* for 10 min. The supernatant was decanted and the sedimented platelets were post-fixed with buffered osmium tetroxide, dehydrated in ethanol and embedded in Araldite (CIBA). Sections of the embedded platelet pellets 0·07–0·09 μm thick were cut on a LKB Ultratome and stained with lead citrate (Reynolds, 1963). Grids of sections were taken with 10 μm of tissue being discarded between each grid to randomize the selection of platelets. Sections were photographed in a Philips EM-300 electron microscope at a magnification of 8000× in such a manner that no fields were less than 10 μm apart. Other samples of platelet-rich plasma were treated similarly at various times after initiating the shape change by the addition of ADP or 5-HT or after reversing the shape change by the addition of inhibitors.

The morphological features of control platelets and platelets treated with shape change inducers or inhibitors were quantified on electron micrographs by measuring parameters of sufficiently large numbers of platelet images to provide statistically significant results. Two methods were used for making these measurements. In one method, the circumference of every platelet section on a series of photographs was measured with a D-Mac pen-follower, omitting sections that were cut by the margin of the photograph. The circumference lengths were grouped at 0·2 μm intervals and their distribution was plotted against the percentage of total segments measured. For each experimental condition about 500 sections were measured. Changes in the proportion of sections with small circumferences, representing sections of long thin filaments or spikes, were compared with untreated controls at various intervals after the addition of aggregating agents or inhibitors. Asymmetry of platelet sections, excluding those with circumferences less than 0·2 μm, was quantified by measuring the longest diameter of each complete section and the widest diameter at right angles to it with a hand rule and expressing the result as a long-to-short-diameter ratio.

In the other method, a number of electron micrographs of platelet sections were projected onto a television screen. On each photograph four fields were scanned and the number (n) of

platelet sections lying completely within each field was counted by an image analysing computer Quantimet 720. The instrument carried out two further measurements on these images from which additional indices of changes in platelet shape were calculated. The unit of measurement was the distance between adjacent sweeps. Enlargement of the initial electron micrographs together with the magnification introduced by the electronic display system gave a final image size of 21,328 ×. At this magnification each scale unit was 0·015 μm in length. The sum of the length of all chords (C), i.e. of all sweep segments which traversed photographed objects of sufficient optical density, provided a value for the total area (A) of all such images. The instrument also counted the total number of such chords for all objects scanned, a value termed the intercept (I). For an individual platelet image, the intercept was a measure of the maximum dimension of that image in a direction perpendicular to that of the scan, i.e. it represented the 'tangent diameter'. In order to compensate for variation in the orientation of asymmetrical platelet segments, the intercept for each frame scanned was determined for two sweep directions at right angles to one another and the average of these two values was taken as a better estimate of the sum of the true tangent diameters. To test the validity of this procedure, intercepts were determined on fifty frames after rotating the photomicrographs through each of four scanning angles 0, 22·5, 45 and 67·5°. The mean of these four values was within 1 % of the value obtained with two perpendicular sweeps, indicating a high degree of randomness in platelet orientation.

Measurements were accumulated and stored on more than fifty fields for each of the different experimental conditions. These values provided the basis for the following calculations:

(1) the mean area per platelet image, $\bar{A} = A/N$;

(2) the mean intercept or mean tangent diameter, i.e. number of chords per platelet image, $\bar{I} = I/N$;

(3) the mean chord length, i.e. the average distance traversed across the platelet images by the scanning beam, $\bar{C} = A/I$.

Sources of error. The dimensions of individual images within a selected size range could not be measured directly by this image analysis system. Nevertheless the system could be made to reject all chords of less than a specified length, thereby eliminating all objects with smaller maximum dimensions. However, this procedure also eliminated short chords from the 'leading' edges of larger images. An approximate correction for this unwanted loss was made by adding to the measured area a factor Ix, where I was the detected intercept (number of chords) and x was a sizing factor (limiting chord length). This correction is quite satisfactory (error of less than 1%) for projected images of spheres of various sizes provided that the ratio of their minimum to maximum diameters is less than 0·3. For objects with a wider distribution of sizes and with irregular surfaces and image profiles, this error is likely to be somewhat greater.

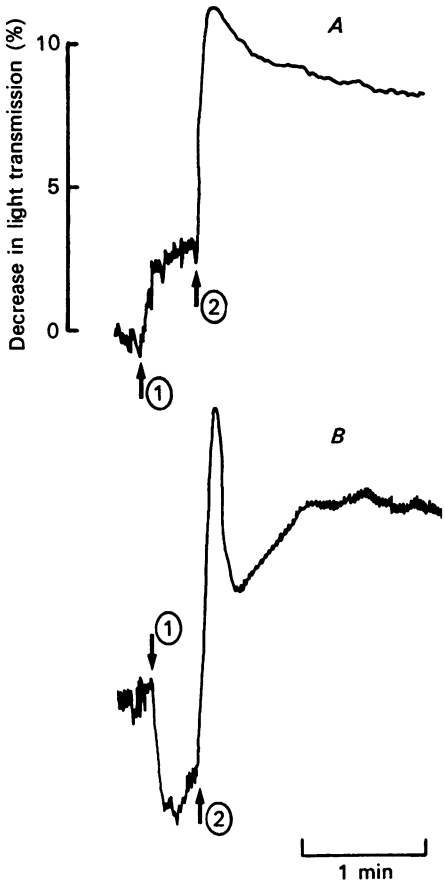
An approximate correction was also made for an error due to vesicles in the platelet cytoplasm by a procedure similar in principle to that just described. This error resulted from (a) an excessively large intercept because chord segments interrupted by vesicle images were included in the total count and (b) from deficits in the measurement of area because parts of chords which traversed vesicles did not count towards the total measured length. To estimate the magnitude of these errors, areas of low optical density were measured rather than the high-density platelet images. The values so obtained included the vesicles within the platelet images as well as the pale background between platelet sections. Sizing factors were applied to these measurements and a plot of $A + Ix$ against x showed a peak at $x = 0{\cdot}225$ μm. This peak identified the mean chord length of the vesicles where $x = \bar{C}x$ and as $\bar{C}x = Ax/Ix$ the total intercept of the vesicles $Ix = Ax/\bar{C}x$ could be calculated. On the assumption, only partially valid, that the vesicle images were circular, calculations gave the mean intercept (tangent diameter per vesicle) $I = 1{\cdot}273\, A/I$; and, as $\bar{I} = I/N$, also the number of vesicles $N = I/\bar{I}$ as well as the mean area per vesicle A/N.

From photomicrographs of platelets under control conditions, the corrected values for vesicles were: $A/I = 0{\cdot}225$ μm; $I/N = 0{\cdot}225 \times 1{\cdot}273 = 0{\cdot}286$ μm; and $A/N = 0{\cdot}65$ μm^2. The average area occupied by the vesicles was about 10 % of that of the platelet images which contained them. A correction for this was applied to all relevant results on the assumption that the size and numbers of vesicles remained statistically constant when changes in shape were induced by aggregating agents; the validity of this assumption is under investigation.

RESULTS

Optical effects of adding EDTA to stirred platelet-rich plasma

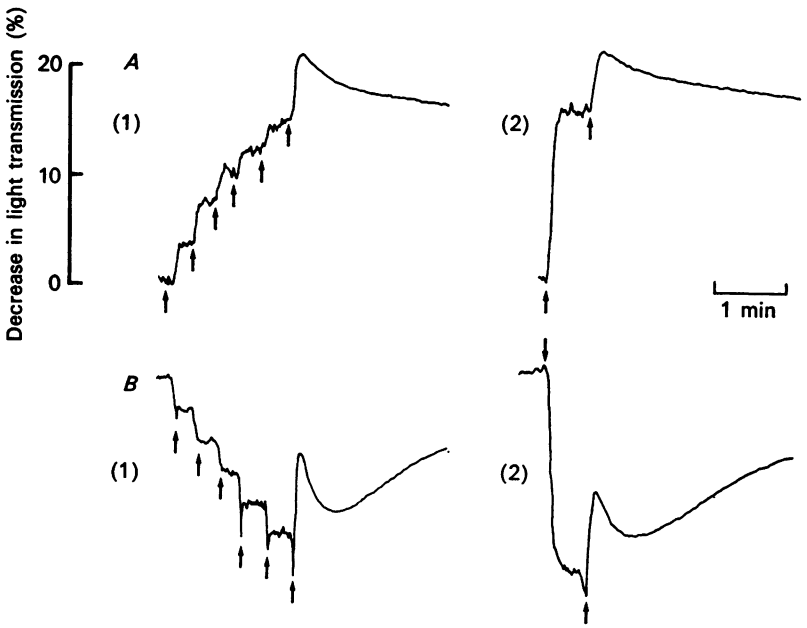
In freshly prepared citrated plasma, the platelets were almost all flat disks with smooth surfaces. When EDTA was added to a concentration of 4 mM, the plasma's light transmittance decreased; simultaneously there was an increase in light scattered horizontally at right angles to the incident beam and to the axis of stirring (Text-fig. 1). These optical effects of EDTA were rapid (reaching their greatest extent in about 5 sec) and persistent; but their magnitude was variable with different plasmas containing similar concentrations of platelets. EGTA produced effects similar to those of EDTA.



