

TRANSFORMATION AND MOTILITY OF HUMAN PLATELETS

Details of the Shape Change and Release Reaction

Observed by Optical and Electron Microscopy

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ABSTRACT

Blood platelets from 10 normal human subjects have been examined with a sensitive differential interference contrast (DIC) microscope. The entire transformation process during adhesion to glass is clearly visible and has been recorded cinematographically, including the disk to sphere change of shape, the formation of sessile protuberances, the extension and retraction of pseudopodia, and the spreading, ruffling, and occasional regression of the hyalomere. The exocytosis of intact dense bodies can be observed either by DIC microscopy, or by epifluorescence microscopy in platelets stained with mepacrine. Details of fluorescent flashes indicate that the dense bodies usually release their contents extracellularly, but may do so intracytoplasmically under the influence of strong, short wavelength light on some preparations of mepacrine-stained platelets. The release of one or more dense bodies leaves a crater of variable size on the upper surface of the granulomere. Such craters represent the surface component of the open canalicular system and their formation and disappearance can be directly observed. Because these techniques permit quantitation of several parameters of motility which are not readily observable by other techniques, it is suggested that high extinction DIC microscope examination may become a rapid and useful method of studying congenital and acquired platelet disorders. Many features of platelet transformation have been confirmed and extended by scanning electron micrographs. These can in turn be interpreted by reference to time-lapse films of living platelets.

KEY WORDS platelets · transformation ·
release reaction · exocytosis · pseudopodia ·
motility · DIC microscopy · scanning electron
microscopy

It has been known since the last century that platelets (thrombocytes) undergo a change of

shape (transformation) as part of their role in hemostasis (for reviews, see references 19, 20, 22, 51, 60, 66, and 67). Light microscope observation established that platelets formed spiky protuberances and gradually spread on glass surfaces in imitation of their spreading on damaged vascular endothelia (9). However, the resolution and sen-

sitivity of light microscopes have generally been considered inadequate for direct observation of the transformation process in single platelets because of their minute size.

Many authors have, therefore, investigated transformation by either scanning or transmission electron microscopy (8, 10, 28, 29, 34–38, 46, 48, 52, 53, 56–58, 64–71). However, the extent to which platelet morphology is altered by fixation in such procedures is unknown. Furthermore, transformation in a population of platelets is not synchronized, since platelets sediment varying distances and settle at different rates (because of differences in density) to the test surface. Any flow near the surface tends to dislodge weakly attached forms causing the population of fixed platelets to be biased toward the later spread forms which are more firmly attached. For these reasons an element of doubt remains regarding the conclusions drawn concerning the transformation process from static representations of fixed specimens.

Recent refinements in microscope design (32, 45) and in the quality of differential interference contrast (DIC) components (4, 23) have not only increased the resolving power of the light microscope by a factor of about two, according to accepted criteria, but have rendered many submicroscopic details in the range of 20–200 nm readily observable by increased image contrast. The result of these improvements is that images obtained with the best DIC microscopes are capable of revealing dynamic changes in the structure of living platelets that could be inferred only indirectly from laborious ultrastructural studies.

The use of high extinction DIC microscopy in studies of platelet structure and function is potentially significant for several reasons. First, some artifacts introduced during fixation for electron microscopy might now be detectable. Second, quantitative information on the motility, transformation, and exocytosis of living platelets is obtainable from photomicrographic and cinematographic records. Such information might be valuable in the investigation of platelets from individuals with congenital or acquired platelet disorders and in assessing the efficacy of pharmacological treatments designed to modify platelet function.

In the present study considerable effort has been devoted to finding conditions for fixation and specimen preparation that resulted in platelet morphologies identical with DIC micrographs of living platelets. A complete description of all experiments with different anticoagulants, various

buffers at different pH values, etc., is beyond the scope of this paper. The results provide what is believed to be a full and accurate report on changes in surface form of platelets during transformation and exocytosis.

MATERIALS AND METHODS

Preparation of Platelets

Blood samples (2.5 ml) from 10 normal human volunteers of both sexes were collected by venipuncture of the cubital vein by 19-gauge siliconized butterfly needles without syringe by allowing blood to flow into plastic vials containing 0.24 ml of 3.5% sodium citrate as anticoagulant. The first blood sample was discarded to remove tissue fragments. Some vials were left unbuffered while others were buffered to pH 7.4 with 0.03 M phosphate. One 2.5-ml sample was allowed to flow into 10 ml of stirred 2% glutaraldehyde at 22° or 37°C buffered at pH 7.2–7.4 with 0.2 M cacodylate to preserve the forms of circulating platelets for later comparison to the state of platelets in platelet-rich plasma (PRP).

Blood samples were centrifuged at 730 g for 3.5 min at 22°C, and the PRP was withdrawn with a polyethylene pipette and placed in clean vials either at room temperature (22°C) or in a 37°C water bath. Unbuffered preparations showed no change in ability to form pseudopodia and spread for at least 1 h, and buffered preparations could be used for at least 3 h after incubation at 22°C and for shorter times at higher temperatures. Some samples remained capable of normal transformation for at least 8 h.

One drop of PRP was deposited on a 24 × 60 mm No. 0 cover glass treated with Siliclad^R and taped to a flat stainless steel frame.¹ The cover glass and frame had been prewarmed to 29°C. A prewarmed, 22-mm square cover glass was placed over the drop and sealed with "valap" (a 1:1:1 mixture of vaseline, lanolin, and paraffin). The preparation was then placed on a rotatable microscope stage, the central portion of which had been warmed to 29°C by a Sage air-curtain incubator (Sage Instruments Div., Orion Research Inc., Cambridge, Mass.). In some experiments, platelets were preincubated with the fluorochrome mepacrine (Hoffman LaRoche, Des Plaines, Ill.) at a concentration of 5×10^{-5} M (39–41).

Optical Microscopy

The microscope was a Zeiss Inverted Axiomat (32, 45) equipped with dark-field (DF), phase-contrast (PC), epifluorescence, and DIC attachments of new design and especially selected for high extinction factor. The DIC attachments with the strain-free 100 × planapochromatic

¹ Platelets do not spread extensively on untreated glass surfaces.

objective and strain-free aplanatic-achromatic condenser were used at a working aperture of 1.10 and exhibited an instrumental extinction factor of about 1.4×10^3 . A high extinction factor is essential in recording fine details. Image contrast was controlled by bias compensation introduced by a Brace-Köhler approx. $\lambda/20$ compensator after the sliding "upper" Wollaston prism was adjusted to extinction. Test photographs taken of one sample of platelets in various mixed stages of transformation at a series of bias compensation settings established that optimal contrast for the platelet as a whole was obtained at a setting of 15 nm or 0.026λ at $\lambda = 546$ nm. This setting exceeded the edge extinction setting for all platelet details. Photomicrographs were taken at an exposure of <1 s with the internal 35-mm camera using Kodak S.0.115 film, which was subsequently processed in D19 (9 min in solutions A and B) to an ASA exposure index of ~ 400 . Cinemicrographic records at 1/4-s exposure were made also with Kodak S.0.115 ciné film and were processed commercially by an extended development process (Cine Service Laboratories, Watertown, Mass.).

Fixation, Dehydration, and Scanning Electron Microscopy

Platelets in various stages of transformation were prepared for scanning electron microscopy in a manner similar to preparations for light microscopy. At predetermined times cover glasses with attached platelets were rinsed briefly (3 s) in neutral phosphate-buffered saline and fixed for 30 min in 2% glutaraldehyde at either 22° or 37°C buffered with 0.2 M phosphate or cacodylate at pH 7.2–7.4. All specimens were postfixed for 30 min in 2% osmium tetroxide at 4°C. Preparations were dehydrated in an alcohol series and critical-point dried (5, 17, 18) in CO₂ in a BoMar SPC=900EX critical-point dryer (Tacoma, Wash.). A coating of gold-palladium ~ 15 – 20 nm thick was applied with a Hummer II sputter coater (Technics, St. Alexandria, Va.). Specimens were viewed and photographed using a Coates & Welter model 106A Cwiskscan² SEM (Coates & Welter Instrument Corp., Sunnyvale, Calif.) at accelerating voltages of 14–18 kV and at tilt angles of 45–63°.

