MORPHOLOGICAL OBSERVATIONS ON BLOOD PLATELETS

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INTRODUCTION

This thesis deals with morphological observations on blood platelets previously recorded in a series of published papers. The experiments detailed were begun under the supervision of Dr. W.E. Stehbens while I was a Research Fellow in the Department of Experimental Pathology, John Curtin School of Medical Research at the Australian National University, and have continued in my laboratory, Department of Pathology, Banting Institute at the University of Toronto. They may be divided into those dealing with observations made on platelets in vivo using rabbit ear chamber preparations, those concerned with defining the ultrastructure of rabbit platelets, and those relating alterations in platelet ultrastructural morphology induced by different aldehyde fixatives used in electron microscopy. They will be presented in these groupings.

The first section of the thesis reviews the accumulated knowledge on platelet morphology. In it I include observations made to the end of 1970 and so incorporate experimental results recorded by others after my papers were published. The next section presents the experiments. It is followed by a bibliography and then a series of appendices containing reprints of the published papers. No illustrations appear in the text. They are reproduced in the appendices,

and the reader is referred to them.

The thesis deals mainly with platelet morphology. Therefore, platelet biochemistry, pharmacology and function are not discussed in depth. I apologise to the reader for this deficiency and refer him to monographs or reviews either written or edited by Marcus and Zucker (1965); Kowalski and Niewiarowski (1967); Marcus (1969); Berger(1970) or Mustard and Packham (1970) to bridge the gap.

HISTORICAL REVIEW

Platelets were not observed in blood until the 19th century. Robb-Smith (1967) has suggested that their late discovery, relative to that of erythrocytes and leukocytes, had to await a solution of the problem of chromatic aberration in compound microscopes, then under development. The problem was solved in 1811 by the Italian optician Marzoli and soon afterwards, in the 1840's, drawings of platelets appeared in reports dealing with observations on the blood published independently by Gulliver and Addison (Robb-Smith, 1967). At about the same time Donné (1842) described a third element in the blood in this manner, "Les globulins sont de petits grains n'ayant pas plus de 1/300 de millimètre de diamètre" and noted that "Ces globulins se réunissert, trois à trois ou quartre à quartre". Although Donné concluded that the "globulins" were fat droplets their size (3.3 microns) is comparable to that of platelets. Indeed, some authors have concluded, in retrospect, that they were platelets (Muir, 1890-1891); Tocantins, 1948; David-Ferreira, 1964). However, Robb-Smith (1967) doubts this opinion because Donné's preparations were of watered blood.

Many articles and treatises appeared in the next 30 years contributing to a lively controversy as to the existence, origin and fate of the new elements in the blood - see Robb-Smith' s review (1967). The arguments were polarized by observations made by Schultze (1865), Osler (1874), Hayem (1878) and others that established a relationship between the platelets and thrombi, and culminated in those of Bizzozero (1882). He described platelets in mesenteric vessels, demonstrated their adhesive quality, their participation in thrombus formation and their role in blood coagulation. He called the new elements "blut plättchen", a term translated into English initially as blood plates or plaques, and later modified to the current term. Thus, as the 19th century drew to an end, platelets were established as a third corpuscular element in the blood.

To that time, observations on platelets had been made directly or by using a type of "phase contrast" illumination. The use of a supravital stain (brilliant cresyl blue) in wet preparations enabled Puchberger (1903) to describe the hyaline (hyalomere) and chromatic (chromatomere) portions of the platelet cytoplasm. Wright (1906a,b; 1910) added a new dimension with his blood stain. He recognized platelets in tissue sections and also described their origin from mega-

karyocytes. Although fat globules (Demel, 1911) and mitochondria (Cowdrey, 1914) were recognized amongst the cytoplasmic granules, the size of the granules lay at the limit of resolution by light microscopy. Thus, while investigations of platelet chemistry and physiology expanded our understanding of platelet function in the early part of this century, knowledge of platelet morphology remained stationary.

Just as the discovery of platelets had to await one technological advance, a better appreciation of their morphology had to await another, the electron microscope. Indeed, the evolution of modern knowledge of platelet fine structure has had three phases, each dependent upon a technological advance.

The first began in 1939, when Wolpers and Ruska (1939a,b) gave their description of platelet morphology, demonstrated by the use of an electron microscope. Their reports, amongst the earliest on biological material examined in such a machine, dealt with human blood platelets that had been collected in one of several anticoagulants, fixed in one of a variety of fluids and then spread on membranes. For more than 15 years, succeeding investigators (Hutter, 1957; Haydon, 1957; Koppel, 1958) and others - see lists prepared by Marcovici and Gautier (1959), Marcovici et al.(1961), and Falcão et al. (1966) - continued to examine platelets in the electron microscope, either directly after their isolation

and fixation, or after spreading them on membranes, with or without heavy metal shadowing, or by the replication technique. Braunsteiner (1961), David-Ferreira (1964) and Marcus and Zucker (1965) have detailed the morphological findings in these experiments. During this period abnormalities of pseudopod formation or platelet spreading in patients with various thrombopathies were also reported - see the review by Hovig (1968) for details.

The results of these early studies are now mainly of historical interest, for the techniques used permitted only a limited examination of platelet morphology. Furthermore, it is now appreciated that platelet structure is markedly altered by contact with the types of membranes used. A better understanding of platelet ultrastructure had to await the mid-1950's and the development of a method to cut very thin sections (Bernhard and Le Plus, 1955). With the advent of this technique, the second phase of discovery opened. During the next ten years, many of the intracytoplasmic structures were defined in platelets fixed in osmium tetroxide solutions and embedded in methacrylate. The reviews written by David-Ferreira (1964) and Rodman, (1967), and the monograph of Schulz (1968) aptly present the knowledge gained.

The third and current phase began in the early 1960's with the introduction of double fixation by glutaraldehyde, followed by

osmium tetroxide (Sabatini et al. 1964) and the use of improved plastic embedding agents (Glauert, 1961; Luft, 1961). Prefixation with the aldehyde revealed cytoplasmic structures not obvious in the platelets when osmium tetroxide was the sole fixative while the new embedding agents permitted better photographic records of the newly revealed morphology.

Platelet Ultrastructure

At this time (1970) ultrastructural studies have established that the mammalian platelet is defined by a membrane, surrounded by an external platelet coat, and has in its ground substance various organelles. I will discuss each of these structures in turn and, unless stated otherwise, the description given will apply to human platelets doubly fixed in glutaraldehyde and osmium tetroxide. It must be remembered that platelet morphology varies slightly from one mammalian species to another. Furthermore, that the ultrastructural morphology of platelets from a particular species may be altered during preparation for electron microscopy. For example, by the anti-coagulants used (White, 1968a), by the method of preparation (Behnke, 1965; Skjørten, 1968) and by the fixatives employed (Gardner et al., 1969; Silver and Gardner, 1970).

Platelet Shape and Size

Platelets in the circulation are thin and disc-like (Silver and Stehbens, 1965) and have a volume of about 5.8 $m\mu^3$ (Marcus, 1969).

When viewed by the scanning electron microscope immediately after fixation, as described by Larrimer and colleagues (1970), the majority are smooth discs with an average diameter of 3.2 mμ. The remainder are discs or spheres with small projections from their surfaces. Rarely, bizarre crescent-shaped or spindle-shaped forms are seen. If, on the other hand, platelets from platelet-rich plasma anticoagulated with acid-citrate-dextrose are viewed by this means, then an equal percentage of smooth discs and discs with small projections are seen.

Sectioned platelets are usually elliptical, rod-like or disc-shaped; a few are round. Their longitudinal diameter varies from 1.5 - 5.0 mμ and their transverse diameter from 0.5 - 2.0 mμ. Occasionally, pseudopods protrude from the platelet surface. The last mentioned change is thought due to platelet activation and is observed more frequently if platelets in anticoagulated blood have been separated from the other cellular elements by centrifugation before fixation (Skjørten, 1968). This activation undoubtedly explains the change in platelet shape noticed by Larrimer and his colleagues (1970) and

described above. Very rarely, giant platelets 5 - 9 mμ in greatest diameter are observed in section (Schulz, 1968).

Platelet-limiting Membrane

Although Bizzozero (1882) described a platelet membrane it was not defined satisfactorily until platelets were examined in the electron microscope. The membrane, 70 - 90 Å wide, has a triple-layered structure like that found limiting other cells (Robertson, 1960). It is thought to have a phospholipid-protein composition like the membrane model proposed by Danielli and Davson (1934/1935). Current evidence favours the acceptance of the Danielli/Davson concept, although the model has some deficiencies. For example, it does not provide structural principles that may be used to explain the differing function of different membranes (Stoeckenius and Engelman, 1969). The structure of the membrane is apparent in platelets doubly fixed in glutaraldehyde and osmium tetroxide, but is better defined if the platelets are fixed in potassium permanganate (David-Ferreira, 1964).

The membrane is derived from that of the megakaryocyte. It is generally agreed that future platelets are delineated in the megakaryocyte's cytoplasm, by the coalescence of an extensive cytoplasmic membrane system called the demarcation membrane system (De Marsh et al. 1955) and that these membranes form the plasma membranes of

the future platelets (De Marsh et al. 1955; Yamada, 1957; De Bruyn, 1964; Han and Baker, 1964; Falcão and Gautier, 1967). The origin of the demarcation membrane system was a matter of controversy (Yamada, 1957; Han and Baker, 1964; De Bruyn, 1964; Schulz, 1966) until Behnke (1968a) in a series of elegant experiments, provided clear evidence, in the rat, that it was derived from the plasma membrane of the megakaryocyte.

External Platelet Coat

Although Roskam (1922) suggested that platelets in plasma might be surrounded by an "atmosphère plasmatique", evidence for the morphological existence of a periplatelet structure was not forthcoming until Rodman and his co-workers (1963) drew attention to a fluffy osmiophilic material present on the outside of platelets fixed in osmium tetroxide. With the use of glutaraldehyde prefixation, the presence of an extra platelet coat has been firmly established. It is seen both in animal platelets fixed in vivo (Stehbens and Biscoe, 1968) and on human and animal platelets fixed in vitro. Both the width (100 - 200 Å) and morphology of the external platelet coat may vary with the method of fixation and preparation used. Behnke (1968b) found that it was amorphous, while Sixma and Molenaar, (1967) using osmium tetroxide fixation reported a thread-like structure. Bull (1966) who examined

negatively stained whole mounts of platelets, observed hexagonal structures approximately 250 Å wide in the area of the external platelet coat.

Rodman and his co-workers (1963) considered the material they described was an external platelet coat but thought that it represented a precipitated plasma protein. Recent work suggests that the coat originates, in part at least, in the megakaryocyte (Behnke 1968a,b; Nakao and Angrist, 1968) rather than being acquired by the fully developed platelet when it enters the general circulation. The experiments of Behnke (1968a,b), Nakao and Angrist (1968) and of Spicer and his co-workers (1969) indicate that the coat consists in part of acid mucopolysaccharides and in part of protein but its exact composition is still not certain. For example, Spicer and his colleagues (1969) suggest that it contains a sialomucin, because the localization of sialic acid on the platelet surface has been indicated by biochemical studies (Madoff et al, 1964). Behnke (1968b) on the other hand, maintains that it contains a sulphated acid-mucopolysaccharide and attributes the adhesive property of platelets to the sulphated mucopolysaccharide and proteins found in their surface coat.

In addition to the external platelet coat, Bull (1966), whose examination of negatively stained platelet whole mounts has been

mentioned above, demonstrated an accumulation of phosphotungstic acid 600 Å wide surrounding each platelet like a halo. He speculated that this morphological finding was produced by the external platelet coat merging into a colloidal cloud formed of the coat and absorbed plasma protein.

Whatever the extent and exact chemical nature of the external platelet coat, its importance cannot be overemphasized. Not only is it a site where proteins, for example, fibrinogen (Nachman, 1965; Salmon and Bounameux, 1958) and coagulation factors (Hjort et al, 1955) may be absorbed, but it is also the site of platelet involved interactions (Mustard and Packham, 1970).

Bull (1966) also drew attention to electron dense bodies that were obvious on the platelet surface in his preparations. He suggested that structures had not been observed previously because they were destroyed or altered when platelets spread themselves on membranes, during preparation for electron microscopy. The bodies Bull described were usually round or oval and were present in 20 - 80% of the platelets examined, whether negatively stained or unstained. Many possessed long whip-like extensions and some showed peculiar loops or whorls. Parts of the bodies extended beyond the cell membrane, indicating, Bull thought, that they were external to it. White (1968b) reexamined

platelets prepared as Bull had done and proved that the dense bodies could be demonstrated on the platelet surface. However, he was of the opinion that the dense bodies were located within the confines of the hyaloplasm normally and that their location in the coat covering the cell membrane, as suggested by Bull, was the result of their partial extrusion during platelet spreading. The nature and function of these bodies has still to be established.