Text-fig. 1. Transmitted (*A*) and scattered (*B*) light passed through a 1·0 ml. sample of stirred citrated plasma containing $11{\cdot}9 \times 10^8$ platelets/ml. Ten μl. of 0·4 M-EDTA was added at arrow 1 to give a concentration of 4 mM. Ten μl. 1 mM-ADP was added at arrow 2 to give a concentration of 10 μM. In this and similar figures of optical recordings (Text-figs. 2–6), change in transmitted light is expressed as a percentage of the change in optical density of the sample attributable to the presence of platelets whereas the change in scattered light is expressed on an arbitrary scale of units. Sensitivity settings of the recorder were 0·2 V for transmitted light, 0·02 V for scattered light, and for the aggregometer 5·0 V for transmitted light and 4·0 V for scattered light.

ADP added after EDTA brought about optical changes already described (Michal & Born, 1971). When the effects of EDTA were large, those of ADP or 5-HT added afterwards were comparatively small.

When a previously unstirred sample of platelet-rich plasma was stirred, light transmission rapidly increased to a new stable level and decreased more slowly to the initial value when stirring was stopped (see also Latimer, Born & Michal, 1977). Scattered light rapidly decreased when stirring began; and when stirring stopped there was at first a further decrease followed by a large increase.



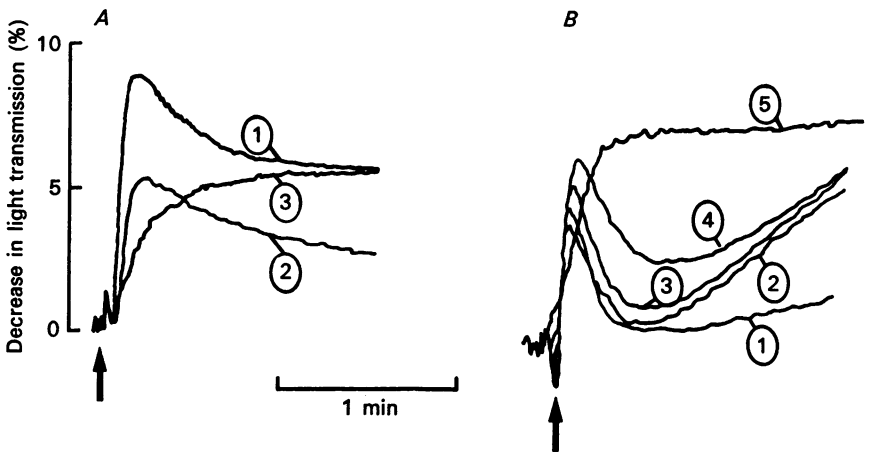
Text-fig. 2. Effects on transmitted (*A*) and scattered (*B*) light passed through plasma containing $6{\cdot}3 \times 10^8$ platelets/ml. when EDTA was added to a final concentration of 25 mM, either in five steps of 5 mM each (1) or in one step (2). ADP (25 μM) was added afterwards. Sensitivity settings of the recorder were 0·5 V for transmitted light and 0·05 V for scattered light, and for the aggregometer, 8·0 V for transmitted light and 5·0 V for scattered light.

After addition of 4–6 mM-EDTA, the optical effects of stirring were either similar or increased, but they were greatly diminished after the further addition of ADP (see also Latimer *et al.* 1977). The optical effects of the chelating agents were directly proportional to concentration over a range of 1–25 mM and the same for a given concentration of EDTA whether it was added in steps or all at once (Text-fig. 2). This is reminiscent of the effect of added calcium on the optical properties of citrated platelet-rich plasma (Born & Cross, 1963). Curiously, the direction of the optical effects on both transmitted and scattered light was the same whether the concentration of calcium was decreased by adding a chelating agent or increased by adding calcium.

EDTA and calcium were the only agents examined which produced opposite effects on transmitted and scattered light. When a maximal optical effect had been

produced by ADP in the presence of 4–6 mM-EDTA, the effect of subsequent additions of more EDTA was undiminished.

The optical effect of ADP (25 μM) added after EDTA was unchanged until the EDTA concentration exceeded 20 mM. With 25 mM-EDTA the amplitude of the change in transmitted light induced by ADP was decreased and with 50 mM-EDTA the velocity was also decreased (Text-fig. 3*A*). On the other hand, the amplitude of the change in *scattered* light caused by ADP increased with increasing EDTA concentration although with 50 mM-EDTA the velocity decreased with the amplitude remaining unaltered (Text-fig. 3*B*).

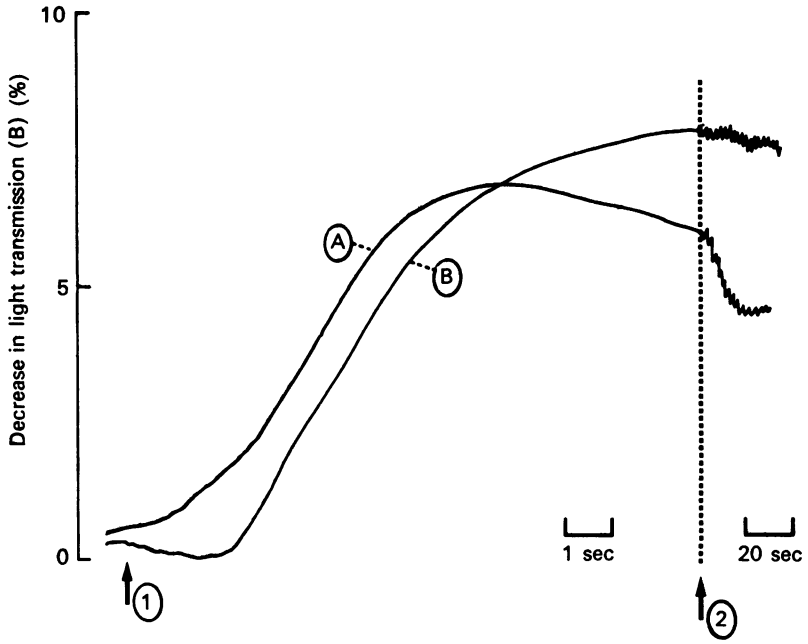


Text-fig. 3. Optical effects of ADP (20 μM) added to platelet-rich plasma in the presence of increasing concentrations of EDTA as follows: on transmitted light (*A*) with 4, 7, 12·5 and 19 mM-EDTA, ADP produced identical tracings and these were superimposed in (1); 25 mM in (2) and 50 mM in (3); and on scattered light (*B*) 4 mM and 7 mM in (1); 12·5 mM in (2); 19 mM in (3); 25 mM in (4) and 50 mM in (5). The plasma and the instrument settings were the same as those in Text-fig. 2.

Comparison of the time courses of the changes in transmitted and scattered light produced by ADP

As a basis for correlating the optical effects of the rapid shape change of platelets with their morphological appearances on electromicrographs, a more detailed analysis than before (Born, 1970; Born, Juengjaroen & Michal, 1972*b*) was made of the effects of ADP or 5-HT on transmitted and scattered light in the presence of enough EDTA (4–6 mM) to prevent aggregation. The time courses of the optical changes and the relative amplitudes of the successive phases were different for transmitted and scattered light (Text-fig. 4). With ADP, the onset of the change in scattered light preceded the change in transmitted light by about 1 sec, and the maximum effect on scattered light was reached 4–5 sec earlier than that on transmitted light. The change in scattered light began to decline rapidly while the change in transmitted light was still increasing (Text-fig. 4). The decrease in the light scattering change from its initial transient maximum was more rapid and proportionally larger than the subsequent decrease in the change in transmitted light. While the change in transmitted light then remained at a plateau (Born, 1970) or decreased

slowly, the scattered light continued to change gradually in the same direction as initially. With some preparations the amplitude of the slow change eventually exceeded that of the initial rapid effect (see Text-figs. 2*B*, 3*B*).