RESULTS

Relative Merits of Different Contrast-Generating Light Microscope Systems for Viewing Living Platelets

While DF, PC, and DIC attachments with the

² The fact that we obtained electron micrographs with this instrument does not constitute a recommendation of its use, because the manufacturers were unable to maintain the instrument to guaranteed specifications.

same microscope objectives all reveal many more features of the structure of transforming platelets than bright-field microscopy, DIC produces by far the most informative images. Although all details are inflated to airy disk diameter, only with DIC (not DF or PC microscopy) has it been possible to record pseudopod shapes that correspond to shapes observed in scanning electron micrographs of well-preserved, fixed specimens. Only with DIC could the shapes of cells be clearly perceived and different particles within the granulomere be recognized in optical sections.

Shapes of Platelets Fixed Immediately on Removal from the Cubital Vein

Living platelets in PRP exhibited a considerable range of morphologies from the disks (which are commonly believed to be characteristic of circulating platelets), to disks with protuberances, spheroids with and without protuberances, bipolar forms, and "sperm-shaped" forms (Fig. 1). The percentage of platelets in other-than-discoform ranged from 5 to 95% in PRP prepared from different samples of blood and at different times.

To determine whether this distribution of shapes was characteristic of freshly drawn blood or was the result of the preparation of platelets in PRP, samples of blood allowed to flow into stirred fixative before the platelets had been separated were examined. Although these platelets showed a higher percentage of discoform, the nondiscoform were seen there as well. In one sample, for example, there were 66.2% disks without protuberances, while disks with protuberances accounted for another 14.2%. Spheroidal platelets with protuberances (8.3%) and without protuberances (5.0%) accounted for all but 6% of all forms. Although the possibility cannot be excluded that the forms of circulating platelets may have been altered during the second or two that elapsed between their removal from the vein and interaction with fixative, it seems likely from this study and others (7, 24) that the form of circulating platelets may be quite variable. In any case, it is clear that in many preparations of PRP the shapes of platelets are different from those of platelets that have been rapidly fixed. What is important in the present work is that subsequent stages in transformation appear to be similar, irrespective of the shape variability of platelets suspended in PRP.

The forms of early transformation stages observed in the SEM not only confirmed those ob-

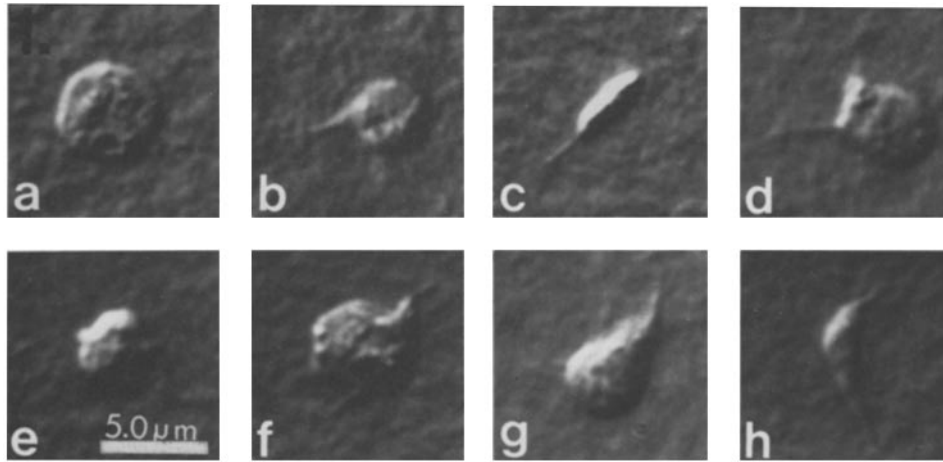


FIGURE 1 Circulating platelet forms preserved by dripping blood from the cubital vein into stirred 2% glutaraldehyde. (a) Discoid form (note dense bodies and open canalicular system); (b) discoid form with tangential protuberance; (c) discoid form with a radial protuberance; (d-f) spheroidal forms with protuberances of variable length (note dense bodies being exocytosed in Fig. 1f; (g) sperm shaped platelet; (h) bipolar form.

served by DIC microscopy, but were, of course, presented in far greater surface detail. Discoid (lenticular) and rounded forms, with and without protuberances (most of which probably were pseudopodia), are shown in Figs. 2 and 9. In most cases these protuberances ranged in diameter³ from 50 to 200 nm and were directed radially or tangentially from the edges of the disk. Many discoid and rounded forms exhibited surface pits or cavities.

The shapes of protuberances were characteristically cylindrical, usually with a slight taper and with hemispherical ends (Figs. 2 and 3). Other forms of pseudopodia observed in prepared specimens appear to be the result of fixation and/or drying artifact.

Nature of Protuberances on Discoid and Spheroid Platelets

Platelets suspended in PRP were observed while sedimenting toward the glass surface. It could be seen that some of the shorter protuberances similar to those in Figs. 1 and 2 were nonmotile, while others, especially on the spheroid forms, were extending or retracting; the latter will be referred to as pseudopodia.

³ Dimensions are corrected for the metallic coatings applied to specimens.

Role of Pseudopodia in the Spreading of the Hyalomere

As sedimenting platelets of either discoid or spheroid form contact siliconized glass, they form greater numbers of pseudopodia that are longer than those characteristic of the suspended platelets (Fig. 4). Some pseudopodia extend into the medium (Figs. 2-4) where they frequently bend and wave before being retracted. Pseudopodia that extend in contact with the substratum are unlikely to be retracted (Fig. 4). Hyalomeres can extend and retract just as cylindrical pseudopodia do and therefore should be considered analogous to the pharopodia (shroudlike pseudopodia) of certain free-living amoebae (2). In addition, in some platelets, they are seen to ruffle just like the lamellipodia, some hyaloplasmic veils, and "ruffled membranes" of many tissue cells (61).

Three common scenarios for spreading of the hyalomere were recognized from repeated projections of ciné films: (a) The hyalomere of a platelet can spread radially either symmetrically or asymmetrically without any preceding pseudopodial activity. The entire spreading process may require as little as 10-12 min from the time of contact with glass. (b) Several pseudopodia may extend from 2 to 10 μm along the substratum before the hyalomere begins to spread radially as a "web" con-