Intraplatelet Components

Granules, mitochondria, glycogen and vacuoles were observed in the earliest ultrastructure studies of sectioned platelets from a number of animal species (Bernhard and Le Plus, 1955; Rinehardt, 1955; Kautz and De Marsh, 1955; Pease, 1956; Watanabe, 1956; Sueyasu and Takeschige, 1956; Fiessly et al, 1957, 1958). The nomenclature of these elements was chaotic until Schulz and his associates (1958) divided the granulomere components into four types (alpha, beta, gamma and delta). Subsequently David-Ferreira (1964) defined a fifth component, the epsilon granulomere. With the passage of time newly recognized or redefined structures have been added to the original list so that the classification defined by Greek letters now has little meaning. At the moment the subcellular structures that may be found in the blood platelets include: 1) ground substance; 2) mitochondria;

3) electron dense granules or alpha granules, including "bulls eye" granules and "hockey stick" granules; 4) very dense granules; 5) vacuoles and vesicles; 6) microtubules; 7) microfilaments; 8) glycogen; 9) ribosomes; 10) siderosomes.

Ground substance

This material, which is homogeneous or finely granular and of moderate electron density forms the platelet cytoplasm. The organelles to be described are found scattered through it.

Mitochondria

Three to six mitochondria are found in each platelet section. They are round or oval and 0.1 - 0.3 mμ in diameter, i.e. about the same size as an alpha granule. Platelet mitochondria, like those in other cells, have outer and inner membranous walls approximately 70 Å thick separated by a space about 80 Å wide. They are smaller than mitochondria found in most other cells and show few cristae mitochondriales (2 - 3 per sectioned mitochondrion). Even though platelet mitochondria seem poorly developed morphologically they function like those in other cells, and are the site of many metabolic reactions (Marcus and Zucker, 1965; Mustard and Packham, 1970). Platelet mitochondria are derived from those in the megakaryocyte. Since they are probably not renewed in the platelet, Marcus (1969) has suggested that their senescence may be a factor in the short life span of the platelet.

Relatively little has been written about the morphological localization of enzyme activity in platelet mitochondria. Braunsteiner (1961) reported the localization of small amounts of succinic dehydrogenase but his observation has not been confirmed. White and Krivit (1965) localized ATP-ase activity in platelet mitochondria using lead ion in the incubation medium. This result has not been confirmed in human (Vethmany and Lazarus, 1967), rat (Behnke, 1967) or rabbit platelet (Wetzel et al. 1967; Silver, 1967). Like Behnke (1967a) I now believe that the reported result was due to a nonspecific lead deposition.

Electron dense or alpha granules

A variety of granules that have in common a lining membrane like that of the platelet membrane and a moderately electron dense matrix exist in the platelet cytoplasm. They form about 80% of the platelet granules (Schulz, 1968) and are found scattered uniformly throughout the ground substance. They are called electron dense granules but have a variety of synonyms including the "alpha granulomere" (Schulz et al, 1958), specific granules, azurophilic granules and "granulations denses" (Feissly et al. 1957).

The most numerous of these granules are round or oval and 0.15 - 0.4 mμ in diameter. Three to eight or more may be found in each platelet section. Their surface usually has a smooth outline but David-

Ferreira (1964) observed small surface lobes in his preparations. The matrix is homogeneous or finely granular. It is separated from the enveloping membrane by a relatively translucent zone 70 - 80 Å wide best seen when the granule is sectioned in certain directions. The translucent zone is made particularly conspicuous when sections are doubly stained with uranyl acetate and lead citrate.

White and Krivit (1967), White (1968c) and Vossen et al (1968) described a tubule-like element 200 - 300 Å in diameter, with an electron transparent core, and surrounded by a dark band 75 Å wide within the matrix of the alpha granule. Usually the structures occur singly, but groups of two or three may be found. For reasons not yet explained, their number may be increased when platelets are exposed to digitonin (White, 1968c). Furthermore, if more than one is present cross bridges can be observed passing between them. Morphologically the tubular structures resemble cytoplasmic microtubules. However, White (1968c) has cautioned against this association until more is learned about the structures. In White's view they may be a tubular form of phospholipoprotein rather than composed of protein subunits, as cytoplasmic microtubules seem to be.

A small percentage of alpha granules, perhaps one or two in each section have an oval or rounded area of increased electron density

located eccentrically in their matrix. These areas, which are 0.25 mμ in diameter, are not further defined from the rest of the matrix. They have been called nucleoids by Wetzel and co-workers (1967). Recently Spicer and his colleagues (1969), using histochemical methods, have shown that the nucleoid region is rich in acid mucopolysaccharide substances, possibly a sulphated mucopolysaccharide. The distinctive appearance that nucleoids give to some alpha granules led to their being called "bulls eye granules" by Jean and co-workers (1963) who found their number increased in certain pathological conditions.

Other elongate electron dense granules up to 1.5 mμ long and 0.8 mμ wide with a matrix like that of an alpha granule may be seen in the platelet cytoplasm but are most uncommon. They have been called "Trommelschlegranula" (drum stick granules) (Schulz et al. 1958), "granules dense giants" (Jean et al. 1963a) or "hockey-stick" granules (Rodman, 1967). Drum-stick granules were first observed in the platelets of patients with von Willebrand Jurgen's thrombopathia and were thought to be a unique finding in that condition (Schulz et al. 1958). This viewpoint is no longer accepted (Jean, 1961; David-Ferreira, 1961) because the granules may be seen in aged platelets and in other pathological conditions. Their origin and function are not known.

Initially several authors suggested that the platelet granules were derived from mitochondria (Rinehardt, 1955; Bernhard and Le Plus, 1955; Sueyasu and Takeshige, 1956; Watanabe, 1956). Others argued against this point of view (Kautz and De Marsh, 1955; Pease, 1956; Jones, 1960) and it is now appreciated that the round and oval forms of electron dense granules found in the cytoplasm of megakaryocytes, are the source of those in the platelet. Probably they originate in the Golgi apparatus of the megakaryocyte (Jones, 1960; Behnke, 1968a) but whether or not all of the forms of electron dense granules described above are derived from the megakaryocyte has still to be established.

Electron dense granules have been shown to contain lipid, lysozomal enzymes, fibrinogen and many other substances (Marcus and Zucker, 1965). However, there is still controversy as to the exact localization of some of these substances (Mustard and Packham, 1970). Nor is it certain if the granules represent a homogeneous group. Schulz (1968) and Hovig (1968) have related them by suggesting that drum stick granules are a modified form of alpha granules. The observations that granules 60 Å in diameter arranged in such a manner as to suggest a periodicity, may be seen within the matrix of hockey stick granules (Rodman, 1967) and that cross striations are apparent in

the matrix of both round and oval electron dense granules (Johnston, 1965; White and Krivit, 1967) could support this viewpoint. However, in my opinion, more substantial evidence than morphological observation alone is needed before one could accept the hypothesis offered by Schulz and Hovig.

Platelet granules have been shown by biochemical (Marcus et al. 1966) and cytochemical methods (White, 1967) to contain hydrolytic enzymes. They have, therefore, been called lysosomes (Førkin, 1963). Some platelet granules may indeed act as lysosomes for, during the uptake of latex particles by platelets, granules appear to fuse with the membrane lining the vacuoles containing the particles (Mustard and Packham, 1968). Unfortunately, fractionation studies have not clearly established the site of lysosomal activity. The experiments of Marcus and co-workers (1966) and of Day and colleagues (1969) indicate that the activity is associated with the alpha granule fraction, while the experiments of Siegal and Lüscher (1967), suggest that the microvesicle fraction best fulfills the requirements for lysosomes. Whatever the truth, no platelet granule can yet be identified with certainty as a lysosome on the basis of morphological criteria alone.

Very dense granules

Very dense granules (VDG) are found in platelets fixed in glutaraldehyde with or without subsequent osmium tetroxide fixation. They are also present in platelets embedded in water soluble material

(Durcupan). Two or three examples are seen in each platelet section. Generally, the granules are smaller than alpha granules but may be of comparable size (0.25 - 0.5 mμ in diameter). They are defined by unit membrane and have a homogeneous matrix that is extremely electron dense, much denser than any part of an alpha granule. The matrix may extend to the organelle' s limiting membrane but much more frequently it forms a rounded or oval body eccentrically placed in a vesicle (vesicular form) and surrounded by an electron translucent zone in which granular material may be scattered or line the vesicular membrane. The VDG occur at random throughout the cytoplasm.

The VDG contain serotonin - see Experiment IIc for further discussion. Their origin is still a matter for debate. We (Silver and Gardner, 1968) have seen VDG-like structures in the outer third of mature rabbit megakaryocytes and assume that their number increase as mature platelets take up serotonin. If the granules in the megakaryocyte really are VDG we are not certain of their origin. White (1968d) suggested that the "platelet dense bodies" (i.e. VDG) develop from hyaloplasmic granules. However, as the author himself points out "the origin of dense bodies from platelet granules cannot be followed dynamically in fixed material". White has also suggested that the granules may discharge through the system of vacuoles and

vesicles in the platelet cytoplasm.

Vacuoles and vesicles

Vacuoles and vesicles, also called the "gamma granulomere" (Schulz et al, 1958) or "elements claires" (Feissely et al. 1960) can be seen in the platelet cytoplasm. Usually, they present as slender membrane-bound tubules, or as microvesicles that may be transected tubules or as vacuoles, but their size and extent may vary markedly with the fixative employed (Gardner et al, 1969; Silver and Gardner, 1970).

Two hypotheses were advanced as to their origin; both have some truth. Some authors considered that the vacuoles and vesicles were derived from the endoplasmic reticulum and Golgi complex of the megakaryocyte (Schulz et al. 1958; Policard et al. 1959). David-Ferreira (1964), on the other hand, while not excluding the first possibility, maintained that most of the vacuoles were the result of active incorporation of material into the platelet cytoplasm. Jean and Racine (1962), on the basis of differential staining with phosphotungstic acid, were the first to indicate that the vacuole and vesicle system did not represent a homogeneous group of organelles, a viewpoint strengthened by Behnke's (1967b) observations on the membrane system in rat platelets. Behnke defined the "surface connecting system "(SCS) as a series of vacuoles and large cavities in continuity with the plasma membrane and proved that the inner surfaces of their membranes have in

common with the plasma membrane a thin coat of amorphous substance which is probably an acid mucopolysaccharide material. He found that the extent of the SCS in individual platelets is highly variable. The other system of vacuoles and vesicles Behnke named the "dense tubular system" (DTS). It consisted of thinner membrane-bound tubules that contained a homogeneous rather electron dense substance. In equatorial platelet sections tubules of the DTS system are usually observed immediately deep to the circumferential bundle of microtubules and sometimes accompany the bundle along the outer one third of the platelet circumference to form a "submarginal dense tubule". The rest of the DTS is scattered through the granulomere. In Behnke's view the DTS does not connect with either the plasma membrane or the SCS. One might suppose that the morphological difference in the two vacuole systems would be reflected in different enzyme activity. A test of this hypothesis by morphological studies at the ultrastructural level has not yet been done.

Platelets have the ability to take up materials from the environment (Schulz, 1961; David-Ferreira, 1961; Movat et al. 1965a,b; Schulz and Landgrüber, 1966). When this occurs platelets both aggregate and degranulate. Mustard and Packham (1968) have pointed out the similarities between this process and phagocytosis as seen in leukocytes. However, Behnke (1967b) and White (1968e) have suggested that some of

the uptake of particles by platelets is due to a process other than phagocytosis. Behnke (1967b) demonstrated that particulate matter added to a platelet-rich plasma in vitro or infused into animals appeared in the canalicular system without apparent alteration of platelet morphology. White's (1968e) evidence is contradictory for it shows that platelets incubated with thorium dioxide for a period of 20 - 30 minutes may degranulate. Possibly the process observed by Behnke does not really differ from phagocytosis.

Microtubules

These structures are discussed fully under Experiment IIa and b.