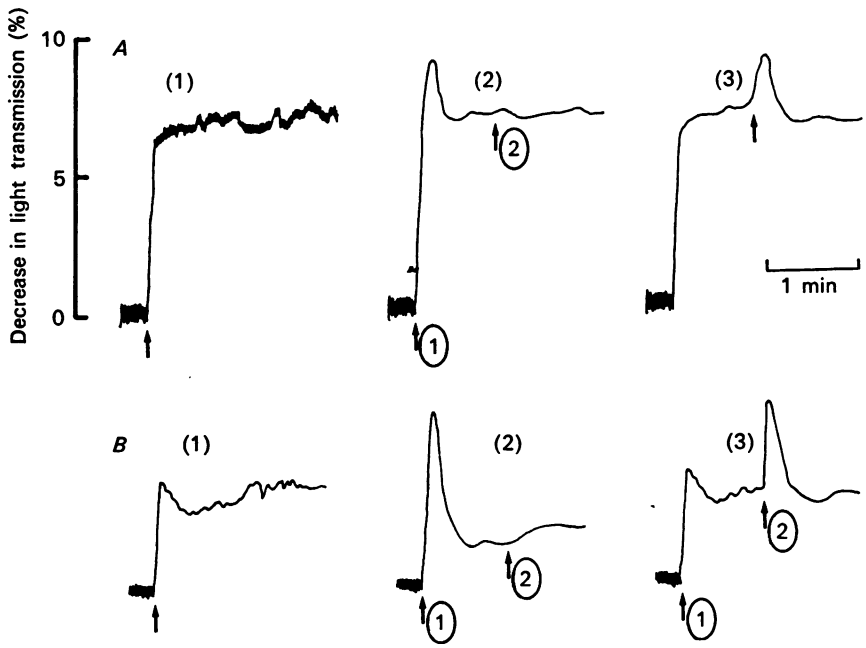
Text-fig. 4. A high-speed recording of the optical effects of ADP (0·2 μM) added at arrow 1 to platelet-rich plasma in the presence of 4 mM-EDTA. The tracings of the changes in transmitted (*A*) and scattered (*B*) light have been superimposed in order to permit a comparison of their time courses. At arrow 2 the paper speed was reduced from 1 to 0·05 cm/sec. Sensitivity settings of the recorder were 0·2 V for transmitted light, 0·02 V for scattered light, and for the aggregometer 5·0 V for transmitted light and 6·0 V for scattered light.

Comparison of the optical effects of ADP and 5-HT

The optical effects of 5-HT differed in some respects from those produced by ADP (see also Born *et al.* 1972*b*). With 5-HT, the initial transient maximum was absent from the record of transmitted light and either absent or very small on that of scattered light. The amplitude of the plateau was about the same as that produced by ADP (Born, 1970) but less well maintained. When 5-HT was added first and the optical change allowed to reach its plateau, addition of ADP then produced a rapid rise and fall in the tracings for both transmitted and scattered light (Text-fig. 5). This effect of ADP closely resembled that part of the early optical change produced by ADP alone which was absent from the effect produced by 5-HT alone. This suggested that the morphological reaction to ADP, unlike that to 5-HT, had at least two components which were distinguishable by optical measurements. With 5-HT concentrations which produced less than the maximal optical effect, the transient change produced by subsequently added ADP was greater than when ADP was added after a higher 5-HT concentration but the subsequent plateau was little changed. 5-HT added after ADP produced small optical changes only on the scattered light tracing.

Reversal of the optical effects of ADP on platelet shape

The changes in transmitted and scattered light produced in platelet-rich plasma by ADP in the presence of EDTA can be prevented or reversed by inhibitors of aggregation including ATP, adenosine and 2-chloroadenosine (Born, 1970). The corresponding optical effects produced by 5-HT can be reversed by methysergide or

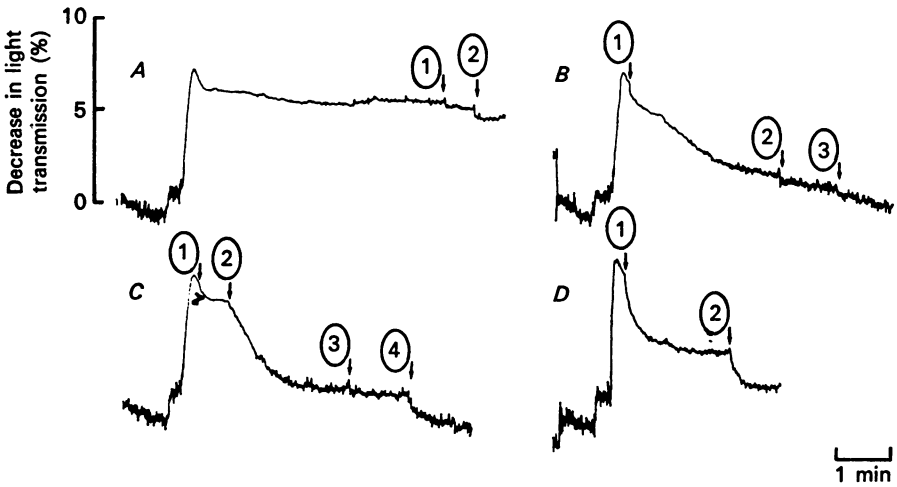


Text-fig. 5. Effects of 5-HT and ADP alone or together on transmitted (*A*) and scattered (*B*) light passed through platelet-rich plasma in the presence of 4 mM-EDTA. For each sequence, 0·3 ml. plasma was mixed with 0·7 ml. saline to give a concentration of $3{\cdot}0 \times 10^8$ platelets/ml. All additions were made in 20 μl. volumes, 5-HT alone (2 μM) produced tracing (1); ADP (20 μM) (arrow 1) followed after 45 sec by 5-HT (2 μM) (arrow 2) produced tracing (2); and 5-HT (2 μM) (arrow 1) followed after 45 sec by ADP (20 μM) (arrow 2) produced tracing (3). Sensitivity settings of the recorder were 0·5 V for transmitted light, 0·02 V for scattered light, and for the aggregometer, 7·0 V for transmitted light and 5·0 V for scattered light.

again by adenosine or 2-chloroadenosine (Born *et al.* 1972*b*). These observations suggested that inhibitors of aggregation can cause reversal of the gross morphological changes in the platelets. This interesting possibility was therefore investigated by a combination of optical and volumetric measurements with methods for quantifying morphological changes recorded on electronmicrographs.

Reversal of the optical effect of ADP was followed with transmitted light, the records of which were larger and more precisely reproducible than those of scattered light. A typical experiment is shown in Text-fig. 6. The first part (Text-fig. 6*A*) shows the sustained decrease in light transmission produced by ADP at the concentration of 10 μM which was used in all of these experiments. The small increase in light transmission recorded first indicates the change produced by EGTA (6 mM)

added 15–20 sec before ADP. The first record also shows the inconspicuous effect of adding 10 and 20 μl. of 154 mM-saline, indicating the absence of significant dilution artifacts in the optical records when reagents were added. Text-fig. 6*B* shows that when 0·2 mM-2-chloroadenosine was added immediately after the optical effect of ADP had passed its peak, light transmission increased again almost to the original base line. The optical effect of ADP was reversed more rapidly by prostaglandin E_1 (1 μM) added shortly after RA-233 (100 μM) (Text-fig. 6*C*), a combination of agents which raises platelet cyclic AMP through respectively activating adenylate cyclase



Text-fig. 6. The optical effect of ADP (10 μM) on light transmitted through platelet-rich plasma (*A*) and its reversal by 200 μM-2-chloroadenosine (*B*); by 100 μM-RA-233 (arrow 1) followed by 1 μM-PGE_1 (arrow 2) in (*C*); and by 1 mM-ATP (arrow 1) in (*D*). In each experiment, addition of ADP was preceded by that of EDTA to a concentration of 6 mM. All inhibitors were added in volumes of less than 20 μl. The control tracing (*A*) also shows the effect of adding 10 μl. (arrow 1) or 20 μl. (arrow 2) of isotonic saline. Repetition of same dose of each of the reversing agents had little additional effect with 2-chloroadenosine (*B*) or with PGE_1 after RA-233 (*C*). However, a second addition of ATP produced a further reversal (*D*). Sensitivity setting was 0·5 V for the recorder, 8·0 V for the aggregometer.

and inhibiting phosphodiesterase. The optical effect of ADP was reversed even more rapidly by the addition of ATP (1 mM) (Text-fig. 6*D*) which competes with ADP at its receptor (MacFarlane & Mills, 1975). When the inhibitors were added a second time, only ATP produced a considerable further reversal (Text-fig. 6*D*).

Volumetric manifestations of the morphological changes

It was shown previously (Born, 1970) that the change in platelet shape produced by ADP is not accompanied by a change in mean volume of the platelets themselves but by increases in the volume of plasma trapped between the platelets when they are packed together by centrifugation at 10,000 *g* for 3 min. This suggested that the average distance between packed platelets is increased by the blebs and spikes on their surfaces. The increase in this trapped plasma volume varied from 24 to 60% (Born, 1970). At the time, the reason for this variation was not investigated further.