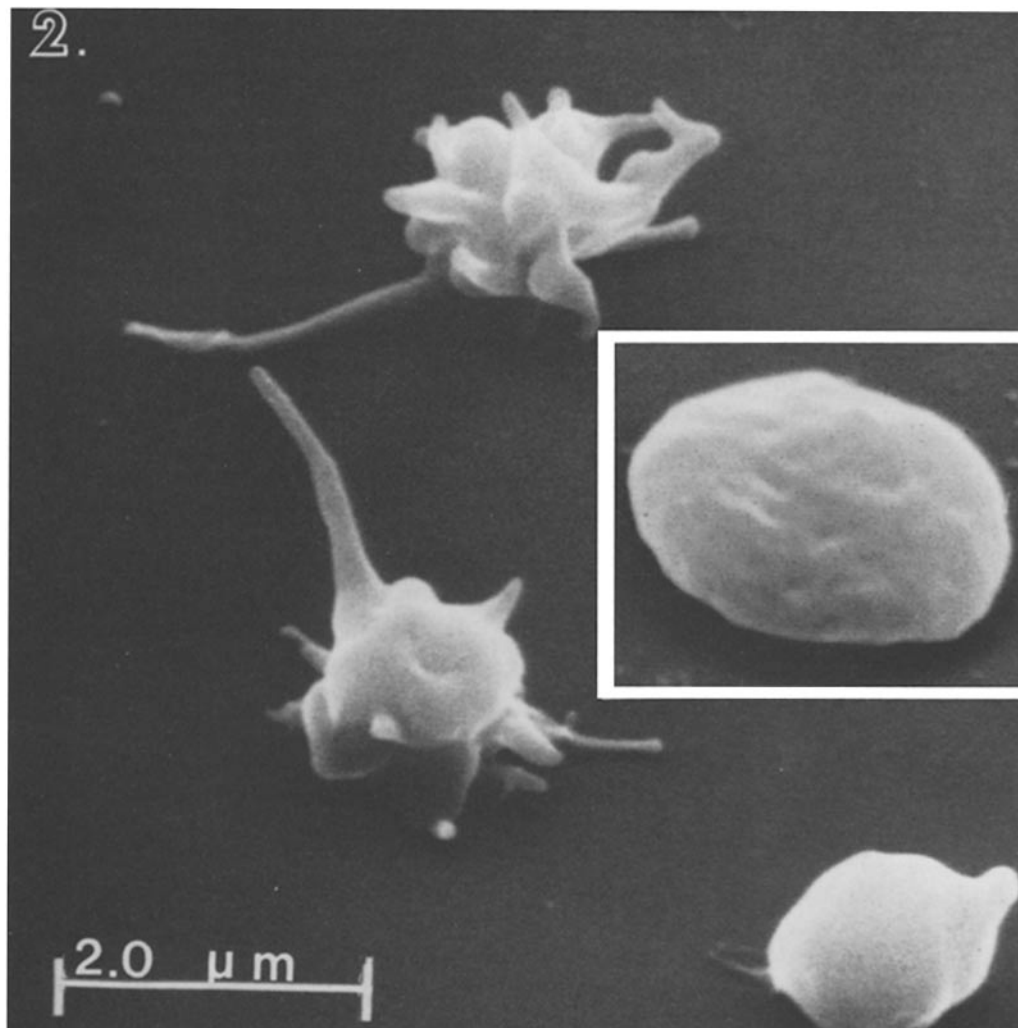


FIGURE 2 Discoid forms (*inset* right, center) frequently show small surface depressions. Rounded form at the lower right has a protuberance (perhaps a pseudopod) forming. The other two platelets are in early stages in forming pseudopodia both in contact with the substratum and directed away from the substratum.

necting them (Figs. 4–7). (c) One or more thick pseudopodia may extend and then stop; the hyalomere then begins to spread laterally from one or more of these. Sometimes more than one of these scenarios take place in different regions of the same platelet.

Because the pseudopodia vary from <50 to >200 nm in diameter (as measured from both scanning and high voltage electron micrographs), light micrographs do not faithfully register local differences in their thickness, but do accurately show changes in their length and orientation. Al-

though the fully spread hyalomere is only 50–100 nm thick (Fig. 8), DIC microscopy is sufficiently sensitive to record both the occasional presence of internal particles and evidence of parallel filamentous substructure. The particular manner in which the hyalomere has extended can frequently be recognized in retrospect by either radial or lateral “striations” in the hyalomere (Fig. 4g and h). These textural features of the hyalomere that are sometimes visible in living platelets can be studied in fixed material in greater detail with the SEM (Fig. 9).

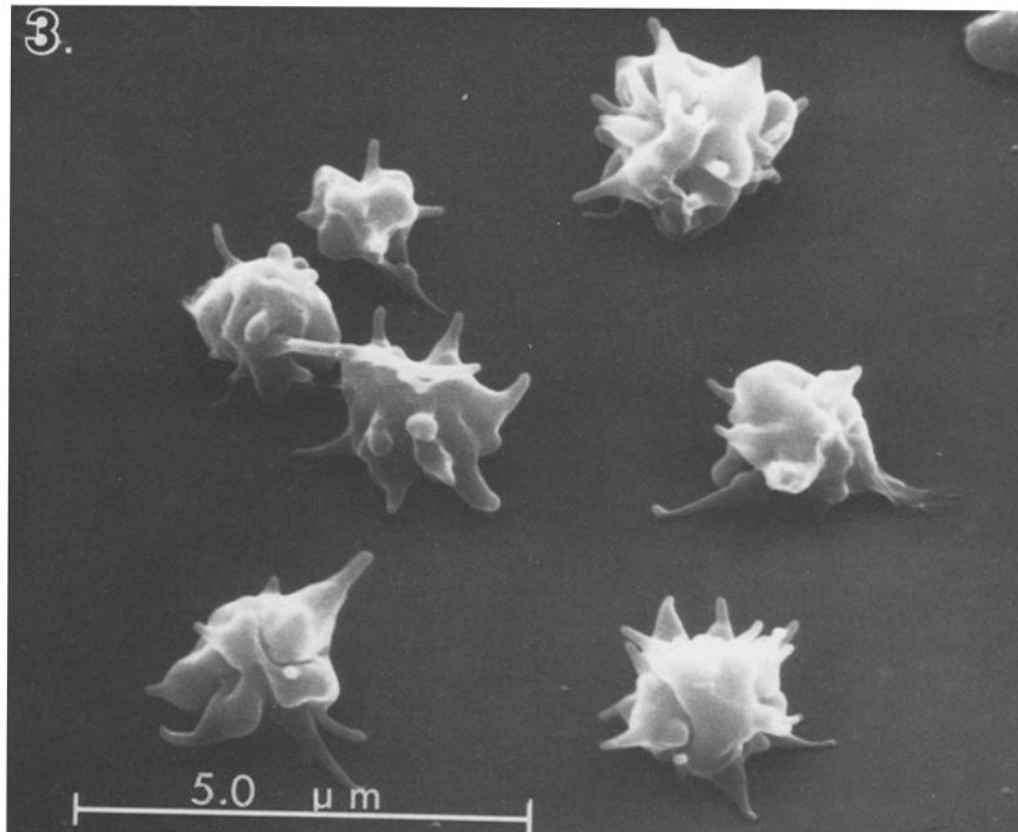


FIGURE 3 Early polypodial stages that have just attached to the siliconized glass surface.

Velocities of Pseudopod Extension and Retraction and of the Spreading of the Hyalomere

Fig. 10 illustrates the range of platelet pseudopod extension and retraction velocities exhibited by 10 pseudopodia from several platelets of one representative normal subject. Two pairs of curves, A_1 and A_2 and B_1 and B_2 , were data from pairs of pseudopodia on two platelets. It can be seen that the variability in pseudopod extension velocity covers the range between 0.75 and 7.5 $\mu\text{m}/\text{min}$. The maximum retraction velocity observed was $-1.9 \mu\text{m}/\text{min}$.

Fig. 11 shows the simultaneous extension and retraction behavior of four pseudopodia from the same platelet. It is obvious that extension and retraction of pseudopodia are local and uncoordinated events. The velocity range in this one platelet was -4.0 to $+2.0 \mu\text{m}/\text{min}$, and pseudopodia could undergo either smooth or abrupt

changes in velocity within this range.

The spreading behavior of platelet hyalomeres shown in Figs. 11 and 12 is a much slower process than pseudopod extension. The time required for a platelet to spread completely once it contacts the glass surface is sometimes as little as 10–12 min and seldom >30 min. Platelets exhibiting the most rapid hyalomere extension show a high initial velocity ($\sim 0.5 \mu\text{m}/\text{min}$) which diminishes steadily as spreading progresses. Platelets that spread more slowly do so at a more uniform velocity corresponding to about half the maximum velocity of rapidly extending hyalomeres. Fully spread hyalomeres sometimes continue to exhibit local regression or ruffling activity reminiscent of that in lamellipodia.