Microfibrils

Microfilaments, 60 - 80 Å in diameter first described in the cytoplasm of sectioned platelets by Bessis and Breton-Gorius (1956). These structures, which are also called microfibrillae (Sixma and Molenaar, 1966; Zucker-Franklin and Bloomberg, 1969) are not obvious in intact platelets fixed in osmium tetroxide or doubly fixed in glutaraldehyde and osmium tetroxide, either because of the electron density of the ground substances or alternatively because they do not normally exist in unstimulated platelets. They become evident if acetaldehyde or formaldehyde prefixatives are used (Silver and Gardner, 1970), or if the platelet is altered in some way before whole mount examination (Bull, 1966) or before fixation (Sixma and Molenaar, 1966;

Zucker-Franklin and Bloomberg, 1969). The microfibrils can be seen forming parallel bundles in the pseudopods or distributed randomly in the cytoplasm. Zucker-Franklin and Bloomberg (1969) described two morphological forms. Some had a diameter between 50 - 70 Å and were scattered through the cytoplasm. Others, 80 - 120 Å in diameter, were found in bundles or dense aggregates. Zucker-Franklin and Bloomberg (1969) could not establish the length of any of the fibrils. A suggestion of a periodicity was apparent in some of them. More recently Zucker-Franklin (1970) has shown microfilaments associated either with the platelet membrane or the membranes of the vesicular system. In cross section the filaments are approximately 75 Å in diameter and are separated by a space approximately 100 Å wide. They are located 200 - 250 Å from the inner aspect of the platelet membrane or on the cytoplasmic aspect of the vesicular membrane. Thus it seems that the plasma membrane and the vesicular system are, under certain circumstances, surrounded by a network of microfilaments.

In other experiments, Zucker-Franklin et al.(1967) found that the ultrastructure of the microfilaments seen in platelets strikingly resembled that of partially purified thrombosthenin, a contractile protein isolated from human platelets (Bettex-Galland and Luscher, 1959, 1961).

Possibly the thicker aggregates of fibrils noticed by Zucker-Franklin (1970) in her preparations are aggregates of the thinner filaments or alternatively a protein compound comparable to an actin/myosin compound. Such a theory would be compatible with the report that thrombosthenin is composed of proteins with the characteristics of actin and myosin (Bettex-Galland et al., 1963). Behnke (1971) has morphological evidence for the existence of both proteins in the platelet. Thus, if the microfilaments are concerned with contraction and they have the orientation Zucker-Franklin (1970) noted, they may be responsible not only for platelet contraction but also the contraction of vesicles and vacuoles within the cytoplasm.

Glycogen

Electron dense granules, 150 to 300 Å in diameter that have an irregular outline can be observed scattered throughout the platelet ground substance, either as single granules or in clumps that may contain hundreds of granules (David-Ferreira and David-Ferreira, 1962). David-Ferreira (1964) named the granule the epsilon granulomere. The granules have the same morphology as glycogen granules described in other cells. Indeed, Jean and Racine (1962) concluded that the granules were glycogen because they lost their "staining" property after incubation with diastase. For some reason, yet to be explained, the electron density of the ground substance may be considerably reduced where

these glycogen granules occur in clumps. Although glycogen appears as individual granules in present day preparations, they tended to adhere and form aggregates when platelets were fixed in osmium tetroxide and embedded in methacrylate (David-Ferreira, 1964).

While some platelet granules may be derived from the megakaryocyte, other granules are probably synthesized in the platelet (Karpatkin et al. 1970).

Ribosomes

Ribosomes, seen as dark granules, 100 - 200 Å in diameter, may be found attached to fragments of endoplasmic reticulum in large rabbit platelets (Silver and Gardner, 1970) that are, because of their size, assumed to be immature (McDonald et al.1964; Detweiler et al. 1962). Thus while immature rabbit platelets may contain ribosomes, there is doubt about their presence in mature human platelets (Hovig, 1968; Behnke, 1969). Probably mature platelets do contain ribosomes since they are able to synthesize protein. Possibly some of the single granules seen in the cytoplasm and interpreted as glycogen granules, are in fact ribosomes.

Siderosomes

Vesicular structures, that have a clear interior with dense granules the size of ferritin particles lining their membrane were first described in the platelet cytoplasm by Schulz et al. (1956).

Such granules form the delta granulomere of David-Ferreira (1964).

Their origin, nature and function are unknown.

EXPERIMENT I

Platelet behaviour in vivo

Although leukocyte behaviour in small blood vessels has been investigated extensively in various species (Addison, 1843; Waller, 1846a,b; Wharton Jones, 1846; Purves, 1874; Conheim, 1889; Metchnikoff, 1891; Florey, 1962) platelet behaviour, at the time this experiment was done in 1965, had received scant attention. Therefore, observations were made on platelets under varying experimental conditions in the vessels of fully and partially vascularized rabbit ear chambers.

Materials and Methods

Semi-lop rabbits of both sexes weighing 2.5 - 3.5 Kg were used. The animals were individually housed and fed McMaster's Laboratory Diet (Sydney, Australia) ad lib.

The basic design and dimensions of transparent ear chambers were published by Sanders et al. (1954). Those used had glass cover slips and three buffers on the central table (5 mm diameter). They were inserted as described by Ebert et al. (1939) and used, in the main, four to five weeks later when fully vascularized. Some partially vascularized chambers were studied two to three weeks after insertion. The rabbits were lightly anaesthetized with sodium pentobarbital

(sodium nembutal, Abbot, Sydney, 50 mg per Kg initially and 10 to 15 mg per Kg as required afterwards), and observations, at a magnification of x 400, were made of fully vascularized chambers, partially vascularized chambers, chambers mildly injured by animals jerking their ears during observation, and chambers before and after the intravascular injection of the various agents.

The agents that altered platelet behaviour were infused through either the carotid artery supplying the ear chamber or the marginal ear vein of the contralateral ear. A 25 gauge hypodermic needle was inserted into the vessel and connected by polyethylene tubing to a syringe in a slow infusion pump. An infusion of physiological saline was started immediately and the agents infused subsequently.

Agents injected intravascularly

Thirteen rabbits were infused for 10 minutes with 10^{-2} molar solution of adenosine diphosphate (ADP) (Sigma Chemical Co., St. Louis) in physiological saline (pH 7.3 - 7.4) at 0.28 ml per minute. Nine received the infusion through the carotid artery and four through the contralateral marginal ear vein. Another three rabbits had ADP infused through a femoral vein. In each instance the ADP infusion was preceded and followed by the administration of physiological saline for

a control period of at least half an hour at the same or at a faster rate than used to administer the ADP. At half-hourly intervals, two rabbits received two infusions of ADP and a third was given three.

From six of the animals (four with venous infusions and two with carotid infusions), 0.5 ml samples of blood were collected at set periods before, during and after the infusion from a polythene cannula inserted into the femoral artery. The samples were mixed with 1 mg of dried disodium ethylene - diaminetetraacetic acid as an anticoagulant. Platelet counts,using the Brecher-Cronkite method, (1950) and leukocyte countswere made.

Methyl cellulose U.S.P. 400 c.p.s. ("Methocel", Fluka, A.G. Switzerland) was prepared as a 1% solution in physiological saline and sterilized by autoclaving in sealed bottles subsequently stored at 5°C. Twenty ml of the solution at room temperature were slowly injected intravenously into 5 rabbits. Second and third injections were administered to one animal at intervals of 8 and 10 days respectively.

A carbon suspension, (Pelican Ink, Ink No. C11/14130 Günther Wagner, Hanover, Germany) was diluted 1:4 in physiological saline and centrifuged lightly. Only the upper four fifths of the suspension was used for the infusion. Three rabbits received 20 ml of the diluted suspension given within four minutes.

Two mg of bacterial lipopolysaccharide (E. coli 026:B6 or S. typhosa 0901, Difco Laboratories, Detroit,) dissolved in 2 - 5 ml of physiological saline were administered intravenously into the contralateral ear of 12 rabbits within 12 minutes. Six were given E. coli and 6 S. typhosa endotoxin.

After observation histological blocks were prepared from the heart, lung, liver, spleen, kidney and ear chamber. They were embedded in paraffin, sectioned and stained with haematoxylin and eosin and with Mallory's trichrome stain.

Thrombin, (topical thrombin of bovine origin, Parke Davis & Co., Detroit) was dissolved in physiological saline and administered intravenously at the rate of 3 NIH units per minute to 4 rabbits. The total dose varied from 240 - 436 units.

Results

The behaviour of platelets differed considerably from that of leukocytes and erythrocytes.

Non-inflamed ear chambers

It was common to see leukocytes sticking to endothelium in partially and fully vascularized chambers that were not specifically inflamed. In some vessels leukocytes did not adhere to the vessel wall, nor were they visible in the stream. In other vessels they rolled along

the endothelium, often at an irregular rate. Some were washed free and adhered again a short distance downstream. When rolling, leukocytes moved slowly and were visible in profile at the side of vessels; they had a wide area of attachment and were slightly distorted. Even when their speed did not permit these observations, their rate of movement, which was slower than that of the passing stream, indicated some degree of adhesion. Some leukocytes adhered to each other as they rolled along. It was less common to find leukocytes fixed to vessel walls for long periods.

Erythrocytes did not stick to the endothelium in the same manner as leukocytes, but passed by at the speed of the stream. They were deformed momentarily when caught on spikes and were only occasionally fixed to leukocytes (fig. 1, appendix 2) or to the vessel wall. In the latter instance they usually remained fixed for long periods and either had a tear-drop outline with a very localized point of attachment, or were dumb-bell shaped if part of the cell projected through the endothelium (fig. 2, appendix 2). In contrast to leukocyte sticking, the localized point of attachment suggested mechanical fixation rather than stickiness of apposing cell surfaces. Moreover, passing cells did not adhere to them. Rouleaux formation in stagnant vessels provided the only instance of mutual attraction of red cells.

Platelets never rolled along endothelium and only rarely adhered to vessel walls. Where this occurred their point of attachment was localized and they were easily washed away. Subsequently one or a small chain of platelets was at times seen adhering to the identical site. Such attachment did not appear to be related to vessel branchings. Most commonly one or several platelets adhered to leukocytes, which were stationary on the vessel wall or rolling along it (fig.3, appendix 2). They also adhered to endothelial spikes, but never to circulating red cells. Occasionally a platelet adhered at the point of fixation of a red cell to the vessel wall. In stagnant vessels platelets were sometimes loosely grouped together in small numbers but like red cell rouleaux they quickly disintegrated when washed into a moving stream. Immediately after positioning the chamber on the microscopic stage, occasional aggregates of 2 - 4 platelets were seen in vessels. They were only present for short periods and may have been due to trauma.

Mild trauma

The response of platelets to trauma was quite dramatic. They adhered to leukocytes in increasing numbers, forming small chains of platelets. As other platelets adhered, the chain gradually lengthened or fragmented and was reformed. Small detached groups of platelets

at times adhering to a leukocyte, were swept downstream, only to cling to other leukocytes or platelet clumps. Additional leukocytes and platelets adhered, and so large aggregates of platelets, leukocytes and an occasional red cell were built up only to wash away or gradually disintegrate. This phenomenon of platelet aggregation and thrombo-embolism was a continuously recurring process which persisted for varying periods of time until it eventually disappeared as the vessels reverted to normal. Such change was widespread but now and then it was restricted to only one or two vessels in the chamber. Platelets sticking to endothelium was not accentuated.

ADP infusion

Preliminary infusion of physiological saline did not produce an alteration in platelet behaviour or any other morphological change. Within 20 - 40 seconds of commencing ADP infusion into the carotid artery, circulation in the chamber slowed temporarily, and interfered with observations in three animals. Within 2 - 4 minutes, large platelet aggregates, at times incorporating red and white cells, were seen in the stream adhering to leukocytes and endothelial spikes, or impacted in vessels, thus obstructing flow (fig. 1, appendix 1; fig. 4, appendix 2). Some were swept into the chamber, possibly coming from thrombus formed at the injection site in the carotid artery (Honor and Ross Russell, 1962). However, other aggregates formed in vessels

under observation. Platelet thrombi and emboli were seen throughout the period of infusion. Platelet adhesion to leukocytes was accentuated, but the tendency to adhere to endothelium was unchanged. The behaviour of other blood elements did not alter nor was any morphological change observed in platelets, blood cells or endothelium. When ADP infusion was stopped, blood flow rapidly improved and platelet clumps became less prevalent. Most aggregates disappeared within 10 minutes, but a few continued to circulate or remained in stagnant or impacted vessels. Second and third infusions produced similar changes but aggregates and emboli took a few minutes longer to develop and were not as frequent.