In view of our new finding that EDTA or EGTA alone cause rapid concentration-dependent changes in transmitted light in the same direction as those produced by ADP but variable in different preparations, it seemed possible that the chelating agents might themselves induce the formation of extrusions varying in number in different experiments, and this might influence the effectiveness of subsequently added ADP in producing further morphological and optical changes. In three experiments the mean platelet volume was not significantly altered by the presence of EDTA or EGTA (4–6 mM) for up to 15 min at room temperature (Table 1). On the other hand, in the same experiments the chelating agent alone caused a considerable increase in the volume of plasma trapped between platelets which had been packed together by centrifugation at 10,000 *g* for 3 min as compared with untreated controls (Table 1). This supported our conclusion that the rapid optical effects of the chelating

TABLE 1. Effect of shape change and its reversal by inhibitors on the volume of packed platelets and trapped plasma

Addition	Concentration (μM)	No. of experiments	No. of determinations	Mean platelet vol. (μl./10^8 platelets)	Mean trapped plasma vol. (μl./10^8 platelets)
None	—	3	5	0·49 ± 0·02	0·19 ± 0·02
EDTA	4000	—	5	0·49 ± 0·03	0·33 ± 0·02
EDTA	4000	2	3	0·50 ± 0·04	0·30 ± 0·04
5-HT	2	—	2	0·51	0·34
EDTA	4000	6	11	0·48 ± 0·02	0·33 ± 0·02
ADP	10	—	15	0·49 ± 0·02	0·50 ± 0·05
ATP	1000	—	7	0·46 ± 0·03	0·41 ± 0·05
ADP	10	4	10	0·47 ± 0·02	0·45 ± 0·04
PGE_1	2	—	4	0·53 ± 0·02	0·31 ± 0·01
ADP	10	2	7	0·52 ± 0·01	0·40 ± 0·04
2-chloroadenosine	200	—	3	0·50 ± 0·02	0·24 ± 0·01

Comparisons	Differences between means	S.E. of differences	*P*
None *vs.* EDTA	0·14	0·03	0·001
EDTA *vs.* ADP	0·17	0·05	0·005
ADP *vs.* ATP reversal	0·09	0·07	0·20
ADP *vs.* PGE_1 reversal	0·14	0·04	0·008
ADP *vs.* 2-chloro-adenosine reversal	0·16	0·02	< 0·001

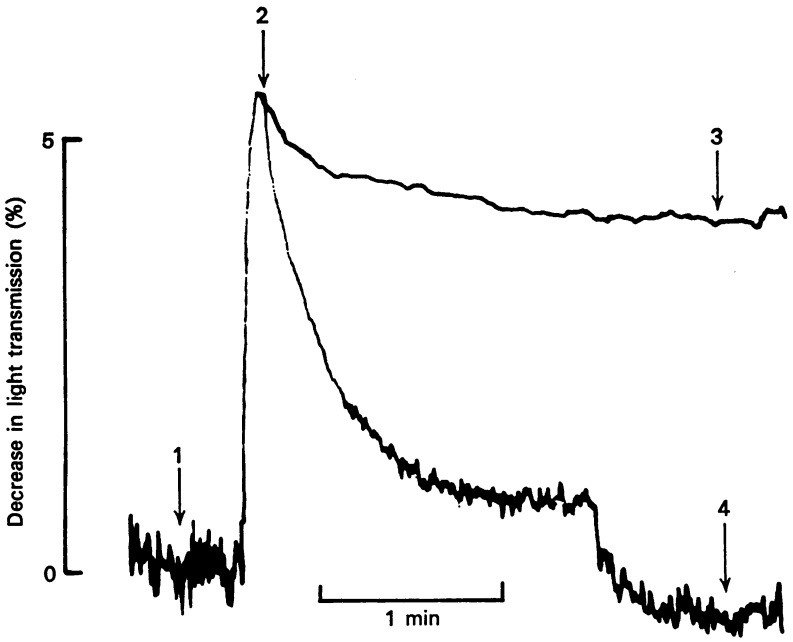
agents were due to the formation of extrusions on the surfaces of platelets which kept them apart during centrifugation. The earlier observation (Born, 1970) was confirmed that ADP causes no significant change in mean platelet volume but increases the volume of trapped plasma (Table 1). In two experiments in which 5-HT was added to produce changes in platelet shape, there was little or no change in mean platelet volume or in trapped plasma volume.

The reversal of the optical effects of ADP in platelet rich plasma by 2-chloroadenosine, prostaglandin E_1, or ATP raised the possibility that these agents are able to reverse the morphological changes caused by ADP, whereby the spikes would

disappear and the platelets revert to their original discoid shape. This expectation received indirect support from volumetric measurements which showed that each of these inhibitors decreased the volume of plasma trapped between sedimented platelets (Table 1). Under these conditions the volume of the platelets themselves remained almost constant at about 5 μl./10^9 platelets.

Morphological measurements

Circulating platelets are normally disk-shaped and are most correctly described as oblate spheroids (Frojmovic & Panjwani, 1976). In order to characterize the sequence of morphological transformations responsible for the optical effects, platelets were fixed at different times after the addition of activating agents and processed for electron microscopy (Text-fig. 7, Pls. 1–5). The appearance of platelets under control conditions, i.e. stirred for 2–5 min at 37 °C in the presence of 6 mM-EDTA, is shown in Pl. 1; and 10–15 sec after the subsequent addition of 10 μM-ADP, i.e. at a time



Text-fig. 7. Superimposed recordings of the optical effect of 2 μM-ADP (upper tracing) alone and of its reversal by 2 mM-ATP (lower tracing) added at the peak of the optical change produced by ADP. The numbered arrows 1–4 indicate when fixative was added for preparation of the electronmicrographs shown on the correspondingly numbered plates.

when the initial change in the optical records was near its maximum, on Pl. 2. In the control samples most platelet profiles were smooth ellipsoids of various sizes, as expected for randomly orientated discoid objects. In addition there were some small round profiles about 1 μm in circumference when cut transversely or long and narrow when cut longitudinally. A plot of the frequency distribution of sectioned objects according to their circumferences as determined with the D-Mac pen-follower showed two peaks. One was low and broad between 5 and 7 μm and represented the

maximum expected statistically for sections of platelet bodies. The other was tall and narrow at 0·5–1·0 μm and comprised about one-third of the total number of separate image profiles (Text-fig. 8*A*); it evidently represented sections through long slender spikes on platelet surfaces. These spikes are not seen in freshly fixed whole blood (Hovig, 1968); their presence on these electron micrographs was presumably caused by the preparatory treatment (centrifugation, pipetting and stirring in the aggregometer in the presence of EDTA).

Ten seconds after the addition of ADP (Pl. 2), the platelets showed three principal modifications. (1) None of the profiles retained the disparity of dimensions which is characteristic of disks. (2) There was a large increase in the proportion of small objects representing the spikes typical of activated platelets. In some sections the roots of these extrusions could be seen to arise from platelet bodies. (3) The surface of the platelets was completely irregular and displayed numerous rounded blebs. The first two changes were also seen 8 sec after addition of 2 μM-5-HT although there were no blebs (Pl. 5*A*).

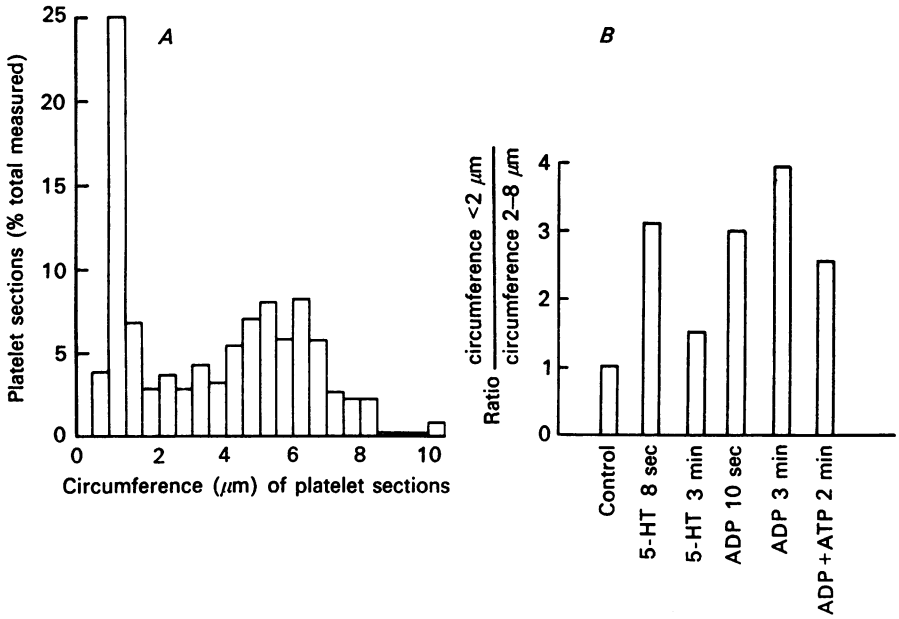
Pl. 3 shows the characteristic appearance of platelets 3 min after the addition of 2 μM-ADP, at a time when the optical changes had stabilized at a plateau (Born, 1970). There were, if anything, more sections of spikes, but the platelet profiles were smoother in outline with none of the surface blebs which characterized the initial phase when the optical effects were maximal.