Shape and Granular Composition of the Granulomere during Spreading

During the pseudopodial and early spreading

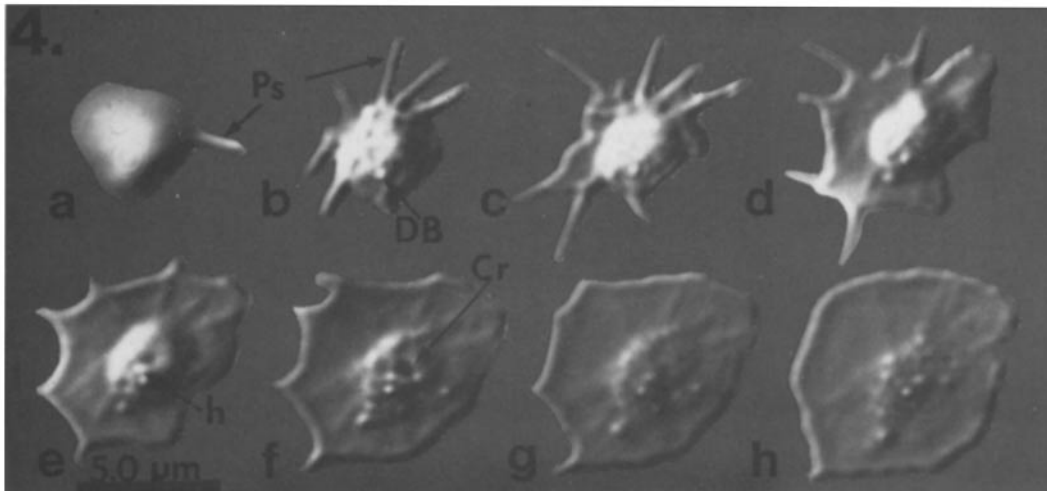


FIGURE 4 The same platelet at various stages in its transformation from a spheroidal form just after making contact with the substratum (*a*) to its fully spread form (*h*). The times at which photomicrographs were taken are 0, 1, 2, 3, 4, 7, 8, and 13 min for stages *a-h*. Structures visible include pseudopodia (*Ps*), dense bodies (*DB*), and craters (*Cr*). Note also some radial striations in the hyalomere of Fig. 4*h*. Photographs were cut out and mounted on the same density background to save space.

stages of platelet transformation, the dome-shaped granulomere hillock contains many granules of different size and dry mass concentration. As the hyaline cytoplasm flows from the hillock into the spreading hyalomere, the hillock gradually flattens. Not all platelets flatten, but some do so completely.

At bias compensation settings of less than ~ 12 nm, the bright and dark shadows cast over the slopes of the hillock obscure the contrast in DIC images generated by the particles within. However, at settings of over 15 nm, the shadows over the hillock are reduced sufficiently in contrast that most or all granules can be seen (and counted if desired) by optical sectioning. From 5 to 10 "large" dense granules ($\geq 0.3 \mu\text{m}$ in diameter) and a comparable number of smaller less dense ones ($0.2 \mu\text{m}$ or less in diameter) can be seen. Direct comparison of platelets stained with the fluorochrome mepacrine (5×10^{-5} M for 30 min) in photomicrographs of the same microscope field in DIC and epi-fluorescence showed that nearly all of the larger granules stained with mepacrine (Fig. 13). Therefore, these will be referred to as dense bodies (39-41).

Observation of the Release Reaction and its Timing in Relation to Spreading

The dense bodies within the granulomere ap-

pear as phase-retarding bodies showing a positive phase gradient seen as a shadow cast in the same direction over that feature as the shadow at the platelet surface (Fig. 4). When each dense body is discharged, it is replaced in its former position by a negative phase gradient that resembles a "crater" because it is shadow-cast in the direction opposite from the platelet surface, as shown in Figs. 4, 5, and 13. In time-lapse films, such craters are seen to appear on the upper surface of the platelet, immediately after each degranulation event. The exocytosed particle can usually be followed as it rises out of the plane of the optical section and then settles again on, or in the vicinity of, the platelet. Most craters gradually disappear. It is apparent from careful optical sectioning that some of the negative phase gradients probably correspond to internal passages of the open canalicular system extending deep into the granulomere. Several dense bodies can frequently be seen either to be expelled intact or to discharge their contents through the same crater opening into the medium. In accelerated time-lapse images, platelets resemble miniature volcanoes spewing forth particles. Arrows in Fig. 12 are time markers indicating single degranulation events which occurred at various times in relation to the spreading of the hyalomeres of platelets. It is clear that degranulation can occur throughout the transformation process.

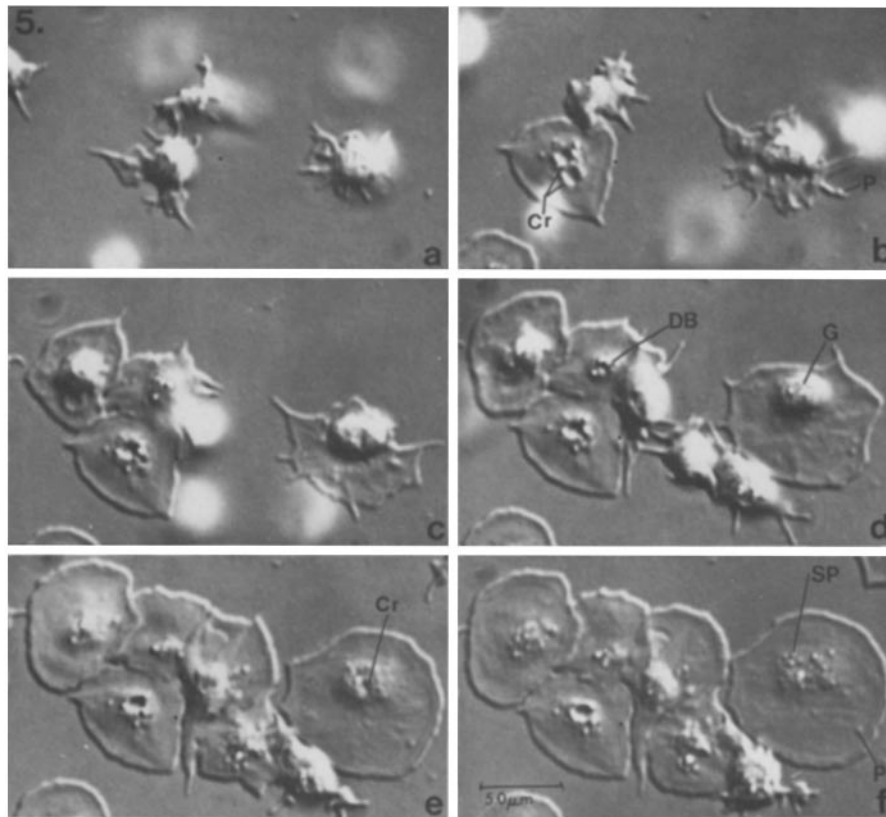


FIGURE 5 A series of photomicrographs of several platelets settling on a siliconized cover glass and beginning to extend pseudopodia. Times were 3, 13, 18, 20, 26, and 31 min after the preparation was made. In frame *a* the platelet on the left exhibits spreading of hyalomeres laterally from two extended pseudopodia, while the right-hand platelet exhibits radial pseudopod formation and radial spreading of the hyalomere, as shown on later frames. The platelet on the left in frame *b* has developed two deep craters (*Cr*, seen in the same focal plane as the hyalomere) and from which two intact dense bodies have been released. The two craters fuse in frame *c*, and one disappears (frame *e*). The right-hand platelet in frame *a* discharges one dense particle (*P*) which lands on a pseudopod and can be seen in frame *f* 18 min later. Particles within the hillock of the granulome (*G*) become progressively more visible as degranulation and flattening occur. Note the tendency of the hyalomeres of crowded platelets to overlap. *DB*, dense bodies.