The site of ADP infusion affected the intensity of the observed reaction. Infusions into the femoral or the marginal ear vein of three rabbits also caused platelet aggregation in the ear chamber, but it was much less prominent than when ADP was injected into the carotid artery.

A 30 - 50 % reduction in the platelet count was recorded during the infusion of ADP. A parallel drop in the leukocyte count was also recorded.

Methyl cellulose

Methyl cellulose induced platelet aggregation within 5 - 15 minutes in the 5 rabbits injected (fig. 5, appendix 2). Platelet aggregates were small at first but increased in size and were still present in 3 rabbits

21 hours after injection. Aggregates caught on endothelial spikes and leukocytes adherent to the vessel walls. Many platelets, in stagnant vessels, or those with slow flow, lost their disc-like shape, and became globular, others developed spikes. Platelet aggregates were seen in 3 animals 24 hours after injection but were not present after 48 hours.

Methyl cellulose also caused red cell aggregation (figs. 5 and 6, appendix 2). This striking change occurred within 5 minutes of injection. The red cells were usually of normal shape but became attenuated when aggregates were stretched at vascular junctions. They did not adhere to endothelium. Red cells were still in aggregates in one rabbit after 5 days but had reverted to normal within a week. This rabbit received two further injections and similar changes occurred.

Leukocyte behaviour was unaltered.

Carbon injection

Platelet aggregation was induced by the carbon suspension but the behaviour of leukocytes and erythrocytes was not altered. The observations did not differ from those reported by Stehbens and Florey (1960) except that platelet aggregates, associated with varying amounts of carbon, occurred within 5 - 10 minutes of commencing the injection.

Bacterial endotoxin

Platelet aggregates were observed within 10 minutes in each of the six rabbits following the injection of S. typhosa lipopolysaccharide. During the first 30 minutes erythrocytes adhered in clusters to leukocytes fixed to the vessel wall. Later this was not so pronounced, perhaps because of the great diminution in circulating leukocytes. Microthrombi and emboli formed and continued to do so as previously described. They enlarged, and within 2 hours large hyaline globular thrombi were seen (figs. 7 and 8, appendix 2). They were being washed slowly into large venules. Platelets could be distinguished on their surface and a few leukocytes and erythrocytes also adhered from time to time. Transitional stages between packed aggregates and hyaline thrombi were seen. The latter were more translucent than platelet aggregates and did not fragment. They frequently impacted in vessels because of their large size. Others adhered to the vessel wall. Some of the thrombi developed tails which extended distally in the stream and were composed of platelets (fig. 9, appendix 2). The tails slowly lengthened or shortened when fragments broke off to form emboli. The tail was sometimes in the central stream, on other occasions it rested against the wall without adhering to endothelium, apposition appearing to be fortuitous. Platelets, often spherical or with spikes, rolled along the surface of these thrombi and some, before being washed away,

appeared to retain an attachment to the tail, as if by an invisible thread. Immediately distal to the tail of the thrombus, some platelets appeared to roll along the endothelium for a short distance in much the same manner as do leukocytes in early inflammation. It could not be determined whether these platelets had retained some thread-like attachment to the thrombus.

Only one animal survived overnight. At 22 hours it still had hyaline thrombi and atypical platelets in the vessel of the chamber. Numerous thrombi were present in histological sections from all animals.

Within 5 minutes of the injection of E. coli lipopolysaccharide platelet aggregation was seen in four of the six rabbits. Hyaline thrombi, similar to those occurring with S. typhosa, were found in two of the three rabbits observed for more than 2 hours, and were still present in one animal after 23 hours. The other five rabbits died during the night. Thrombi were found histologically in the four rabbits which developed platelet aggregates (fig. 10, appendix 2).

The adhesion of red cells to white cells was less pronounced than following S. typhosa endotoxin and there was a decrease in the number of circulating leukocytes in five of the rabbits.

Thrombin

Thrombin produced changes similar to those seen with the endotoxin. Platelet aggregation occurred and hyaline thrombo-emboli were present in all of the animals within an hour. As in the endotoxin treated rabbits, platelets appeared to roll along the vessel wall in the immediate vicinity of the thrombi. A number of platelets were spherical and a few had spikes on their surfaces.

Thrombi were present histologically in the tissue of all animals.

Additional observations

On rare occasions, small round hyaline bodies, slightly larger than platelets, rolled along the endothelium in the same manner as leukocytes. They were not common but were seen at least once following each agent injected. It is possible that they were atypical or large platelets.

Discussion

These observations indicate the essential differences in behaviour of platelets, leukocytes and erythrocytes in the small vessels of rabbit ear chambers, with particular reference to their sticking to endothelium. Adhesion of platelets to the vessel wall is an integral part of thrombosis and is generally regarded as the first observable change

in experimental thrombosis induced by injuries to the vessel (Bizzozero, 1882; Eberth and Schimmelbusch, 1886; Welch, 1887;1889; Beattie et al. 1948a; Cappell, 1958a; Payling Wright, 1958a; French, 1962; Honor and Russell, 1962). Yet it is also stated that, apart from preliminary vasomotor changes, the first tissue response in acute inflammation produced by similar injuries is the sticking of leukocytes to endothelium (Conheim, 1889; Beattie et al. 1948b; Cappell, 1958b; Payling Wright, 1958b; Florey, 1962), and platelet sticking is not a feature. This variation in response may be accounted for by a difference in the intensity of the injury (Stehbens, 1965).

Platelet sticking in this investigation can be divided into three stages. The first was seen in ear chambers not specifically inflamed, though mild inflammatory changes cannot be completely excluded in this experimental model. Most commonly, a few platelets adhered to the surface of the leukocytes which were either fixed to or rolling along the vessel wall. However, such changes were not always present. Only Florey (1962) and Bränemark (1964) have mentioned that platelets adhere occasionally to endothelium. A mechanism for the observed phenomenon could be a temporary reversible separation of endothelial cells with adherence of platelets to the denuded basement lamina, as occurs following mild trauma (Jørgensen et al, 1967a). Tranzer and Baumgartner

(1967) have distinct ultrastructural evidence that this can occur.

The second stage of platelet sticking is manifested by platelet aggregation and thrombo-embolism. This stage is primarily an augmentation of platelets sticking to leukocytes and to each other and is induced by mild trauma, injection of adenosine diphosphate, Pelican ink and methyl cellulose. The microthrombi and emboli formed do not persist and are similar to those observed by Sanderson (1931) in an injured ear chamber. He saw platelets adhered to leukocytes initially rather than to endothelium and the thrombus disappeared within 24 hours.

The third stage, heralded by the formation of hyaline thrombi is a more advanced stage of platelet aggregation. The greater degree of translucency and hyalinization of the thrombi is probably due to the close packing of platelets and alteration in their structure, as observed in electron micrographs of recently formed thrombi (French and Poole, 1963) and is analogous to the hyaline appearance of closely packed erythrocytes in vascular spaces. This phase does not appear to be reversible, at least during the first 24 hours since thrombi were found in the tissues histologically.

The results also indicate that intravascular thrombosis may be produced without gross trauma to the vessel wall which is the usual

technique adopted in the experimental production of thrombi (Bizzozero, 1882; Eberth and Schimmelbusch, 1886; Welch, 1887, 1899; Honor and Ross Russell, 1962). However, there is a continuous gradation, from the sticking of isolated platelets to leukocytes and endothelium on the one hand, to the eventual formation of hyaline thrombi on the other. One cannot necessarily conclude that adhesion of isolated platelets to leukocytes or endothelium as observed above necessarily represents the earliest stages of thrombosis. This may be so in these experiments in which there was no gross disruption of the endothelium, but in severe endothelial injury, platelet adhesion at the site of trauma is the first response (Bizzozero, 1882; Eberth and Schimmelbusch, 1886; Welch, 1887, 1899; Honor and Ross Russell, 1962).

The experiments with adenosine diphosphate demonstrate that the intravascular administration of ADP is associated with the intravascular agglutination of platelets, and also with their adhesion to leukocytes. Platelet thrombi adhere to leukocytes and endothelial spikes and on other occasions become impacted in small vessels, but in the rabbit their adhesion to endothelium is not a prominent feature. Recently Jørgensen et al (1970) have contradicted this opinion. They showed focal vessel wall damage, the formation of endothelial gaps and blood vessel extravasation following ADP-induced platelet

aggregation in vivo . The authors suggest that transient platelet aggregation in the microcirculation could be an important factor in causing vascular lesions and mural thrombosis.

The drop in platelet count recorded during ADP infusion confirms the work of Regoli and Clark, (1963). The change is probably due to the trapping of aggregates in blood filters and also to their sequestration in small vessels as occurs when other substances are injected intravenously (Stehbens and Florey, 1960), rather than to their pavementing on endothelium. Perhaps the incorporation of leukocytes in the platelet thrombi explains the simultaneous leukopenia recorded.

At the time these experiments were done, the pharmacodynamics of platelet aggregation and thrombus formation had not been explained although several authors had suggested that ADP release might be an essential step or the "final common pathway" in the mechanism (Clayton and Cross, 1963; Born et al. 1964; O'Brien, 1964). Subsequently much has been learned of the mechanism of ADP induced aggregations and of its importance in physiological and pathological processes - see review by Mustard and Packham (1970).

EXPERIMENT II

The first study of platelet ultrastructure (Experiment IIa) was done in 1963-64 soon after the introduction of glutaraldehyde/osmium tetroxide double fixation (Sabatini et al. 1963). This and subsequent experiments were aimed at defining the ultrastructure of rabbit platelets doubly fixed in this manner. Use of the aldehyde prefixative revealed cytoplasmic microtubules in the platelets and led to Experiment IIb in which their morphology was better defined.

Experiment IIa. Ultrastructure of rabbit platelets

Materials and Methods

Blood collection.

All hypodermic needles and glassware used to prepare platelet-rich plasma was siliconized (200 fluid - Dow-Corning, Toronto), and in some instances, chilled to 4°C before use. Using 20 ml syringes and 18 gauge needles, blood was obtained by cardiac puncture from healthy rabbits of both sexes anaesthetized with sodium pentabarbital administered intravenously (Sodium nembutal, Abbott, Montreal, diluted 1:1 with physiological saline and given in a dose of 30 mg/Kg). In some instances the blood was collected without an anticoagulant. In others, 9 volumes of blood were mixed with one volume of an anticoagulant -

either 3.8% trisodium citrate made up in physiological saline (pH 7.4, 350 mM) or a 2% solution of disodium diaminoethano-tetraacetic acid made up in physiological saline and containing 5% dextran (Glaxo-Allenbury's, Toronto) - pH 7.4. In other instances the heart blood was drawn directly into a glutaraldehyde/anticoagulant solution devised by Behnke (1965).

Processing for electron microscopy.

Where appropriate, samples of platelet-rich plasma separated by centrifugation (500 g for 5 minutes), or samples of blood were diluted in up to 3 to 4 times their volume of 5% glutaraldehyde (50% w/w glutaraldehyde - Fisher Scientific Co., New Jersey) made up in Sorensen's phosphate buffer (pH 7.4; 603 mM). After 1 - 2 hours fixation, the plasma-fixative or blood-fixative solutions were centrifuged once or twice as needed, to obtain a platelet pellet. The pellet was washed in Sorensen's phosphate buffer with or without 5.2 gm% sucrose (pH 7.4; 314 mM) and post-fixed for 1 - 2 hours in 1% osmium tetroxide solution containing sucrose (Caulfield, 1957) - pH 7.4; 274 mM. In some experiments the blood or platelet-rich plasma was fixed in osmium tetroxide solution alone. After fixation, the platelets were washed (in water in early experiments, and in Sorensen's phosphate buffer in later ones), then dehydrated in graded ethanol, transferred through mixtures of propylene oxide which contained increasing amounts of

embedding material and eventually were embedded in an epon-araldite mixture. Ultrathin sections, cut on an ultramicrotome (LKB Co., Sweden) equipped with glass knives, were stained with a saturated solution of uranyl acetate in 50 or 70% ethanol, or with a lead solution (Karnovsky (B method) 1961; Reynolds, 1963) or sequentially with both heavy metals; some were not stained at all. They were then examined in an electron microscope, (JEM 6C; Siemen' s Elmiskop 1; RCA 3F; Philips 200 or 300).

In other experiments, blood was collected from the anticubital vein of humans, and, following ether anaesthesia, by cardiac puncture from guinea pigs or from the retro-orbital sinus of mice. Blood from 6 - 12 mice was pooled. Platelets from the blood of these species was obtained and processed for electron microscopy in the manner outlined above.