Pl. 4 shows the typical appearance of platelets after such an addition of ADP followed at the peak of the optical change by the further addition of ATP at a concentration (2 mM) sufficient to reverse completely the optical effect of ADP. Most of the platelets had reverted to a discoid shape and the number of spike sections appeared smaller. The morphological changes produced by 5-HT (Pl. 5*A*) reverted similarly but spontaneously after an interval of 3 min or more (Pl. 5*B*).

These impressions were confirmed by quantification of the numbers of spike sections, i.e. those with circumferences less than 1·5 μm; and by measurements of the length–width ratios of platelets profiles with circumferences greater than 2 μm. The proportion of spike sections less than 1·5 μm in circumference was calculated by planimetry on 360–480 sections for each of the six experimental conditions already described. The results (Text-fig. 8*B*) show that the proportion of these sections increased to about 3 times the control value immediately following the addition of either ADP or 5-HT. Three min after addition of ADP the proportion of spike sections increased further to 4 times the control whereas 3 min after 5-HT the proportion was greatly decreased. Reversal by ATP of the changes produced by ADP was accompanied by decreases in the number of small sections to about 2·5 times that in control samples.

In control samples which contained only EDTA, the length–width ratio of platelet profiles ranged from 1·0, i.e. circular, to 10 with an approximately normal frequency distribution (Text-fig. 9*A*). The ratio was clearly diminished 10 sec after 10 μM-ADP; but the irregularities of the platelet profiles due to bleb formation made measurement of length–width ratios somewhat imprecise. Three minutes after adding ADP the distribution of the ratio was highly skewed towards low values (Text-fig. 9*B*) and the mean was decreased (Table 2). The ratios were increased (Table 2) although return toward the control distribution was incomplete after reversal of the optical effects

of ADP by 2 mM-ATP (Text-fig. 9*C*) or by 600 μM-2-chloroadenosine (Text-fig. 9*D*). The effect of 2 μM-5-HT on the length-width ratios of platelets segments after 8 sec, was similar to that produced by ADP (Text-fig. 9*E* and Table 2) but, unlike ADP, the 5-HT effect reverted spontaneously after 3 min (Text-fig. 9*F*, Table 2).

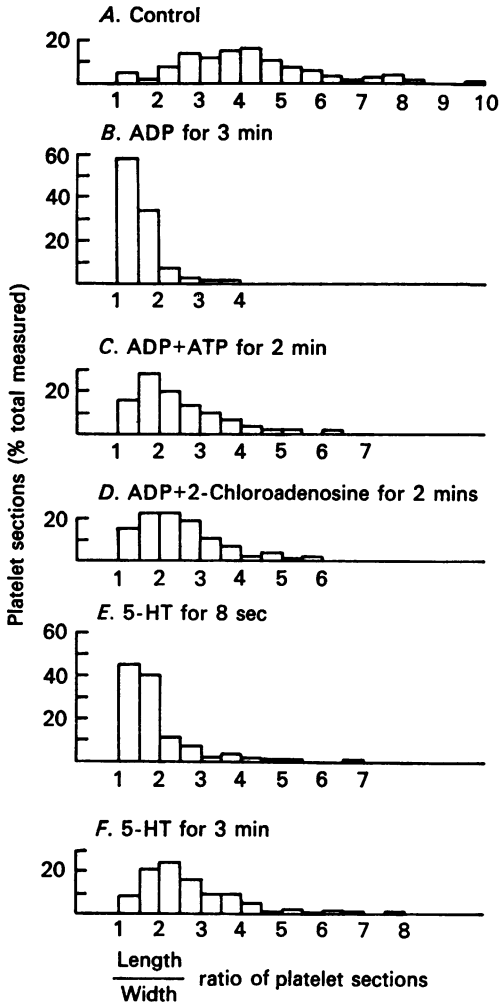


Text-fig. 8. *A*, histogram showing the frequency distribution of control platelet segments of various sizes according to their circumferences as measured by the D-Mac pen-follower on electron micrographs such as the one presented on Pl. 1*A*. *B*, bar graph showing the changes in the proportion of slender platelet extrusions (with circumferences less than 2 μm) after treatment with 2 μM-5-HT (8 sec or 3 min), 10 μM-ADP (10 sec or 3 min) and after reversal of the effects of ADP by 2 μM-ATP.

TABLE 2. Length–width ratio of platelet profiles under different conditions

Condition	No. of determinations	Mean	S.E.
Control	189	4·0	± 0·11
After ADP for 3 min	113	1·5	± 0·04
After reversal by ATP	115	2·4	± 0·10
After reversal by 2-chloroadenosine	137	2·4	± 0·08
After 5-HT for 8 sec	342	1·7	± 0·13
After 5-HT for 3 min	545	2·6	± 0·02
	Comparisons		
	Difference between means	S.E. of difference	*P*
Control *vs.* ADP (3 min)	2·5	0·12	< 0·001
ADP *vs.* reversal by ATP	0·9	0·11	< 0·001
ADP *vs.* reversal by 2-chloro-adenosine	0·9	0·09	< 0·001
Control *vs.* 5-HT	2·3	0·17	< 0·001
5-HT (8 sec) *vs.* 5-HT (3 min)	0·9	0·13	< 0·001

The image-analysing computer provided more precise quantitation of the morphological changes produced by ADP (Table 3). The increase in the number of spikes following the addition of ADP was reflected in a doubling of the ratio of their combined area to that of the total area of all platelet images; this increase persisted



Text-fig. 9. Histograms showing the frequency distribution of varying degrees of axial asymmetry of platelet segments expressed as length:width ratios obtained from direct measurements on electron micrographs. Results are presented for the following conditions of treatment: *A*, control with 6 mM-EGTA; *B*, 3 min after the addition of 10 μM-ADP; *C*, 2 min after the addition of 10 μM-ADP followed by the addition of 2 mM-ATP; *D*, 2 min after the addition of 10 μM-ADP followed by the addition of 600 μM-2-chloroadenosine; *E*, 8 sec after the addition of 2 μM-5-HT; and *F*, 3 min after the addition of 2 μM-5-HT.

throughout the 3 min period during which samples were taken. The frequency distribution of image sizes before and at increasing times after the addition of ADP confirmed the results obtained with the D-Mac pen-follower (Text-fig. 10). The

increase in mean intercept and the decrease in mean chord length observed 3 sec after ADP but not later (Table 3) reflected the addition of many short chords to the images of platelet bodies (the small images of spikes were excluded from this computation). These changes were expected from the irregularities of the image profiles due to the blebs which ADP caused to appear transiently on the platelets.

TABLE 3. Platelet parameters determined with the image-analysing computer

Condition	Area of processes: Total area (2)	Platelets (1): No. of images	Mean area (μm^2)	Mean intercept (μm)	Mean chord lenth (μm)
Control	0·1090	739	1·595	1·598	0·998
After ADP for 3 sec	0·2094	480	1·587	2·273	0·698
After ADP for 20 sec	0·1897	411	1·569	1·521	1·031
After ADP for 3 min	0·1847	459	1·585	1·566	1·012

Condition	Area of processes: Total area (2)	Slender processes (2): No. of images (3)	Mean area (4) (μm^2)	Mean intercept (μm)	Mean chord length (μm)
Control	0·1090	360	0·058	0·308	0·188
After ADP for 3 sec	0·2094	1340	0·072	0·383	0·188
After ADP for 20 sec	0·1897	803	0·074	0·393	0·188
After ADP for 3 min	0·1847	952	0·077	0·410	0·188

(1) The measurement for platelets was made by using a sizing factor which excluded all values of less than 0·375 μm.

(2) The measurement for long slender processes was made by obtaining the differences between the values made with a sizing factor of 0·375 μm and one which excluded only background noise (i.e. values of less than 0·075 μm).