The large particles that are seen to be discharged intact have corresponded, in every case examined, to particles that accumulate mepacrine. This has been established by DIC and fluorescence photomicrographs of the same platelets.

It is interesting that exocytosed dense bodies are visible not only in sequential light micrographs either on or in the vicinity of platelets, but also in scanning electron micrographs (Figs. 6-9), where the specimens had been rinsed before fixation and subjected to a number of fluid transfers.

When platelets of most individuals are stained with mepacrine and examined with the fluores-

cence microscope, exocytosis events are seen as brief (<1 s) flashes which appear to be localized very close to the position of the dense body being released. Examination of the same platelet immediately afterward by DIC microscopy always shows a crater where the dense body had been (Fig. 13). Discharged dense bodies are occasionally but rarely fluorescent.

The platelets of some individuals become highly sensitive to short wavelength (blue-violet irradiation from an HBO-200 lamp) after staining with mepacrine. When these platelets are observed by fluorescence microscopy at any time during trans-

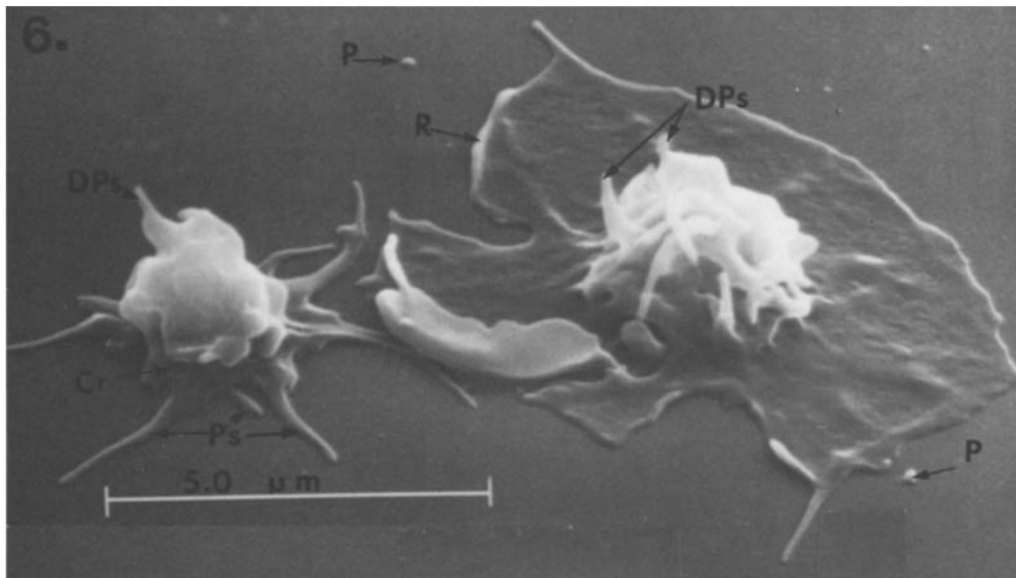


FIGURE 6 *Left:* Early spreading stage with attached pseudopodia (*Ps*), dorsal unattached pseudopodia (*DPs*), and several surface pits or craters (*Cr*) of the open canalicular system. *Right:* Platelet with hyalomere partly extended before the retraction of dorsal pseudopodia (*DPs*). Exocytosed particles (*P*) are visible in the vicinity of these platelets. *R*, ruffle.

formation they discharge one or more dense bodies within ~10–20 s of illumination by the blue-violet excitation irradiation. However, this photodynamically induced release reaction is different from the brief flashes described above. In this case the fluorescent flash spreads from the dense body throughout the entire cytoplasm of the platelet and, although diminishing in intensity, is visible for ~10 s above the background brightness. The photodynamically induced release reaction is therefore apparently intracytoplasmic release in contrast to the normal release which is extracellular.

Smaller unidentified particles “disappear” from ciné images, but it has not yet been possible to see whether they are exocytosed intact or whether only their contents are expelled. Many scanning electron micrographs (see especially Fig. 4) show small pits or cavities (100–150 nm in diameter) that may be too small to be detected by DIC microscopy. Because these openings are much smaller than the dense bodies or the craters left by their exocytosis, it is tempting to speculate that they might be the openings through which the smaller granules or their contents are either exocytosed or secreted.

Interaction of Platelets

Two platelets that contact the substratum in close proximity usually both spread, even though the hyalomere of one may overlap that of a neighbor (Fig. 7). This shows that there is no absolute contact inhibition as far as overlapping is concerned. However, a platelet that falls on top of another that has already spread is blocked in its access to the glass surface and, as an apparent consequence, rarely spreads at all. It may, however, form free pseudopodia and partially degranulate despite its failure to spread. In aggregates of platelets, it is only the bottom layer that exhibits fully spread hyalomeres. Layers of platelets above are only partially degranulated and tend to have interlocking pseudopodia holding the aggregate together. The activity of these pseudopodia can be followed by DIC microscopy.

DISCUSSION

Light Microscope Observation of Platelet Transformation

Tocantins (60) and Robb-Smith (51) pointed out that Donné (24), Addison (1), and Gulliver

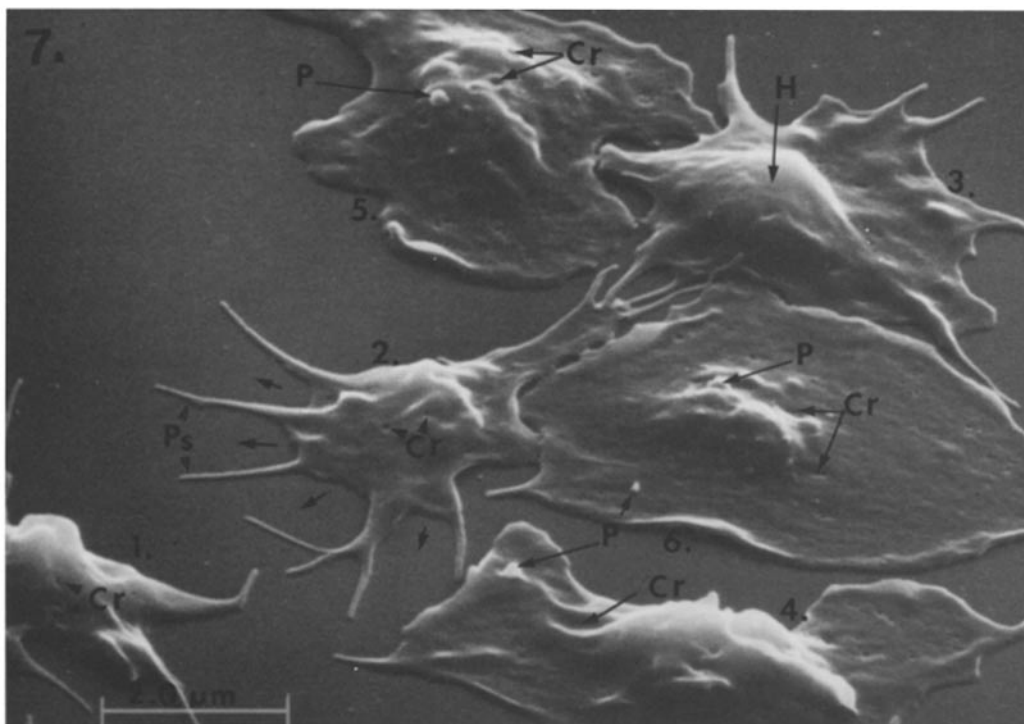


FIGURE 7 Spreading platelets numbered in transformation stage sequence 1-6 on the basis of cinematographic records of similar preparations. 1, Early pseudopodial stage. Note small craters (*Cr*) on the surface. 2, Late pseudopodial stage with the hyalomere beginning to spread between pseudopodia (*Ps*) (arrows). Note craters (*Cr*) in the surface. Note that there is no pronounced granulomere hillock, but surface irregularities and cavities suggest recent active exocytosis of particles. 3, Later spreading stage with granulomere hillock (*H*). 4, A still later spreading stage showing a particle (*P*) that may have been exocytosed from a nearby surface crater (*Cr*) of the open canalicular system. 5, A still later spreading stage showing one particle (*P*), perhaps an exocytosed dense body, and two nearby craters (*Cr*). 6, An almost completely flattened and spread platelet.