Observations

In general, sectioned rabbit platelets have an elliptical profile, but some are round or oval (fig. 1, appendix 3, 6, 7, 8; figs. 3 and 4, appendix 3). Their size varies considerably, but in the majority the greatest dimension is between 3 - 5 mμ. Exceptionally large platelets 5 - 8.8 mμ in diameter are found infrequently in normal rabbit blood. They are slightly more numerous in venous than arterial blood and are present in increased numbers following blood regeneration (Silver and Silver, 1971).

The platelets are bounded by a double layered membrane 70 - 85 Å wide (fig. 5, appendix 3). Most have a smooth external surface but pseudopodia-like structures, occasionally with expanded club-like tips, project from some activated platelets (fig. 1, appendix 3). Small membrane-bound fragments of finely granular protoplasm that contain a few vacuoles and may represent transected pseudopods are seen occasionally, also "clear platelets" like those described by David-Ferreira (1964).

A fuzzy, electron dense external platelet coat, 100 - 200 Å wide, is found adherent to the external surface of the platelet membrane in some preparations (fig. 1, appendix 3). It is better preserved when other preparative techniques are used, for example, if alcian blue is added to the glutaraldehyde solution.

The ground substance of doubly fixed platelets is finely granular and has scattered through it various granules and vesicles.

Several mitochondria (beta granulomere) are usually found in each section. They show only 2 or 3 cristae per mitochondrion.

Round to oval alpha granules, 0.2 - 0.3 mμ in diameter are the most numerous (figs. 1 - 4, appendix 3). Their morphology is like that of alpha granules found in human platelets. They may contain nucleoids but the small surface nodules described in human platelet

alpha granules by David-Ferreira (1964) were not apparent in my preparations. Furthermore, I have not convincingly demonstrated in the alpha granule of rabbit platelets the "microtubule-like" structures clearly pictured by White (1968c) and by Vossen et al. (1968) in alpha granules of human platelets. Nevertheless, alpha granules in some of my preparations show vaguely outlined tubular structures within their matrix.

Elongate drumstick granules, with a matrix like that of the alpha granule, are occasionally present in rabbit platelets but are never numerous. They are up to 1.6 mμ long and are defined by parallel single membranes, 40 - 60 Å wide (fig. 2, appendix 8).

Very dense granules (VDG) like those found in human platelets occur at random throughout the platelet cytoplasm (fig. 1, appendices 3, 6, 7, 8; fig. 2, appendix 6). They are however more numerous in rabbit platelets than in those from man, guinea pig or mouse. Their morphology is not altered by the anticoagulants used. The granules can be distinguished in unstained sections, and in platelets collected from rabbits that have not been anaesthetized. We have seen them in platelets embedded in water soluble material (Durcupan). They occur in both isolated platelets and in those preserved in vivo in bone marrow. Exactly similar structures are seen occasionally in megakaryocytes in the outer third of the cytoplasm of mature cells (fig. 3, appendix 6).

Vacuoles and vesicles are also observed in the rabbit platelet cytoplasm. Their size and number varies slightly with the method of fixation used. Some are lined on their inner surface by electron dense material comparable to that seen forming the external platelet coat. These form the surface connecting system of vacuoles defined by Behnke (1967b). Others present the same morphology as the dense tubular system described by that author.

Double fixation with glutaraldehyde and osmium tetroxide reveals a well defined system of microtubules in the platelet protoplasm. The microtubules have a diameter between 200 - 250 Å and in platelets where they extend into pseudopodia-like projections, could be up to 2 mμ long. Their walls, which are 70 - 80 Å thick and more electron dense than their interior, are smooth surfaced on both inner and outer aspects, and cannot be resolved as a unit membrane. Microtubules are observed most frequently cut in cross section at opposite ends of the elliptical platelet profile where they form a compact bundle containing on average 15 tubules (range 10 - 30). However, the number of tubules varies between the bundles at either pole of a single platelet and from platelet to platelet. Within the bundles they appear to be arranged regularly in 3 - 6 roughly parallel rows, each containing 3 - 5 microtubules spaced approximately 100 Å apart. In some instances,

the microtubules are surrounded, in a defined area 0.15 - 0.2 mμ in diameter, by a ground substance that is less electron dense than elsewhere, possibly indicating a different or distinct composition. Much less commonly single microtubules or a group of 3 - 4 are seen in cross section at a site other than at the platelet poles. Other platelet sections having an elliptical profile show longitudinal sections of 3 - 5 parallel microtubules at opposite poles, while in platelets with a round profile and presumably sectioned in an equitorial plane, 4 - 6 of the structures describe arcs of varying length parallel to the plasma membrane and 500 - 600 Å deep to it. Single microtubules pass between the peripheral band and the centre of the platelets but none pass towards the plasma membrane nor do microtubules come in contact with it. No microtubules are seen to branch although the possibility of this happening can not be completely excluded. Nor is any structure seen that could be interpreted as a central point of fixation for the tubules.

Cytoplasmic microfilaments (Bessis and Breton-Gorius, 1965) or microfibrillae (Sixma and Molinaar, 1966; Zucker-Franklin and Bloomberg, 1969) are not seen when rabbit platelets are pre-fixed in glutaraldehyde.

Single glycogen granules up to 200 Å in diameter and large clumps containing many granules are seen in the cytoplasm. As in human

platelets the surrounding cytoplasm is usually less electron dense where the granules occur in clumps.

Very rarely endoplasmic reticulum is found in large, possibly immature platelets. Probably some of the electron dense granules 200 Å in diameter found in the platelet cytoplasm may be free ribosomes.

Vesicular structures that have a clear interior with dense granules the size of ferritin particles lining their membrane (siderosomes or delta granulomere) are most uncommon in rabbit platelets . They are best seen when osmium tetroxide is used alone for fixation.

Guinea pig platelets are remarkably elongate. They, like human and mouse platelets, contain the cytoplasmic organelles described above. On average 13 microtubules were present in the groups found at the poles of human and mouse platelets, and 10 in guinea pig platelets.

Discussion

The organelles found in the cytoplasm of rabbit platelets in our experiment are comparable to those described by other authors following osmium tetroxide fixation, (Sueyasu and Takeshige, 1965; David Ferreira, 1961; Kjaerheim and Hovig, 1962) with the exception that cytoplasmic microtubules had not been observed.

Under particularly favourable circumstances, fibres may be seen in platelets examined by phase contrast microscopy (Bessis, 1956).

Radially placed fibres have also been recorded in the hyaloplasm of platelets spread on films and examined with the electron microscope (Bessis, 1948; Bessis and Bricka, 1948; Haguenau and Bernhard, 1952). These appeared to be composed of granules 50 - 70 mμ in diameter (Bessis and Bricka, 1948). Bessis (1950) described some images as showing granular radial fibres lying very close to each other and connecting the opaque centre with the edge, and also stated that other peripherally placed circular fibres, 150 - 200 mμ thick, according to Bessis and Bricka (1948), were made up of a dozen elementary fibrillae. Although Bessis, 1956, subsequently concluded that the granules did not pre-exist before fixation, he found that the general arrangement of the fibres was always reproducible and argued that if an artifact was involved, its effect would intensify the appearance of pre-existing structures without rendering them unrecognizable. No equivalent structures were recorded in reports on the fine structure of platelets seen in section after fixation in osmium tetroxide (Rinehart, 1955a,b; Sueyasu and Takeshige, 1956; Yamada, 1957; Schulz and Hiepler, 1959; Rodman et al., 1962; Kjaerheim and Hovig, 1962; David-Ferreira, 1964). The fibres described by Bessis and others in spread platelets actually resemble the microtubules described here; Bessis and Breton-Gorius (1965) drew the same conclusion at about the time my paper was published.

An ability to demonstrate the microtubules in this instance was probably dependent upon the use of glutaraldehyde as a fixative, for it is apparent that the structures are best preserved when glutaraldehyde is used (Ledbetter and Porter, 1963; Sandborn et al. 1964) and when the fixative contains calcium ions or has a pH less than 7.4 (Maser and Philpott, 1964). However I have seen similar structures, though not as well defined, in platelets fixed 4 - 6 hours in Caulfield's fixative which contains osmium tetroxide. Although the fibres in spread platelets and microtubules are both located in similar regions of the cytoplasm, the recorded diameters of the two groups of structures varies. Part of the variation may be due to the different methods of preparation used; for example, Maser and Philpott (1964) found that the diameters of frozen dried microtubules in nucleated erythrocytes were about twice those observed in thin section, and part may depend upon the number of microtubules with or without specialized ground substance surrounding them, that are present in each fibre. Alternatively the fibres demonstrated in spread platelets could correspond to microfibrillae and microtubules.

Fawcett (1962, 1969) described groups of transected microtubules present at opposite poles of sectioned nucleated erythrocytes and suggested that they corresponded to the marginal band described many years earlier by Dehler (1895). Maser and Philpott (1964) confirmed this opinion. By analogy then, rabbit platelets possess a

marginal band. In the comparative sphere, microtubules were described at, or about the same time in the platelets of hamsters (Hayden and Taylor, 1965), humans and rats (Behnke, 1965). These, with my own observations (Silver, 1966) and later ones (Sandborn et al. 1966;Stehbens and Biscoe, 1969) indicate that microtubules are probably a universal finding in mammalian platelets.

The presence of the radially running bands makes difficult interpretation of the configuration of the microtubules. It is hard to reconcile the concept of a series of loops with the image obtained. Rather, one could imagine a series of tubules, perhaps anchored to a point in the centre of the platelets, passing towards the perimeter, joining the marginal band and describing one or several loops. A figure of eight configuration is another possibility.

It has been suggested that microtubules in peripherally placed bands, as in the rabbit platelet, are concerned with maintaining cell shape (Fawcett and Witebsky, 1964). Maser and Philpott (1964) on the other hand, doubt that they have a continuing role in the maintenance of cell shape. In their opinion the structures are only concerned with initiating the asymmetry of nucleated red cells. Present observations are compatible with either viewpoint. Possibly the peripheral band in platelet maintains their shape and the radial fibres provide strengthening for their interior.

Since at that stage (1965) relatively little was known about the microtubule, attempts were made in the next experiment to better define the ultrastructure of the microtubule.

Experiment IIb. Morphology of Microtubules in Rabbit Platelets

Materials and Methods

Blood was obtained from healthy rabbits by cardiac puncture. Each animal had four successive punctures at 6 week intervals. Platelets were separated from citrated blood, doubly fixed in glutaraldehyde and osmium tetroxide solution, and prepared for examination in an electron microscope as outlined above.

Enlarged photographic images of microtubules were subjected to a method of rotational analysis designed to enhance image detail (Markham et al. 1963). This involved moving a sheet of photographic paper through an arc of a circle (360/n degrees), stopping and exposing the tubule image (45/n secs for each exposure) then repeating the procedure until the paper had been completely revolved. In this experiment, n values were increased from 3 - 20 and a complete rotation was made for each value of n.

Observations

Surrounding the wall of each microtubule is a zone that is less electron dense than either the tubule wall or the nearby platelet cytoplasm. Usually, the width of this translucent zone is 70 Å, no matter

whether it separates adjacent tubules or a tubule from the cytoplasm (fig. 1, appendix 5). Occasionally, however, it is 140 Å wide. In some preparations a faint nodularity is apparent in the zone but no image intensification is observed after tubules have been subjected to rotational analysis. Some transected tubules show a dense opacity at their hub. No definite structure could be defined in this opacity by direct microscopy or following the rotational technique.

Generally, the wall of transected microtubules appears as a relatively homogeneous electron dense annulus, approximately 70 Å wide (fig. 1, appendix 5). Sometimes, nodular areas of increased density are seen within an arc of the annulus (fig. 2, appendix 5), or, less commonly, around all of the circumference. The nodules are 35 - 40 Å in diameter and are equally spaced, the centre to centre distance being 60 - 65 Å. On rare occasions, usually in platelets stained for a brief period, regularly arranged continuous circular profiles could be defined in the wall (fig. 3, appendix 5). These are 60 - 70 Å in diameter, and, like miniature microtubules, have an electron dense wall some 15 Å wide enclosing an area of relative electron translucency 30 - 35 Å wide. The spacing between the centres of the small "tubular" structures was 55 - 65 Å.

Only 3 tubules with round profiles in their walls were suitable for rotational analysis. In two, image reinforcement occurred when n was 12. In the third it was evident when n was 13. Twenty-seven

tubules showing well defined nodular densities around part or all of their annular walls were subjected to rotational analysis. Fourteen were round in section (R), suggesting that they were truly transected and 13 were slightly elliptical (E). They came from 11 platelets as indicated.