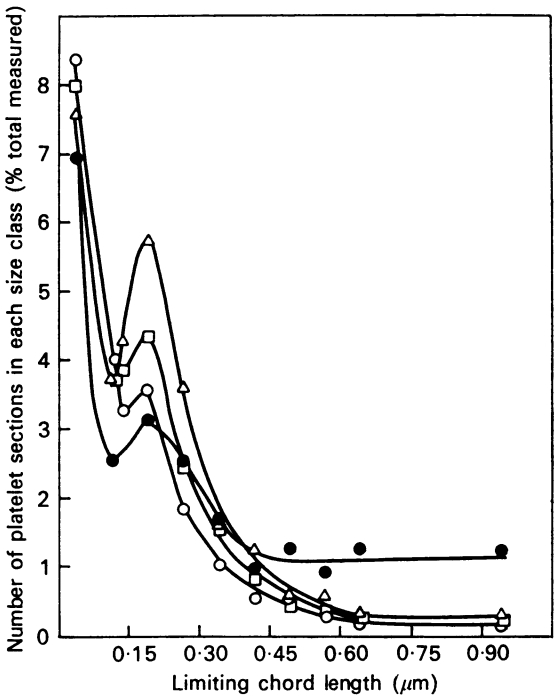
(3) The relative number of slender processes was estimated from the position of the break in the slope of the graph of the total counts against sizing factors as plotted in Text-fig. 9.

(4) The area of all sections through long slender processes, separate from that of other platelet images, was calculated by assuming that the processes constituted a symmetrical population of chord lengths around the peak which was observed between class size intervals of 0·150 and 0·225 μm, i.e. half their total area = measured A + (measured $I \times 0{\cdot}188$). When the area of these processes had been calculated, their intercept was determined from $A/I = 0{\cdot}188$.

DISCUSSION

The morphological changes which accompany activation of platelets by aggregating agents have so far been quantified by optical and volumetric methods. The information which they provide has had to be interpreted on non-quantitative impressions obtained from microscopical observations, usually made in parallel but separately. Quantitative phase-contrast microscopy (Frojmovic & Panjwani, 1976) has permitted the calculation of the axial ratios (thickness/diameter) for rabbit and human platelets in randomly orientated suspension. The mean value so obtained, 5·1, is in reasonable agreement with the value of 4·0 for the axial ratio of normal rabbit platelets which we have determined from electron-microscopic section images. These observations have been limited to apparently normal platelets.

The results in this paper are based on quantitative techniques for the direct measurement of changes in platelet morphology, by measurements made on electron micrographs of fixed centrifuged platelets. This has enabled us to correlate the indirect, optical and volumetric evidence of the shape changes with the morphological features which give rise to them, namely the overall shape of platelet bodies, the smoothness or irregularity of their surfaces, and the number and sizes of extrusions which appear as blebs and spikes.



Text-fig. 10. Plots of the frequency distribution of platelet images of various sizes calculated with the 'Quantimet' image analyzing computer by obtaining the increment in numbers of images counted with successive 0·075 μm increases in the 'sizing factor' (limiting chord length). The number of segments in each size class is expressed as a percentage of the total count (i.e. the number of images obtained when only the background noise (chord lengths less than 0·075 μm) was excluded). The plots are for (1) control conditions (●), and for platelets fixed at the following intervals after the addition of 10 μM-ADP: (2) 3 sec (○); (3) 20 sec (□); and (4) 3 min (△).

The main results are as follows. (1) The constancy of the mean total area of platelet sections as determined by computerised image analysis corresponded closely with the constancy of platelet volumes as determined by the thrombocrit method (Born, 1970) under various experimental conditions. (2) The large increases of 55–60% caused by EDTA in the volume of plasma trapped with centrifuged platelets was accompanied by the appearance on electron micrographs of large numbers of small images representing sections through the long slender extrusions or spikes. These small images outnumbered sections through the main bodies of platelets by about 3 to 1. It should be remembered that these spikes are comparatively long in relation to the thickness of the electron microscope sections and therefore arise from several

layers of platelet bodies lying in other planes parallel to that of the section photographed. (3) The further increases of 50–100% in the volume of trapped plasma following the addition of 10 μM ADP after the previous addition of EDTA were accompanied by a corresponding increase of about twofold in the percentage of platelet sections comprising the peak of the continuous frequency distribution curve of platelet images. Furthermore, enumeration of all sections with a circumference less than 2 μm indicated an even greater increase of 3- to 4-fold in the proportion of images representing sections through spikes. This effect of ADP confirms earlier volumetric determinations (Born, 1970) and supports the interpretation then proposed. The absence of similar increases in volume of trapped plasma 3 min after addition of 5-HT corresponds with the failure of these preparations to maintain the initial decrease in light transmission and in the number of spike sections counted on electron micrographs of platelets fixed 8 sec. after the addition of this agent. (4) The reversal of the optical effects of ADP by the subsequent addition of 2-chloroadenosine, prostaglandin E_1 or ATP was accompanied by diminutions in both the increase in trapped plasma volume due to ADP and in the proportion of spike images on electron micrographs. At the same time the shape of the platelet bodies indicated by the length–width ratios of their section images reverted from spherical to discoidal. Similar reversals accompanied the spontaneous subsidence of the morphological effects produced by 5-HT.

The morphometrically quantified reversals of the shape changes produced by ADP were always in the expected direction but usually less complete than their accompanying optical records would indicate. This suggests that there is some persistence of the effects of ADP, presumably depending on the continued interaction of ADP with its platelet receptor and on the stimulus–response coupling sequence which this produces. Nevertheless, our results show that the morphological changes produced by ADP can be largely reversed, either as a result of the blocking of the ADP receptor by a competitive antagonist such as ATP or by an increase in platelet cyclic AMP through the activation of adenylate cyclase by 2-chloroadenosine or prostaglandin E_1 together with phosphodiesterase inhibition by RA-233. Thus, the rapid reactions of platelets to an agonist or subsequently to antagonists which have so far been quantified by indirect optical and volumetric methods have now been accounted for quantitatively by direct morphometry.

Our observations call attention to a difference in the time course of changes in transmitted and scattered light following the addition of ADP to platelet-rich plasma. The maximum change in transmitted light was reached later but persisted whereas the change in scattered light subsided rapidly although incompletely from its earlier maximum. This difference can be accounted for morphologically from measurements on the electron micrograph. They showed that the rapid decline of the change in scattered light is accompanied by the disappearance of the blebs which covered the platelet surfaces immediately after ADP was added and which were also associated with a transient decrease in the mean chord length obtained from the computerized image analysis. When 5-HT was added instead of ADP, the morphological reaction did not include the rapid transient formation of such blebs; and the optical peak characteristic of bleb formation could still be elicited by ADP added after 5-HT had produced its maximal optical effect. These observations provide a morphological

basis for differences in the optical and volumetric manifestations of platelet activation by ADP and by 5-HT.

The disparity in the direction of the effects on transmitted and scattered light by the chelating agents EDTA or EGTA and by calcium added to platelet-rich plasma remain unexplained. Our morphological and volumetric observations indicate that addition of EDTA or EGTA alone causes the formation of spikes. Theoretical considerations suggest that the formation of spikes increases light transmission through platelet-rich plasma, thereby opposing the optical effect of sphering of the platelets (Latimer *et al.* 1977). This could account also for the observation that chlorpromazine, which causes sphering but no surface extrusions, produces a greater diminution in light transmission than ADP. Experimentally the percentage changes in optical density produced by chlorpromazine or ADP were reduced equally in unstirred platelet-rich plasma. It was concluded that changes in the optical density of platelet-rich plasma resulting from stirring were limited to the effect of disk to sphere transformation and were not affected by the absence or presence of spikes. Our results now show, however, that calcium chelating agents not only decreased light transmission but also increased the optical effects of stirring of platelet-rich plasma. Of all agents examined only the chelating agents (or added calcium) increased horizontally scattered light. Unlike other agents which caused the extrusion of spikes, the chelating agents did not alter appreciably the overall discoid shape of the platelets. These observations suggest that chelating agents either modify platelet shape to produce thinner discs of greater circumference or bring about a radial distribution of spikes from the outer rim of the platelets so as to increase their responsiveness to flow-dependent orientation.

Dr Dearnley's contribution is published by permission of the Director, Institute of Geological Sciences.