(31) deserve credit for the first light microscope observations of platelets with early achromatic microscopes in the 1840's. However, it was not until late in the 19th Century, when better instruments were available, that Osler (47) and Schimmelbusch (55) dimly perceived and depicted changes in platelet shape and the existence of spiky projections during early clot formation. DF microscopy enabled Stüble (59) to follow some of the details of the role of platelets in clotting. Similar DF studies were performed by Ferguson (26), Best et al. (13), Fonio and Schwendener (27), and Markosian and Kozlov (42). While highly sensitive to sharp phase gradients of isolated objects,⁴ DF microscopy becomes less useful for ob-

⁴ The value of DF microscopy had been proven by the demonstration that sperm tails contained 11 "fibrils" which splayed out on squashing (6).

serving internal cellular detail (e.g., particles or filaments) because of background light-scattering.

PC microscopy has also been used to good advantage to observe the continuity of platelet pseudopod formation and spreading (11, 12, 16, 44). However, for objects the size and shape of platelets, PC introduces several kinds of potentially misleading optical artifacts. First, it is difficult to discriminate between discoid and spheroid platelets, unless they rotate as they settle, because of the well known halo artifact. The halo also obscures short pseudopodia or other protuberances, so that the false impression may be created that nearly all platelets are disks (7, 34). Second, only the longer and thicker pseudopodia are seen protruding beyond the halo, and these are misrepresented as having pointed ends (hence their description in the past as "microspikes" or "tentacles") with incorrect apparent diameters. Third,

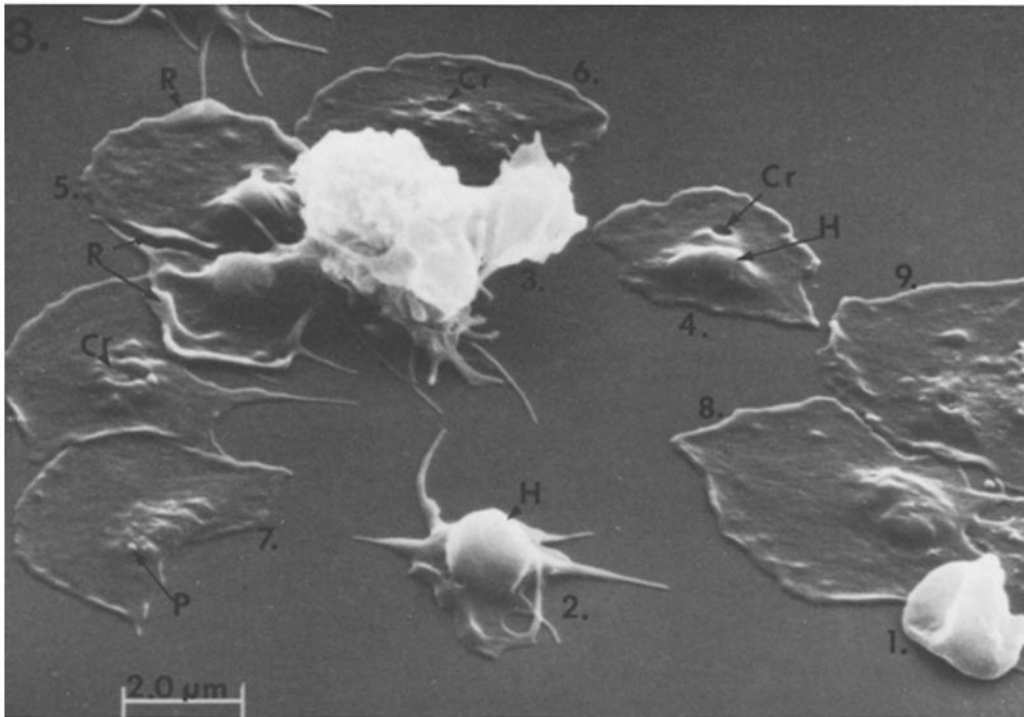


FIGURE 8 Platelets numbered 1–8 in order of spreading sequence. 1, An unattached, activated form. 2, A late pseudopodial stage beginning to spread with its hillock (*H*) forming. 3, A poorly attached aggregate that has accumulated an electric charge in the electron beam. 4, A hillock stage showing hillock (*H*) and a crater (*Cr*). 5, Another hillock stage with a ruffle (*R*). 6 and 7, Flattened platelets with exocytosed particles (*P*) and craters (*Cr*). 8 and 9, Fully flattened stages.

the particles in the granulomere are almost impossible to detect within its hillock until the platelet has almost completely flattened. By then, considerable degranulation will have occurred. The surface craters and other manifestations of the open canalicular system, if seen at all, appear as empty vesicles or vacuoles by phase contrast, and have been misinterpreted in different articles as either contractile or pinocytosis vesicles (11, 12).

Since the introduction of DIC microscopy (4) in the early 1960's, only one publication (8) has reported the use of this method for observing platelets. The reason for this is that early instruments of this type possessed insufficient contrast to demonstrate a decisive advantage over PC microscopy. Although DIC photomicrographs convey phase gradient and therefore height information, they do not offer stereoscopic or "three-dimensional" views as some have believed (8). However, in recent years, components of improved extinction factor made possible results that were clearly superior to those obtained with phase con-

trast (35) which suggest that platelets were worthy of re-examination.

Form of Platelets in Circulating Blood and in PRP

Some of the earliest observers of blood platelets reported them as discoid or lenticular in shape. However, it has been shown by both transmission and scanning electron microscopy that a certain fraction of platelets possess short, marginal projections. For example, Barnhart and Riddle (7) reported that from 20 to 40% of carefully collected canine and human platelets have such projections. Similar results were reported by Hattori et al. (34) who found 78% discoid, 18% spheroidal, and the remaining 4% irregular forms in the single human subject studied. Of the discoid forms, ~15% had marginal protuberances. Our results generally confirm those of the above authors. In contrast, White (66) has claimed to have routinely prepared platelets, 90% of which are discoid. He did not state,