Tubule studied in each platelet	1	2	3	4	6
Number of platelets examined	4	3	1	2	1

Twenty-one tubules showed image intensification in their outer wall. In 16 (8R + 8E), the reinforcement occurred at only 1 value of n. In each instance this value was verified by obtaining reinforcement at values of n/2 or 2n. Five tubules (1R + 4E) showed marked reinforcement at one n value with slight accentuation at values of $n \pm 2$. The distribution of n values in which reinforcement was obtained in the 21 tubules is shown in fig. 4, appendix 5. Most of the values lie within the range of 12 ± 2. Six tubules did not show any definite reinforcement. Intensified images were round and their diameter was about 35 Å. Although measurement was difficult, since boundaries were indistinct, the diameter of the rotated tubules, the size of intensified images, their distance apart, and their radial distances from the centre varied very little from one microtubule to the next.

Discussion

Maser and Philpott (1966) noted a disproportionate number of "hollow" tubular profiles in sectioned erythrocytes from the killifish (Fundulus heteroclitus) stained by floating the sections on a drop of "staining solution". They suggested that stain penetration was incomplete so that the entire depth of the tubule within a section was not defined. Grids carrying thin sections of platelets were immersed in heavy metal solution for staining. As a result, perhaps, most of our sections show short tubular segments of microtubules. Many sections had to be viewed to find a truly transected tubule orientated to allow a clear view down through its centre. Longitudinal sections of microtubules are not seen frequently.

The nature of the electron lucid zone surrounding both individual tubules and those in the marginal band is not certain. It is apparent in separated platelets (Behnke, 1965; Silver, 1965, 1966; Sixma and Molenaar, 1966) and in some preparations of platelets prepared in vivo (Behnke, 1965; Haydon and Taylor, 1965; Stehbens and Biscoe, 1967) although not in all (Sandborn et al. 1966). Behnke and Zelander (1966) prepared isolated microtubules from human platelets, negatively stained them and found that the structures were 400 Å in diameter. Since the diameter of the unit represented by a sectioned tubule and its peripheral electron transparent zone is about 400 Å, the diameter of isolated tubules

may be comparable to that of the sectioned tubules. This would mean that some material at the periphery is not preserved by the method of fixation used, or the processing method extracts it. Maser and Philpott (1964) drew a similar conclusion from observations of freeze-dried, whole mount and sectioned tubules from nucleated erythrocytes. If the electron transparent zone is produced in this way, then the material surrounding one sectioned tubule in a platelet marginal bundle must be able to fuse with that surrounding another to maintain a constant distance between adjacent tubules, or tubules and adjacent cytoplasm. On the other hand, tubular diameter actually may be 400 Å in diameter, and the electron lucent zone be due to contraction during tissue preparation. This does not seem likely however, in view of the relatively constant diameter of both tubule and electron transparent zone.

The dense regions at the hub of some sectioned microtubules are apparent in the preparations of Behnke (1965) and were noted by Stehbens and Biscoe (1967). Their nature is not known. Maser and Philpott (1966) regards as artifacts similar structures seen in fish erythrocyte microtubules.

The variable morphology of transected microtubules is probably due to variations in staining intensity. Thus, double staining with uranyl acetate and lead hydroxide in some instances obliterates all structures in the tubule wall and produces the homogeneous electron

dense "annulus" observed. Less intense staining resolves a sub-structure. Subunits with a circular profile and 70 Å in diameter were best defined, though not infrequently, after brief periods of staining. Ledbetter and Porter (1963), who found similar units in the walls of microtubules from plant cells, also commented on the need for brief staining to preserve their fine structure. We surmise that the nodular densities seen in the tubular wall also delineate circulat subunits and are the result of differential staining. For example, nodular densities about 35 Å wide would result if staining emphasized the area where the walls of adjacent circular subunits came into apposition. Equally, they could be due to selective staining in the central areas of the circular subunits, although this seems less likely since that area is relatively electron dense.

Rotational analysis of three tubules with circular subunits in their wall revealed 12 or 13 subunits. Examination of the nodular densities, which we equate to the circular subunits, provides further evidence that their number in the microtubular walls lies within the range of 12 ± 2. In the comparative sphere, 13 circular subunits have been observed in microtubules from two species of plant cells (Ledbetter and Porter, 1963) and in flagella tubules from insect sperm (Phillips, 1966). Mammalian sperm flagella tubules on the other hand seem to

contain somewhat fewer subunits (André and Thiéry, 1963; Pease, 1963). Gall (1965) found that the negatively stained microtubules from salamander erythrocytes contained 12, 13 or 14 subunits.

Behnke and Zelander (1966) observed 6 to 7 longitudinal filaments about 35 Å wide, running parallel to the microtubule axis within the diameter of negatively stained preparations of isolated human and rat platelet microtubules. If only one surface of the microtubule was strongly contrasted by the negative stain, then this number of units is comparable to that defined in rabbit platelets by rotational analysis. Gall (1966), who showed 6 to 7 units within the diameter of isolated and negatively stained newt erythrocyte microtubules, has advanced a comparable argument to indicate that the real number may be 14. One suspects that selective staining, perhaps of the area between circular subunits, accounts for the filaments measuring 35 Å in diameter.

Our results may mean that the number of subunits in the microtubule wall varies from one tubule to the next, or in different segments of the microtubules. We believe that the variation is due to the variability in tubule shape and/or to irregular density of staining rather than to a true variation. For example, rotation of an elliptical "tubule" drawn on a glass photographic plate with 10 equal-sized opaque dots at equal intervals on its circumference, gave maximum reinforcement when n was 10 with some reinforcement at 8 and 12

respectively. Ledbetter and Porter (1963) attributed the variation in their results to difference in intensity of tubule staining. Furthermore, to add round subunits to the wall and maintain tubule diameter would mean either that unit size would diminish or that units would become packed close together; alternatively tubule diameter would increase. Yet, in this material tubule size, nodular unit diameter, and spacing of tubules seemed to vary very little. The apparent uniformity of tubular diameters is confirmed by Wolfe (1965), Behnke and Zelander (1966) and Gall (1966) who studied long segments of isolated microtubules. However, the supposition that the number of units in the microtubule is constant, may apply only to the particular type of microtubule being examined, and to the species from which the cell came.

Subunits in microtubules may be in the form of straight filaments (Ledbetter and Porter, 1963) or a series of helically arranged globules (Maser and Philpott, 1966). Some negatively stained preparations show straight filaments with indefinite beading along their edge (Behnke and Zelander, 1966; Gall, 1966).

Like Behnke (1969), we have seen microtubules in the cytoplasm of the megakaryocyte at all stages of cell maturation. The microtubules are found throughout the cytoplasm, being numerous in the pericentriolar area, but do not have an obvious ordered pattern of arrangement.

When a megakaryocyte extends its processes into the marrow parenchyma microtubules are seen arranged roughly parallel to the long axis of the extension. Possibly, they have a role in the formation of the extension. The facility with which microtubules are disrupted and reformed (see below) suggests that there is probably a rearrangement of microtubule protein in the extended fragment of megakaryocyte, now a platelet, to form the observed platelet marginal bundle.

The microtubules are heat labile (Behnke, 1967a; White and Krivit, 1967). They disappear when the platelet is chilled and reappear on warming. Supposedly the microtubule protein polymer breaks down into a monomer component in the platelet cytoplasm; it can repolymerise if rewarming occurs within a fixed period. With the loss of microtubules a platelet becomes rounded, supporting the concept that they serve a cytoskeletal function. Behnke (1967d) has discussed the implication of this change when storing platelets for transfusion purposes. The incubation of alkaloids obtained from the periwinkle plant Vinca rosea Lina with human platelets causes microtubules to disappear from the cytoplasm with the formation of characteristic crystals in the cytoplasm (White 1968f; Behnke, 1969); comparable changes occur if rabbit platelets are treated in this manner. Bensch and Malawista (1969) considered the crystals a sequestration of microtubule protein caused by

changes induced in the protein by the alkaloids. However, other proteins in the cytoplasm may be involved in the crystal formation. Colchicine also causes platelet microtubules to disappear (White, 1968f; Behnke, 1969) possibly by binding microtubule monomers so that the monomer-polymer equilibrium, postulated to exist in the cytoplasm, is shifted (Behnke, 1969).

Although White et al (1966) proposed that microtubules could play a role in platelet contraction, later experimental results have dispelled this concept - colchicine depolymerises microtubules but does not interfere with clot retraction (White, 1968f). It is now thought that microfilaments and microtubules are not related. Microfilaments are probably composed of thrombosthenin (Zucker-Franklin et al. 1967) while microtubules are composed of another protein, called tubulin by Mohri (1968). If the findings of Stephenson (1970) on sea-urchin microtubules can be related to platelet microtubules the latter may be composed of either A- or B- tubulin. Both proteins show gross dissimilarities from muscle actin on the basis of tryptic peptides. Further biochemical characterization of platelet microtubule protein can be expected in the very near future. This will permit a better morphological characterization and an indication of the source of microtubule protein in the megakaryocyte.

My attention then turned to the very dense granule (VDG) in the rabbit platelet. Experiments were done to characterise it morphologically and to localise amines in it at the ultrastructural level.

Experiment IIc. The Very Dense Granule in the Rabbit Platelet

Materials and Methods

Heart blood was obtained from rabbits using either 3.8% trisodium citrate or a 2% solution of disodium diaminoethanetetraacetic acid containing 5% dextran (Glaxo-Allenbury, Montreal) as an anti-coagulant. Samples of platelet-rich plasma separated by centrifugation (500 g for 5 minutes) were placed in an equal volume of fixative. Table 1, appendix 6 read from left to right indicates the fixatives or sequence of fixatives used for groups of experiments. The plasma-fixative mixture was centrifuged (2000 g for 10 minutes)to obtain a platelet pellet. If more than one fixative was used, the platelets were washed in Sorensen's phosphate buffer before being placed in the next fixative. After fixation they were washed, dehydrated and prepared for electron microscopy in the manner outlined in Experiment II.

Some amines, for example 5 hydroxytryptamine (5-HT) (Wood, 1966) and norepinephrine (Telkka et al, 1964) when mixed with glutaraldehyde form a product that can reduce a metallic solution to form an electron opaque material. It is thought that the reaction involves

the formation of a Schiff base and that a condensation product reduces the metallic solution (Tramezzani et al. 1964; Wood, 1966). The reactions formed the basis of attempts to localise amines in platelets. All platelets subject to histochemical studies (four series of experiments) were fixed for an hour in 5% glutaraldehyde. They were then divided into two groups. One was exposed to ammoniacal silver hydroxide according to the method of Tramezzani et al. (1964) and the other was incubated overnight in a potassium dichromate solution using the technique described by Wood (1966). Platelets exposed to ammoniacal silver hydroxide were subsequently washed in a 1% solution of sodium sulphate to avoid nonspecific silver precipitation. Some platelets from both groups were postfixed for an hour in osmium tetroxide solution. All were dehydrated and prepared for electron microscopy in the manner outlined above. Stained and unstained sections from these preparations were examined.

Observations

The morphology of the rabbit platelet VDG doubly fixed in glutaraldehyde - osmium tetroxide is like that of the VDG found in human platelets (see historical review). They are, however, more numerous in rabbit platelets.

When platelets are fixed in glutaraldehyde alone, the opaque body within the vesicles of the VDG is smaller than when both glutar-

aldehyde and osmium tetroxide are used (fig. 4, appendix 6). It has a uniform density in most cases, but may be granular. If so, the matrix has a greater density than than of an alpha granule. However the difference is not enough to distinguish an alpha granule from a VDG positively, unless the VDG matrix is eccentrically placed. Acetaldehyde fixation causes a marked change in platelet shape and morphology (Gardner et al., 1969). Only the vesicular forms of VDG can be identified after such fixation and these are not frequent. Often the body in the vesicle is small (fig. 6, appendix 6). As all granules have an increased density after acetaldehyde - osmium tetroxide fixation, it is almost impossible to distinguish between alpha and VDG (fig. 5, appendix 6). Acetaldehyde - glutaraldehyde - osmium tetroxide fixation gives the same results as those seen after glutaraldehyde and osmium tetroxide.

When glutaraldehyde fixed platelets are treated with dichromate, the electron density of the VDG matrix seems to increase (fig. 7, appendix 6), but no electron dense particles are apparent. After treatment with ammoniacal silver hydroxide, platelets show electron dense particles within the matrix of the VDG, but not within alpha granules. The size and opacity of the particles makes them easily distinguished from the granular material found within the vesicular form of the VDG. The particles are difficult to find after glutaraldehyde and osmium tetroxide fixation, because the VDG is so dense; they are better seen if glutaraldehyde alone is used (figs. 8, 9, appendix 6).