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EXPLANATION OF PLATES

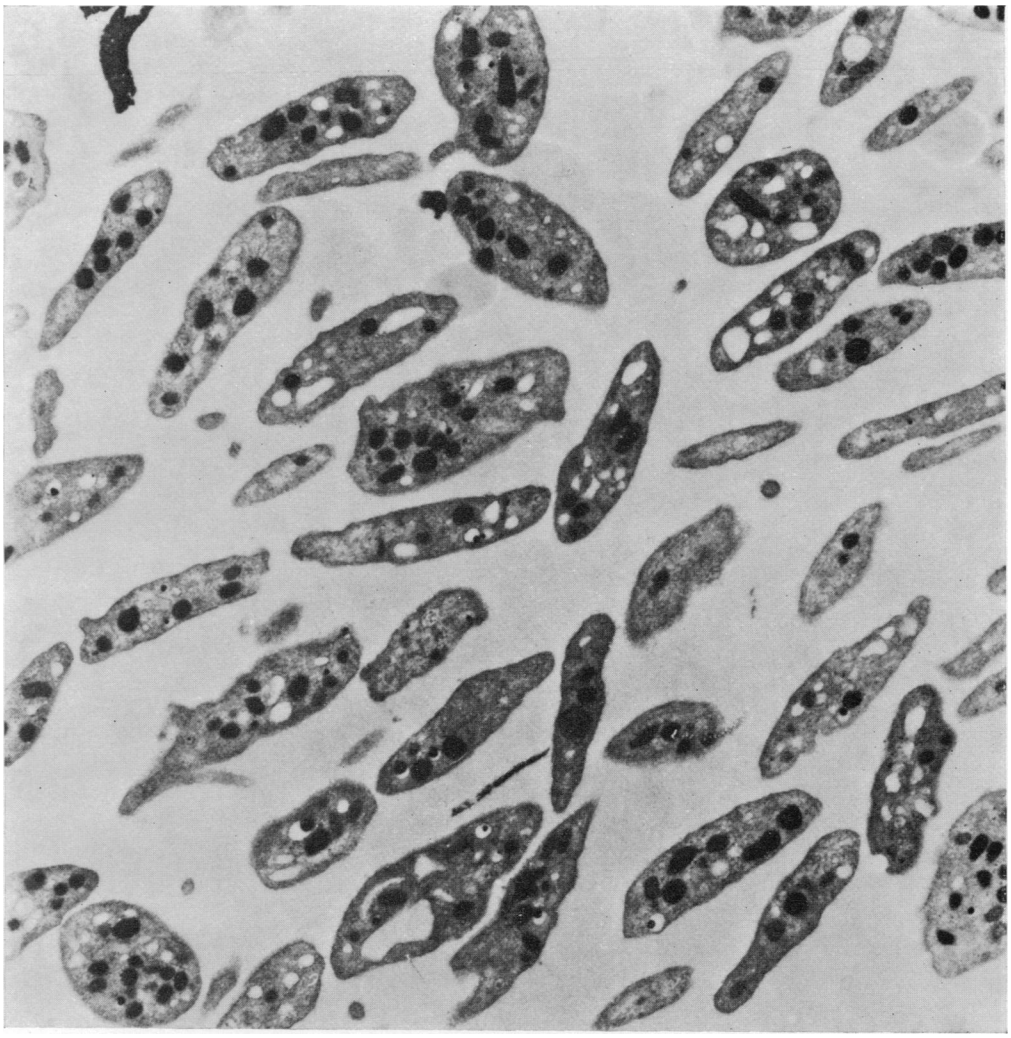
PLATES 1–4

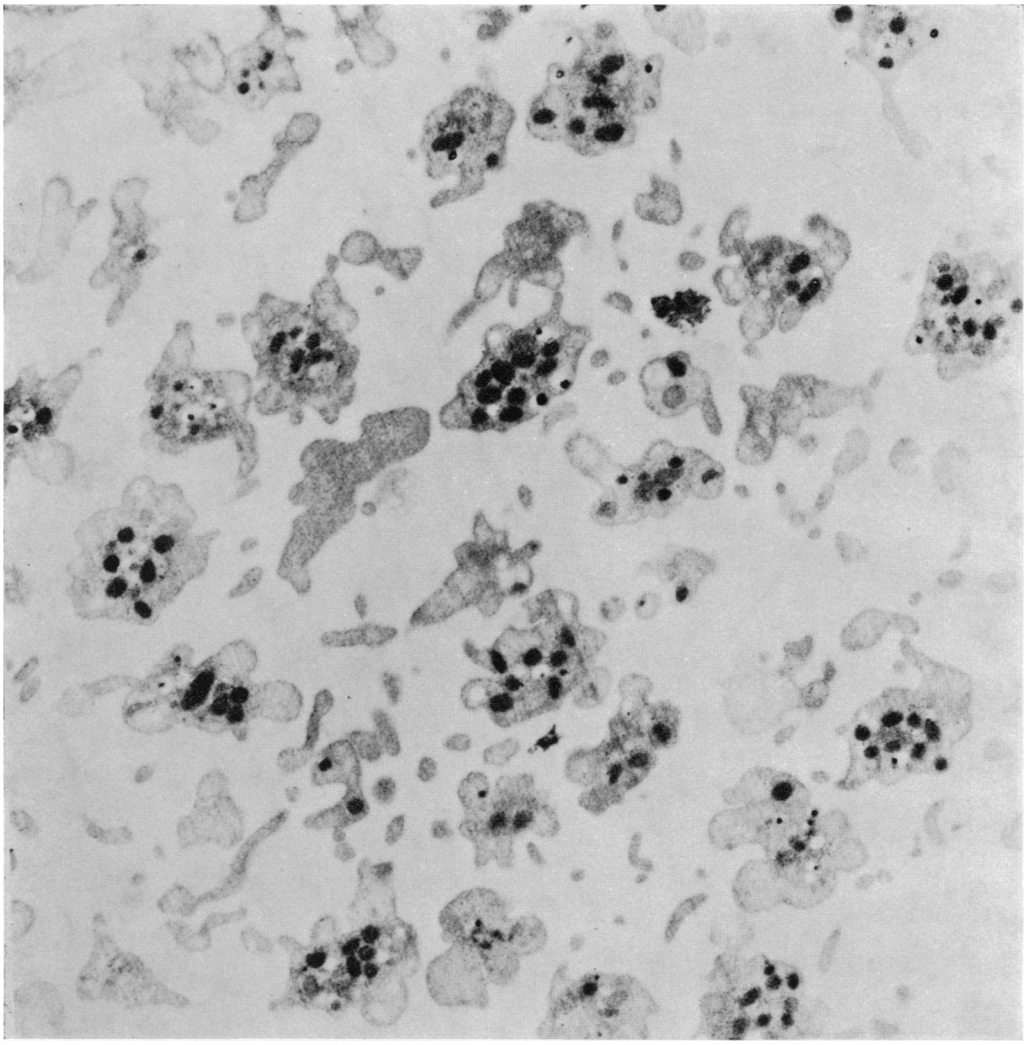
Pls. 1–4 are electronmicrographs of rabbit platelets from a single experiment. Each sample was initially stirred for 2 min at 37 °C with the addition of 6 mM-EDTA during the final 15 sec.

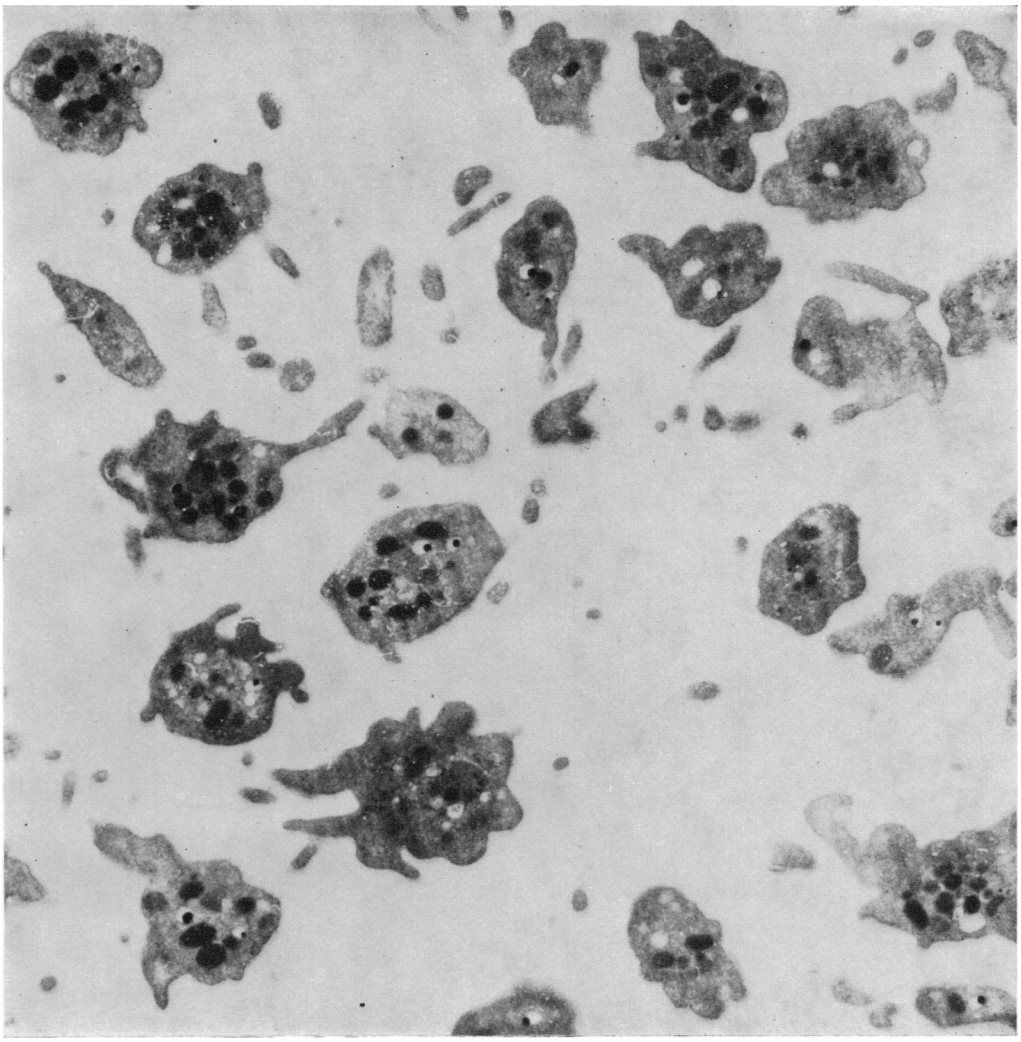
In Pl. 1 the sample was fixed at the end of this preliminary incubation. In Pl. 2 the sample was fixed 10 sec after addition of 10 μM-ADP when the optical recording of the shape change had reached its peak. In Pl. 3 the sample was fixed 3 min after addition of 10 μM-ADP. In Pl. 4 the sample was fixed after reversal by 1 mM-ATP of the optical effect of the shape change produced by 10 μM-ADP. ATP was added first at the peak and again 2 min later to complete the reversal.