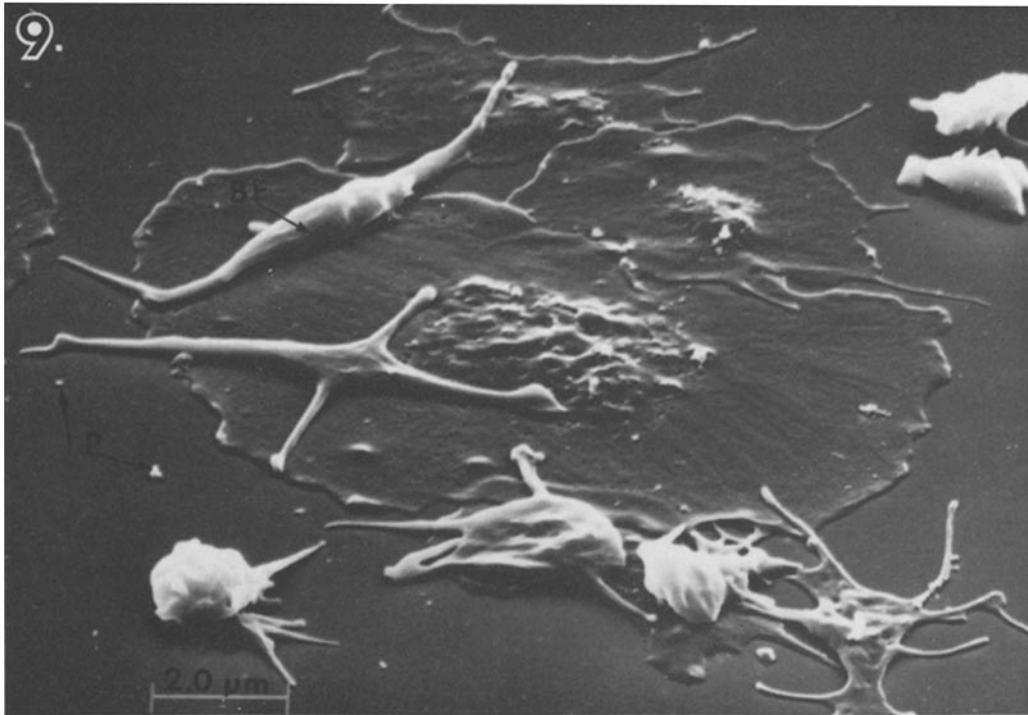


FIGURE 9 Fully flattened platelets with parallel and semi-radial striations reflecting underlying cytoplasmic filaments. Note exocytosed particles (*P*) in the surround as well as on top of platelets. Note many examples of pseudopodial overlap and the activated but unspread bipolar forms (*BF*) and other early stages lying on the flattened platelets.

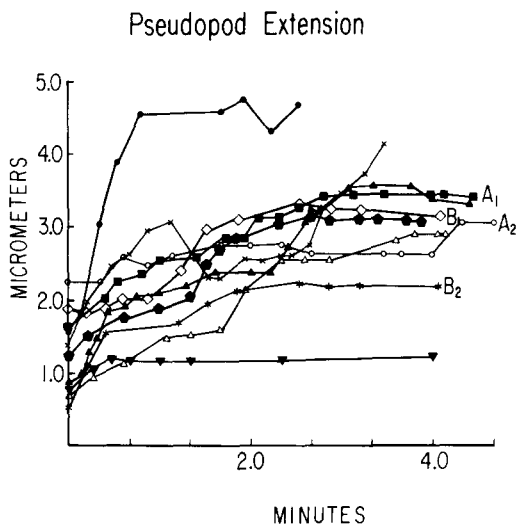


FIGURE 10 Time-course of pseudopod extension and retraction in 10 different platelets from the same subject. *A*₁ and *A*₂ and *B*₁ and *B*₂ were two pseudopodia from two platelets (*A* and *B*).

however, what percentage of these forms had protuberances and whether the method by which they were examined was PC (which we have found to be unreliable) or scanning electron microscopy. In fact, at least five out of six platelets labeled "discoid" do indeed have protuberances by our criteria (e.g., Figs. 5, 7, and 29 in reference 67).

It is significant that DIC microscopy of either living or fixed platelets can be used for rapid and accurate counts of percentages of platelets of various morphological types in blood drawn directly into fixative. Because a much greater percentage of spheroidal platelets with protuberances is seen in unfixed in contrast to fixed platelet preparations, it seems clear that partial activation occurs in PRP as suggested by White (66). Conceivably, this activation might be caused by release of small amounts of ADP from erythrocytes during preparation of the PRP (14). However, it is now equally clear that the subsequent stages of transformation are indistinguishable regardless of whether the platelet initially observed is discoid or spheroid.

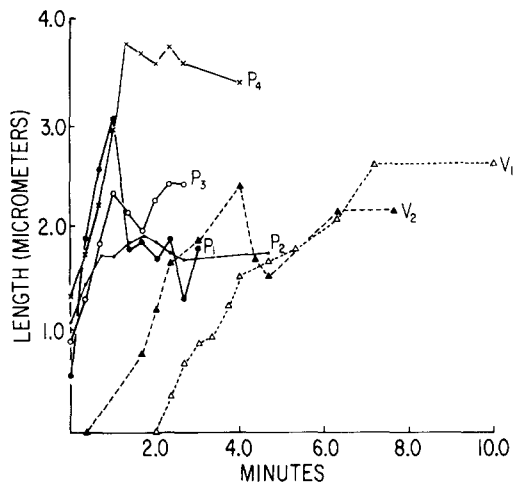


FIGURE 11 Time-course of pseudopod extension and retraction (velocities are given by positive and negative slopes) measured from four pseudopodia (P_1 and P_4) of the same platelet. Also shown are the extension and regression of two segments of the hyalomere that eventually spread between the pairs of pseudopodia. (Hyalomere segment V_1 spread between pseudopodia P_1 and P_2 , and V_2 spread between P_3 and P_4 .)

The possibility exists that the shape of platelets in the circulation might be an indicator of certain pathologic states since it has been reported that individuals who have suffered a stroke or myocardial infarction have a higher proportion of "activated" platelets (50, 54). If this turns out to be generally true, DIC microscopy might allow rapid microscope examination of platelets in these conditions.

It should be mentioned that direct microscope observation of the sizes and shapes of suspended platelets is relevant to the validity of the measurement of size and shape parameters by x-ray scattering (49), because the latter method requires the assumption (shown here to be invalid) that platelets in PRP exist exclusively in the form of disks.

Sessile Protuberances vs. Pseudopodia

Much current evidence from low temperature experiments points to the probable role of the marginal microtubular band in maintaining the discoid shape of the platelet (70). The transition from discoid to spheroid shape is also suspected to be a result of the rupture of the marginal microtubular band (66). It is of interest, therefore, that some short protuberances on suspended platelets have been observed to contain the fractured mar-

ginal band of microtubules (67, 69, 70). It is tempting to suggest that some of these sessile protuberances may be the result of a straightening of components of the microtubular band after it has been partially broken down.

By contrast, there is reason to believe that those protuberances that extend and retract from the surfaces of platelets are true pseudopodia. Their rates of movement, sequence of shapes, etc. are like those of slime mold amoebae (25), many soil amoebae, or some tissue cells (61, 62). Ultrastructural studies have established that many of the protuberances of platelets contain oriented bundles of microfilaments (57, 64, 72), and the contractile protein F-actin. The radial or lateral striations in the hyalomere observed by DIC microscopy are probably a manifestation of parallel bundles of microfilaments in pseudopodia and the spreading hyalomere.

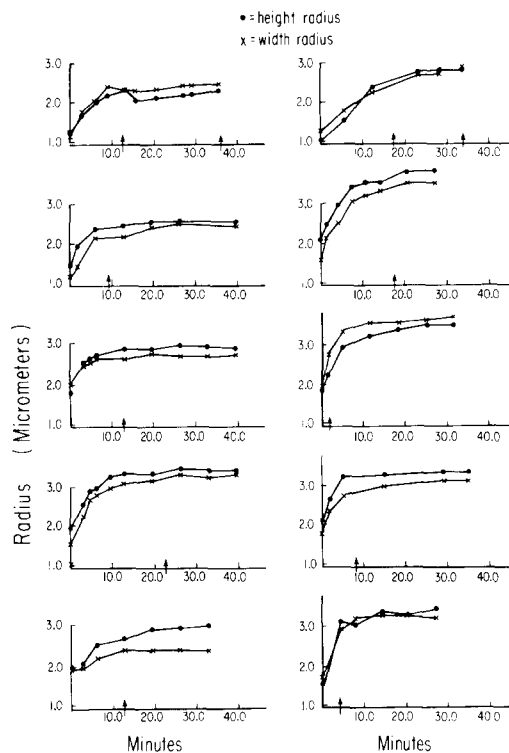


FIGURE 12 The time-course of spreading of the hyalomeres of 10 platelets measured in orthogonal directions (\times and \bullet) to show the degree of directional independence of spreading. Arrows along the abscissa indicate times at which some (but not all) degranulation events were detected.