Discussion

Schulz and Weddell (1962) described large and small fat droplets in platelets from patients who had hyperlipemia as a result of platelet fusion or pathological conditions. The large droplets, like those observed in the cytoplasm of platelets from persons who had thrombopathies (Jean et al. 1963b) were not membrane-bound, and so may be distinguished from VDG. The small droplets, on the other hand, closely resembled VDG morphologically. Most of the blood used in our experiments was collected in the morning. To exclude the possibility that the animals had a postprandial hyperlipemia and that VDG represent phagocytosed lipids, platelets from four rabbits starved from 18 - 24 hours were examined. These were found to contain as many VDG as those from other animals. Furthermore, platelets from rabbits made hyperlipemic as a result of eating a cholesterol fat-rich diet for many weeks, did not contain more VDG than did those from normal rabbits (Silver, 1971). . Thus, while the matrix of the VDG may contain a lipid component, to explain its dense osmiophilia, we do not believe that the VDG itself is the result of phagocytosed fat. The fact that the granules are visible in unstained preparations indicates an innate electron density. White (1969) has suggested that the inherent electron opacity could be due to a nucleation of heavy metals in the matrix.

Reserpine releases 5 HT from platelets both in vitro (Shore et al. 1956) and in vivo (Carlsson et al. 1957). Tranzer and his colleagues (1966) observed a diminution in the number of VDG and of the 5 HT content of platelets when rabbits were treated with the agent, and an increase in both the number of VDG and the 5 HT content when platelets from the reserpinized rabbits were incubated with 5 HT. As a result of these observations, Tranzer and his colleagues concluded that 5 HT was probably stored in the VDG of rabbit platelets. Telkka and co-workers (1964) also examined the fine structure of platelets from reserpinized rabbits and reported that the only structural alteration was a swelling of the platelets. They used 1.2% potassium permanganate as a fixative. In our hands this solution has proved to be a poor preservative for platelet fine structure. All organelles are partly destroyed, and VDG are not apparent. Therefore, it is probable that Telkka et al. could not detect VDG.

Ultracentrifuge studies with rabbit platelets indicate that in the bound form 5 HT is qualitatively localized in the granule fraction (Wurzel et al. 1965), Solatunturi and Paasonen (1966) subfractionated an 18,500 g sediment which contained granules and vacuoles and found that 22% of the recoverable 5 HT and 25% of the histamine were present in one of the subfractions. Morphological examination of this fraction, after fixation in glutaraldehyde and osmium tetroxide, showed that it

contained almost all of the most electron dense particles. An illustration of the granules shows that they closely resemble the VDG in intact platelets. Smaller quantities of 5 HT and of histamine were also present in other subfractions. These, with a large amount of amines in the final fraction that contained particles from all the preceding fractions, may have been due to contamination or to membrane rupture. da Prada and his colleagues (1967) have isolated dense bodies (equivalent to the VDG) by centrifugation in a continuous density gradient. The organelles, with vesicle-like structures, formed a layer which contained the greatest amount of 5 HT and of histamine. The high concentration of the latter suggested to the authors that the two amines might be localized in the same or in similar subcellular structures.

Aldehyde fixation may stabilize some component from the VDG matrix. This would explain the better preservation of the VDG following aldehyde fixation than osmium tetroxide fixation. In this respect, glutaraldehyde would seem to have a greater action than acetaldehyde - perhaps because of its two aldehyde groups. It is possible that some component of the matrix is rendered insoluble. For example, the admixture in a test tube of serotonin (6×10^{-3}M) and 5% glutaraldehyde made up in Sorensen's buffer produces a yellowish precipitate. Addition of ammoniacal silver hydroxide to the precipitate, after it has been washed thoroughly several times in buffer, changes the colour to

a darker red brown. Examination of this material in an electron microscope suggests that it is composed of granules that have the same size as those seen in our platelet micrographs (a mixture of Sorensen's buffer and silver solution produces a clear liquid). A rough brown precipitate is the final result when norepinephrine (6×10^{-3}M) is treated in this manner. It too is composed of granules having a similar size. On the other hand, when glutaraldehyde and histamine (6×10^{-3}M) are mixed, the solution remains clear and colorless. The particulate deposit obtained in electron micrographs (after silver treatment) is distinct and is consistently localized to the VDG. Therefore, we conclude that the matrix of the VDG contains an ubsubstituted amine. Rabbit platelets contain norepinephrine, histamine, and serotonin. Norepinephrine might cause the observed result but it is not certain whether the small quantity present in platelets would trigger the reaction. Our observations <u>in vitro</u> suggested histamine may not be stabilized during the aldehyde fixation. Thus this amine could be leached from the platelets during fixation and/or washing. These considerations, and those discussed above, make it highly likely that the unsubstitute amine demonstrated is 5 HT. However, we cannot state this categorically, because other amines could cause the reaction.

We did not obtain a particulate deposit over VDG when platelets were treated with dichromate but had the impression that the density of the VDG matrix increased. Wood (1965) reported a similar finding. He

presented one electron micrograph of a platelet in tissue from a cat's central nervous system that had been fixed in glutaraldehyde and treated with dichromate. The platelet contained two organelles that could be vesicular forms of VDG, although VDG did not appear to be particularly numerous in cat platelets (Stehbens and Biscoe, 1967). The matrix of both organelles was very electron opaque but no distinct particles are obvious within it.

Davis and Kay (1965) and Davis and White (1968) have attempted to localize 5 HT in rabbit platelets using tritium labelled 5 HT. Their result - the platelets were fixed in glutaraldehyde and osmium tetroxide - showed a significant concentration of radioactivity concentrated in dense, osmiophilic organelles with some radioactivity at other platelet sites. My own observations yielded comparable results, but I was less willing to accept that the radioactivity was concentrated in the VDG (Silver, 1968).

Like Tranzer et al. (1966), we found that rabbit platelets contained far more VDG than did those from other species. This may be because rabbit platelets contain much more serotonin (Humphrey and Jaques, 1954). However, VDG may not be the only site of 5 HT storage in platelets. For example, ultracentrifuge studies suggested there may be unbound 5 HT within rabbit platelets. These results must be interpreted with caution, because organelle membranes are ruptured during

such preparation. Furthermore, it seems likely that any free 5 HT within the platelet cytoplasm would be metabolized by monamine oxidase localized there (Paasonen, 1965). Baker et al. (1959) showed that granules from human platelets contained 5 HT and adenosine triphosphate.

With the aid of a histochemical fluorescence method, Tanaka et al. (1967) have demonstrated 5 HT in peripheral blood platelets and in mature bone marrow megakaryocytes of rabbits and humans. We have seen organelles like VDG in the outer third of the cytoplasm of mature megakaryocytes, a morphological finding that agrees with the histochemical observation of Tanaka et al. (1967). Possibly they are the site of 5 HT storage in the megakaryocyte. If this is so, one is faced with the problem of deciding which of the granules in less mature megakaryocytes is a precursor of the VDG. Furthermore, one must question the source of the 5 HT. Either it is brought to the megakaryocyte, or it is synthesized <u>in situ</u>. Local synthesis seems unlikely because the enzyme that decarboxylates 5 hydroxytryptophane has not been demonstrated in bone marrow cells (Clark et al., 1954; Gaddum and Giorman , 1956).

Crawford et al. (1967) studied platelets from 6 patients with the carcinoid syndrome. The platelets contained far more serotonin than did those from 35 normal subjects or those from 75 patients who did not

have the carcinoid syndrome. Platelets from 6 patients and from 3 control subjects were fixed for electron microscopy in glutaraldehyde and osmium tetroxide. The authors did not describe VDG in platelets from either group. In platelets from the patients, they noticed an increased degree of vacuolation and dilatation of both a granular "microtubule" system and of vesicles. Cytoplasmic granules and mitochondria were not changed. The authors concluded that the serotonin in human platelets, when present in high concentration and when the normal storage compartments have reached capacity, might localize within an extra granular system. White and Davis (1969) also examined the platelets from 4 patients with the carcinoid syndrome. The major anomalies observed were irregular shape, dilatation of the channel system and the presence of unusual organelles showing irregular masses of roughly parallel filaments enclosed within a membrane. Dense bodies (VDG) were increased in number over those found in normal platelets but not to a marked degree. However, it should be pointed out that the authors found far more VDG in their preparation of normal human platelets than others have (Tranzer et al, 1966; May et al, 1968). Thus there may be species difference in the localization of serotonin in platelets.

Although it could be argued that the VDG is one of the recognized cytoplasmic granules altered by preparative methods, our

observations suggest that it is a specific granule which can be distinguished from the alpha granule by its morphology and by its histochemical characteristics.

These experiments triggered a series of in vitro chemical tests to elucidate the chemical basis of the argentaffin technique in localizing 5 HT within tissues fixed by glutaraldehyde. We (Gardner et al, 1971) concluded that glutaraldehyde when mixed with the amine forms a precipitate containing a compound or compounds in which one aldehyde group of glutaraldehyde has been involved in a condensation reaction with 5 HT to form a tetrahydro-B-carboline. The other aldehyde group is available in "crypto" form as a carbinolamine or its dehydration product and is readily released under the subsequent reaction conditions when ammoniacal silver hydroxide is added. Thus, metallic silver is precipitated at the site of 5 HT storage in the tissues. This explanation of the reaction varies from that offered by Tramezzani et al (1964).

The earliest observations on the fine structure of sectioned rabbit platelets were made on material fixed in osmium tetroxide solutions. Subsequently the morphology of platelets doubly fixed in glutaraldehyde and osmium tetroxide was described. Other aldehydes have been used as platelet prefixatives (White and Krivit, 1965; Rodman, 1967; Nathaniel and Chandler, 1968), but the reports give inadequate details of the resultant platelet morphology. Therefore experiments were done to establish the fine structure of rabbit platelets prefixed in acetaldehyde or in paraformaldehyde and to compare the findings with those of platelets prefixed in glutaraldehyde. Osmium tetroxide postfixation was used in all experiments.

EXPERIMENT III

Variations in rabbit platelet morphology induced by different aldehyde prefixatives.

Materials and Methods

Using techniques described in Experiment II platelet-rich plasma was obtained from the heart blood of healthy rabbits. Aliquots of the plasma were diluted in 3 - 4 times their volume of an aldehyde solution and prefixed for varying intervals. Tables I in Appendices 7 and 8 provide details. In the experiments involving acetaldehyde

prefixation, both the temperature and the duration of fixation was varied - see Table I, appendix 7 -, not only to establish the optimum time of fixation, but also to divorce the changes produced by the low temperature from those due to the fixative itself. Some of the platelets prefixed in acetaldehyde were not postfixed; others were postfixed in Caulfield's (1957) osmium solution. Still others were postfixed in 5% glutaraldehyde in M/15 Sorensen's phosphate buffer (pH 7.4 603 mM) and, after a wash in phosphate buffer, incorporated in osmium solution. Where other aldehydes were used as prefixatives, platelet pellets were prepared by centrifugation (2000 g for 10 minutes), washed 15 minutes in phosphate buffer and postfixed for 1 hour in Caulfield's osmium tetroxide solution. A further 15 minute phosphate buffer wash followed postfixation. Then the platelets were dehydrated, embedded and sectioned for electron microscopy as outlined in Experiment II.

Observations

The morphology of rabbit platelets doubly fixed in glutaraldehyde and osmium tetroxide has been described (experiment IIa). A pictorial concept of a sectioned platelet doubly fixed in this manner is shown in Fig.3, appendix 8.

The platelet pellet obtained after fixation in acetaldehyde is soft and sticky. Ultrathin sections are very difficult to cut and are of

poor quality. Postfixation in osmium tetroxide makes the pellets firm and easily handled.

When acetaldehyde alone is used to fix the platelets, membranes are poorly preserved, so that the cell and its organelles seem to be defined by granular margins. Cytoplasmic organelles are not well delineated except for the very dense granules which appear shrunken (fig. 2, appendix 7). With osmium tetroxide postfixation, platelet preservation is considerably improved. The results are equally good whether acetaldehyde fixation proceeds for 30 minutes or 2 hours. We found 1 hour fixation convenient.