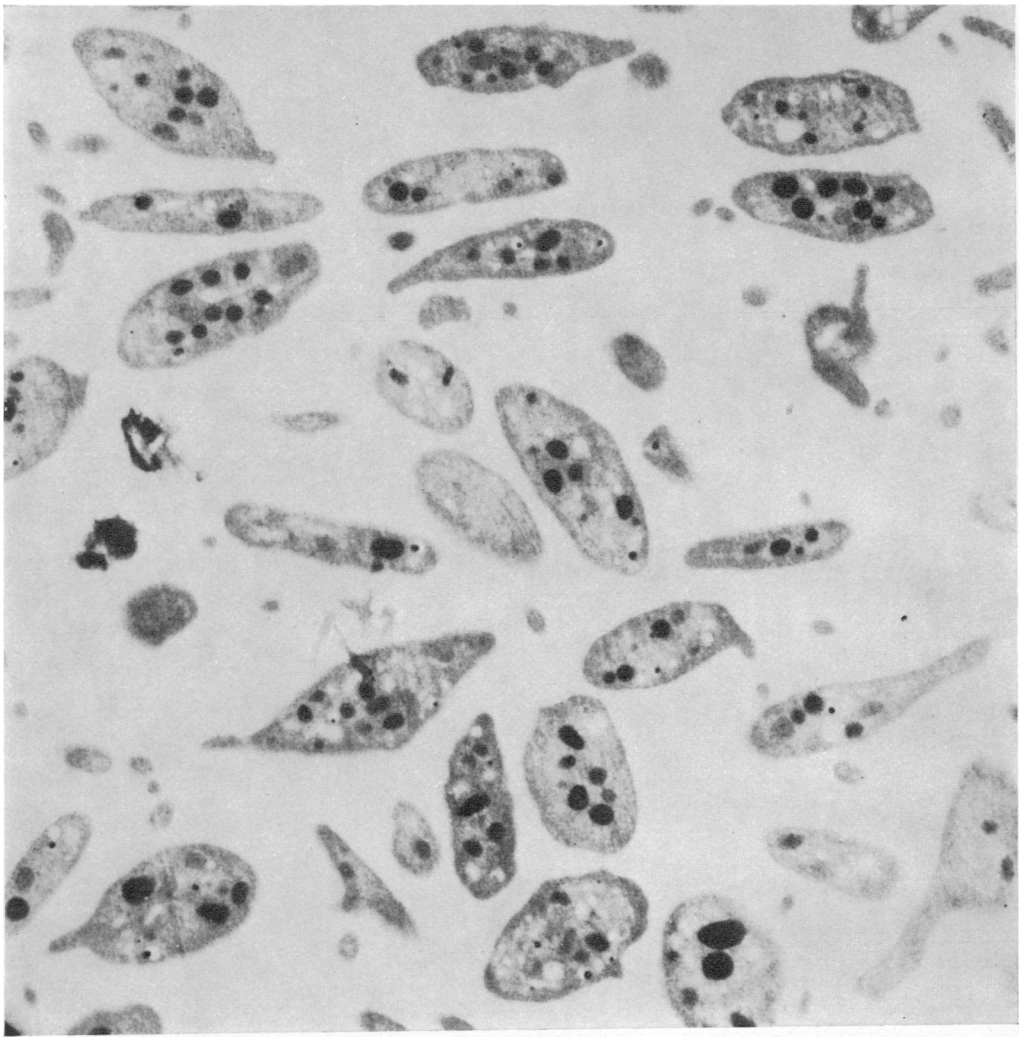
PLATE 5

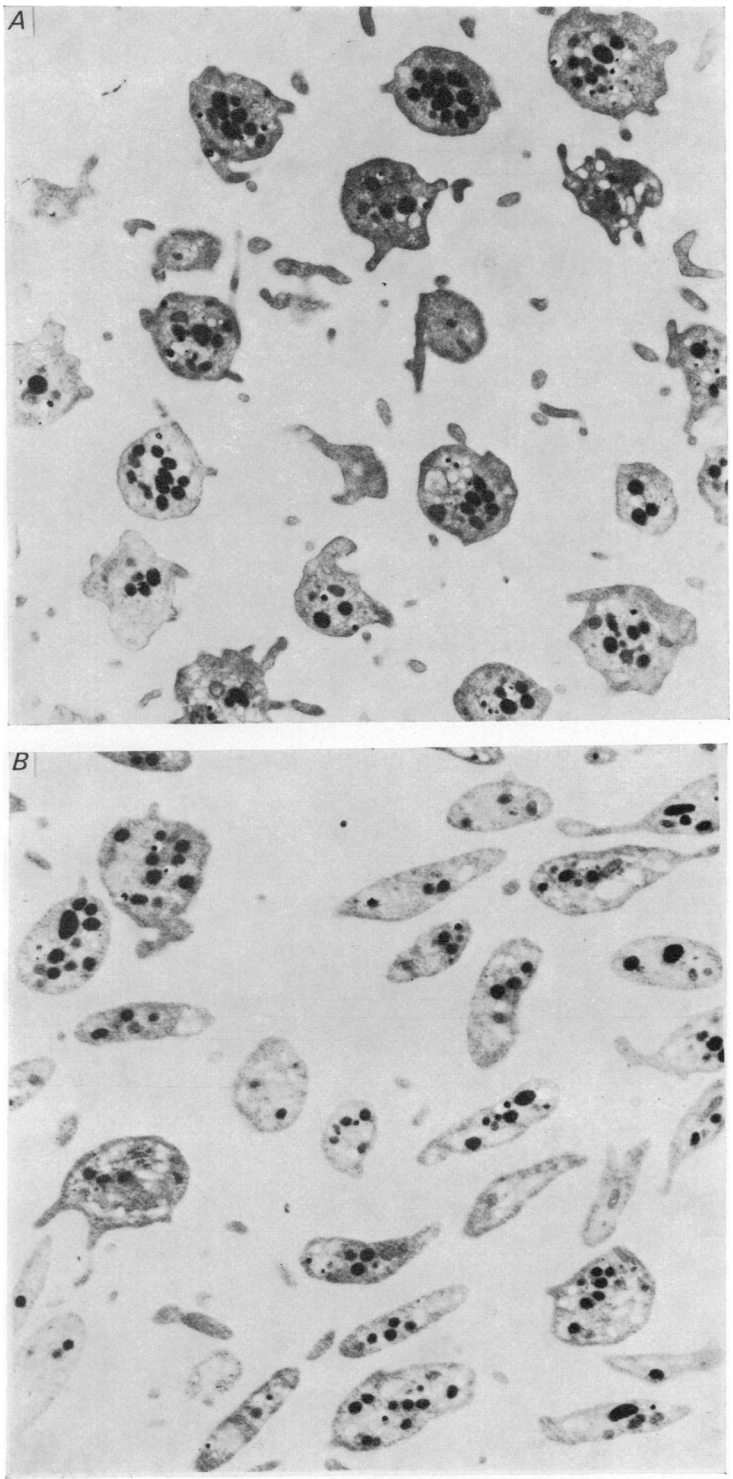
This shows electron micrographs of rabbit platelets after similar preliminary incubation followed by the addition of 2 μM-5-HT. The sample shown in photograph *A* was fixed 8 sec later and that shown in *B* 3 min later.







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