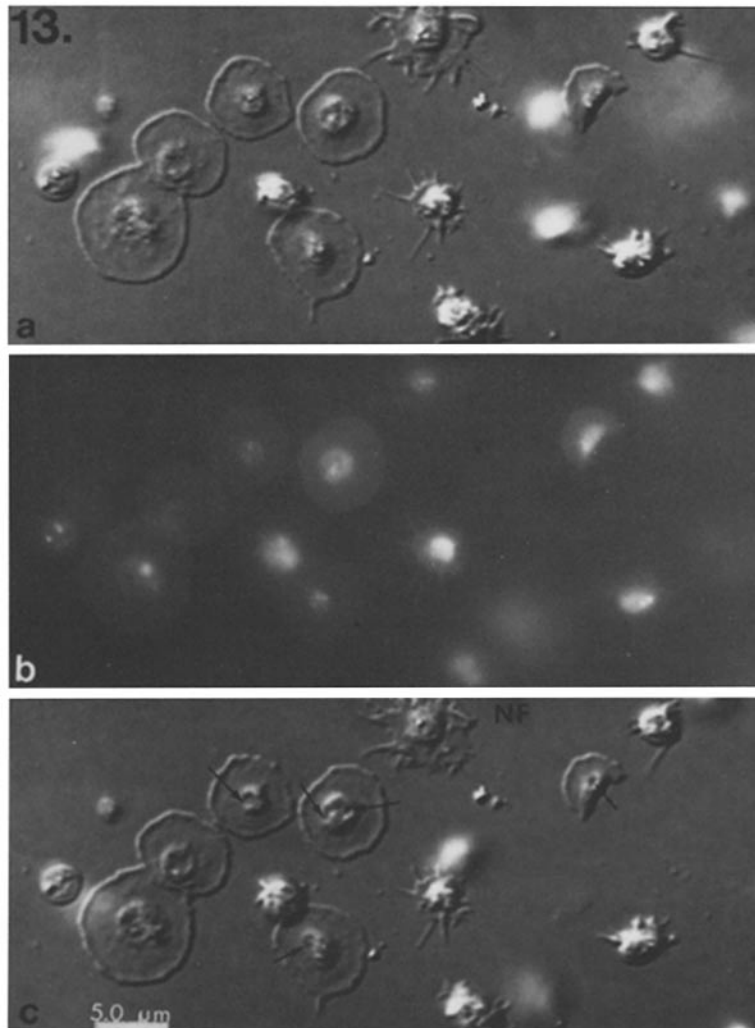


FIGURE 13 The induction of intracytoplasmic release of dense bodies by exposure to strong blue-violet light. (a) A DIC micrograph (<1-s exposure) of a field of spreading platelets using narrow band mercury green illumination. (b) A 20-s exposure by fluorescence microscopy, during which all but one of the platelets exhibited a prolonged photodynamically induced (~10 s) flash of variable brightness. (c) A DIC micrograph taken immediately after the fluorescence micrograph to show the presence of new craters (arrows) in all platelets except the one that exhibited no flash (NF).

Observations of the extension and retraction of pseudopodia and of hyalomeres of platelets suggest that both processes could be manifestations of similar mechanisms. The leading edge of the hyalomere is usually its thickest part, and it sometimes shows ruffling activity. In small, free-living amoebae as well as tissue cells, pseudopodia can take the form of thick filopodia, broad cylindrical lobopodia, or a flat pharopodium or lamellipodium at different times, or under different physi-

ological conditions (2, 61, 62). The interpretation of hyalomere spreading in platelets as a type of pseudopod formation is strengthened by the finding that it is inhibited by cytochalasin B (15, 66, 71), suggesting the causal involvement of actin or an actin-binding protein in the process (3). On the other hand, these phenomena are not identical because the extension velocity of platelet pseudopodia is characteristically 1.5–150 times the velocity of hyalomere spreading.

Platelet Spreading and Exocytosis

As platelets begin to spread, their rounded central region or hillock very slowly flattens. The various particles in the granulomere remain clustered in the center of the cell as the cytoplasm streams outward to form the hyalomere. Dense bodies, recognized by their accumulation of the fluorochrome mepacrine (39–41), are discharged either singly or in clusters, giving rise to a crater, seen as a negative phase gradient. This process may begin before the hyalomere appears, but is more frequent during spreading. The number, width, and depth of craters is highly variable, as is their duration. Craters may be observed to extend deep into the hillock of the granulomere and therefore probably communicate with, or constitute the surface component of the open canalicular system in transforming platelets.

Because the dense bodies that stain with mepacrine can be recognized also by DIC microscopy, both DIC and fluorescence microscopy offer alternative and complementary ways of observing their release. By combining these methods, the release of dense bodies has been established as a concomitant of crater formation. Normal extracellular release (exocytosis) of the contents of dense bodies causes a brief (~1 s) flash in the immediate vicinity of the former location of a dense body. Photodynamically induced intracytoplasmic release is also associated with formation of a crater, but the fluorochrome diffuses throughout the cytoplasm, where it flashes more slowly (~10 s). Photodynamically induced intracytoplasmic release occurs only in platelets of some individuals.

The exocytosis of dense bodies occurs throughout the transformation process. However, by 30 min when nearly all platelets from normal subjects have both settled and spread, most will have discharged as many of the particles as they will ever discharge.

In a recent study of hydrated platelets in a special wet chamber used for high voltage electron microscopy, the disappearance of dense granules between one sequential electron micrograph and the next has been interpreted as direct observation of the release action (21). Our techniques appear to offer a decided advantage over this approach.

Besides surface craters, small cavities or pits are seen on the surfaces of spreading platelets by scanning electron microscopy. These might represent sites where the α granules or other small granules are either exocytosed or their contents

secreted. Although many particles smaller than dense bodies, presumably α particles, do “disappear” from DIC ciné images, it has not yet been possible to study their release by optical microscopy.

Much is known from careful ultrastructural and biochemical studies about the release reaction in platelets stimulated by thrombin, or inhibited by cytochalasin B or low temperature (67, 68). It is becoming clear that not all that is known about chemically activated platelets applies to platelets undergoing transformation after attachment to siliconized glass. During transformation, the visible manifestations of the release reaction take the form of single exocytosis events that may extend throughout the entire period of transformation.

Platelet-Substratum vs. Platelet-Platelet Interactions

Platelets that sediment onto the upper surfaces of already spread platelets show activation by forming long pseudopodia that extend and retract. However, their hyalomeres do not spread and pseudopodial contacts are temporary until one pseudopodium touches the siliconized glass whereupon it then spreads. In contrast to the condition of cells cultured in monolayers (23), there is no contact inhibition of the release reaction or of overlapping. Platelets that contact siliconized glass in adjacent locations usually spread completely, degranulate at least partially, and often overlap one another's hyalomeres. The factors that control cell interactions with solid surfaces and then with other cells are complex and incompletely understood (43, 63), and the reasons for contact inhibition or the lack of it are speculative. The pseudopodia that form in suspended activated platelets interact to form aggregates in a manner analogous to the sorting of embryonic cells.

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