When doubly fixed in acetaldehyde/osmium tetroxide, most platelets become rounded although a few remain lentiform (fig. 3, appendix 7). The diameter or length is from 3 - 5 mμ. The external platelet coat is not seen as clearly as it is following glutaraldehyde/ osmium tetroxide fixation. Membranes have a normal morphology and are very well delineated, especially those of mitochondria, whose cristae are excellently preserved (fig. 4, appendix 7). Cytoplasmic organelles are also well preserved. Their size and shape is comparable to that obtained with glutaraldehyde/osmium tetroxide fixation. However some morphological differences are seen. For example, areas of increased density within the matrix of the alpha granules (nucleoids) are not as obvious. Glycogen granules are preserved but very dense granules are not. Cytoplasmic microtubules are not seen - even if the platelets

are rewarmed to 37°C for one hour before postfixation. However, fine delicate filaments 79 - 106 Å wide are found just deep to the plasma membrane and aligned parallel to it (fig.5, appendix 7). The most striking morphological alteration is an increase in the number and size of the cytoplasmic vacuoles. These form prominent, wide channels that course through the platelet cytoplasm. The plasma membrane is frequently continuous with the membrane delineating the vacuoles. Occasionally, grey amorphous material similar to that seen on the external surface of the platelets is apparent adherent to the inner wall of the vacuole. The increased prominence of the vacuolar system is observed irrespective of the duration or temperature of acetaldehyde fixation. A pictorial concept of the findings when platelets are doubly fixed in this manner is shown in Fig. 5, appendix 8.

Platelets sectioned following prefixation in paraformaldehyde are swollen and have a rounded contour. Any that are oval are wider and shorter than those seen after glutaraldehyde prefixation. Relatively little external platelet coat is preserved and the plasma membrane outline is often blurred and may show discontinuities (fig.6, appendix 8). The cytoplasmic matrix is less electron dense than that obtained by glutaraldehyde or acetaldehyde prefixation and is comparable to that seen when osmium tetroxide is the only fixative. Generally, organelles

are dispersed throughout the cytoplasm but occasionally they come towards the centre of the sectioned platelet as occurs after osmium tetroxide fixation (David-Ferreira, 1964). The overall number of organelles per section is reduced because very dense granules (Silver and Gardner, 1968) are infrequent; those present have a small dense-staining matrix within their vacuoles. Glycogen granules are also reduced but other organelles are preserved in their usual number. The morphology of alpha granules is like that seen after glutaraldehyde prefixation, but their matrix shows a much more variable electron density. Microtubules are not observed but microfibrillae are sometimes present in the cytoplasm. The number of cytoplasmic vacuoles both of the surface connecting system and dense tubular system (Behnke, 1967b) is comparable to that seen after glutaraldehyde prefixation. However, vacuoles that are continuous with the surrounding environment at the platelet surface are more common. A pictorial concept of these findings is shown in Fig.7, appendix 8.

Discussion

Acetaldehyde is very unstable (boiling at 20°C) and its vapour is extremely pungent. It is easily oxidized to acetic acid and reduced to ethanol. Thus stock solutions must be stored in a freezer. Furthermore, acetaldehyde,although readily soluble in many solvents, evolves

considerable heat of solution. Therefore, to prevent its loss, pre-cooled glassware and solutions must be used.

The soft sticky consistency of the pellets after fixation solely in acetaldehyde, and the poorly preserved morphology of those platelets, suggests that acetaldehyde is a poor tissue fixative. Further evidence for this was provided by the following observation. By accident, hypotonic Sorensen's solution (160 mM) was used for washing in one experiment. Most of the acetaldehyde fixed platelets disappeared during the wash; those salvaged showed marked ballooning and distortion. Platelets fixed in glutaraldehyde were not affected by this wash. Thus, if platelets are to be fixed in acetaldehyde they must be handled with care and hypotonic solutions must be avoided.

The low freezing point (less than -15°C) of the acetaldehyde solution should permit platelet chilling without formation of ice crystal artifacts. In these studies, ice crystals did not form, even if platelets were placed directly into the fixative at -15°C. However, a preliminary chilling at -0°C which allows acetaldehyde diffusion of the platelets before they are subjected to a lower temperature, seems preferable. The ultimate effect of subzero fixation is to halt internal metabolic reaction, thus placing platelets in a state of "suspended animation". The excellent preservation of mitochondria, the best that we have yet seen, suggests that this might have been accomplished.

The changes in platelet morphology apparent after acetaldehyde fixation principally affect microtubules, platelet shape, and the intercytoplasmic vacuolar system. Platelet microtubules are thermolabile structures (Behnke, 1967c; White and Krivit, 1967). Behnke (1967c) has shown that microtubules disappear after exposure to cold and reappear when chilled platelets are rewarmed. However, their ability to reform is dependent upon the duration of chilling. Platelets maintained at 4°C for 24 hours had virtually no microtubular reformation, even when warmed to 37°C for two hours.

Because platelet microtubules are best preserved by glutaraldehyde (Silver, 1965; Behnke, 1967c), we employed glutaraldehyde postfixation in some experiments. In no instances were microtubules seen, even when platelets were rewarmed after initial acetaldehyde fixation. The absence of microtubules is probably due to chilling of platelets during weak fixation by acetaldehyde. The failure of reappearance of microtubules when platelets were rewarmed may be due to the extreme low temperature (-15°C) or, alternatively to the action of acetaldehyde itself. We believe it is essentially due to the temperature of fixation. Microtubules are thought to provide a "skeletal system" maintaining the platelet in lentiform shape (Silver, 1965; Behnke, 1965; Sixma and Molenaar, 1966). For example Behnke (1967c) and White and Krivit (1967) showed that platelets become

rounded when chilled; an alteration they relate to the disappearance of cytoplasmic microtubules. Our observations confirm this association.

The loss of microtubules may also account for changes in the vacuolar system. The affected vacuoles are undoubtedly equivalent to the surface connecting system described in rat platelets by Behnke (1967b). In natural circumstances they may be maintained in a semi-collapsed state, possibly by the action of the microtubular "skeleton". Loss of the cytoskeleton would cause the vacuoles to expand and become more prominent. A consequence would be an alteration of platelet shape. Some acetaldehyde fixed platelets have such an exaggerated surface connecting system that they appeared to have "exploded" (fig.6, appendix 7). The vacuolar system is much less prominent following glutaraldehyde fixation. This could be related to the ability of glutaraldehyde to preserve microtubules. In addition, glutaraldehyde may stabilize membranes better than acetaldehyde and render them more resistant to alterations when microtubules disappear. Acetaldehyde, a weaker fixative, may not be able to prevent this change.

We have not seen filaments in the cytoplasm of rabbit platelets fixed in glutaraldehyde followed by osmium tetroxide unless the platelets were chilled to 0°C for 30 minutes before fixation. These structures

are much more obvious after acetaldehyde/osmium tetroxide fixation. We think they are comparable to the microfilaments (Zucker-Franklin and Nachman, 1967; White, 1967) or microfibrillae (Bessis and Breton-Gorius, 1965; Sixma and Molenaar, 1966) described by others in the cytoplasm of platelets. In our preparations the microfilaments lay close to the position normally taken by microtubules. White (1967) stated that microfilaments are associated with microtubules. Perhaps chilling destroys the platelets during acetaldehyde prefixation, depolymerizes the microtubules and, as they disappear their associated microfilaments are revealed. It should be mentioned however that neither Behnke (1967c) nor White and Krivit (1967) observed these structures in their experiments on cooled platelets.

The absence of very dense granules after acetaldehyde/osmium tetroxide fixation may be another manifestation of acetaldehyde's weak fixative action. These granules are preserved in acetaldehyde alone (fig.2, appendix 7) and when both glutaraldehyde and osmium tetroxide follow acetaldehyde fixation (fig.6, appendix 7). They are not present when platelets are fixed solely in osmium tetroxide (Tranzer et al, 1966). It is probable therefore that acetaldehyde as contrasted with glutaraldehyde cannot sufficiently stabilize the dense granules to withstand processing through osmium tetroxide. Further studies are necessary to determine the fate of serotonin during the action of these

different fixatives.

These investigations indicate that platelet fixation in acetaldehyde causes changes in platelet morphology although a good preservation of platelet ultrastructure may be achieved. However in view of the excellent results obtained by glutaraldehyde/osmium tetroxide fixation, the use of acetaldehyde is not recommended.

Nathaniel and Chandler (1968) used cold buffered 10% paraformaldehyde as a primary fixative for the study of platelet aggregates induced in the rat by adenosine diphosphate. The authors did not define the effect of the aldehyde on the morphology of normal platelets, but their description of the aggregated platelets varies only slightly from that given here. Since aggregation induced by adenosine diphosphate causes little discernible alteration in platelet structure other than pseudopod formation (Ashford and Frieman, 1967; Jørgesen et al., 1967b) their findings are probably comparable to ours. Rodman's (1967) only comment on the morphology of human platelets fixed in 1% buffered paraformaldehyde was that "microtubules were poorly preserved ..... they have an appearance similar to microtubules in platelets fixed primarily with osmium tetroxide". In our experiments, no microtubules were seen in the platelets. Nathaniel and Chandler (1968) did not mention the presence or absence of microtubules and none were apparent in their illustrations. If the structures (Rodman, 1967) described as

microtubules in paraformaldehyde-fixed platelets are the same as those labelled in the first figure of his paper (where the platelet was fixed in osmium tetroxide), we doubt that they were preserved at all. In our experiments, paraformaldehyde prefixation was done at room temperature. Thus, the microtubules were not depolymerized by cold. Rather we postulate that paraformaldehyde either destroys the microtubules or, more likely, does not stabilize some microtubule components for subsequent passage through osmium tetroxide. For example, Roozemond (1969) found that the amount of phospholipid extractable from rats' hypothalamus was greater and the type of lipid extracted differed when the brain was fixed in formaldehyde rather than in glutaraldehyde/formaldehyde mixture. Possibly a comparable mechanism plays a part in destroying microtubules when platelets are prefixed in acetaldehyde. Again, a variation in the extraction of platelet components during fixation may cause the difference in matrix density observed after prefixation with each of the aldehydes. The cause of the diminished numbers of very dense granules seen after prefixation with either acetaldehyde or paraformaldehyde is not certain. Possibly the granule's matrix is not stabilized by the aldehyde. Alternatively, the very dense granules may discharge their contents to the external environment. Such a mechanism could explain the great number of vacuoles found opening on to the platelet surface. In our

opinion, paraformaldehyde/osmium tetroxide fixation results in no better preservation of platelet fine structure than that obtained with osmium tetroxide if used alone. Platelet morphology does not vary if the concentration of the paraformaldehyde is either 4% or 10%.

White and Krivit (1965) used varying concentrations of glutaraldehyde, phosphate and hydroxyadipaldehyde as prefixatives in the study of the fine structural localization of adenosine triphosphatases in platelets and blood cells. The authors did not elaborate on the morphological changes induced by the prefixatives. The sixth and seventh illustrations in their paper showed platelets prefixed in hydroxyadipaldehyde. Even though the platelets had been incubated in the modified Wachstein Meisel medium before fixation, they are poorly preserved.

Of the methods of platelet fixation for electron microscopy that we have used, prefixation in glutaraldehyde and post fixation in osmium tetroxide preserved platelet ultrastructure best. If one may extrapolate from the results of White and Krivit (1965) hydroxyadipaldehyde is not a good fixative. Thus, till better fixatives are available, glutaraldehyde/osmium tetroxide fixation should be mandatory in any study planned to define changes in platelet ultrastructure. If other fixatives are employed, their effects on the morphology of normal platelets must be clearly defined to prevent confusion between changes

induced by the fixative and those induced by the fixative and those induced by the experiment.

The method of platelet collection outlined above is convenient. The presence, in the final result, of occasional platelets showing pseudopod formation, indicates that it may be less than ideal. Platelet aggregation could be induced during preparation of platelet rich plasma or by the use of glutaraldehyde. Skjørten (1968) observed more pseudopod formation in platelets obtained from platelet rich plasma prepared at 37°C and then fixed in glutaraldehyde than in those obtained from native blood fixed instantly in an aldehyde. Thus, use of an anticoagulant/glutaraldehyde mixture such as the one introduced by Behnke (1965) may be the best method of collecting platelets for fine structural studies.

We found no great difference in platelet morphology whether 2.5% or 5% glutaraldehyde was used. Nor did a period of platelet fixation between 15 and 60 minutes alter the result. Occasionally, when citrated platelet-rich plasma and glutaraldehyde were mixed, the mixture clotted. This occurred less frequently when platelet-rich plasma was added to glutaraldehyde than vice versa. Apparently, a rapid dilution or inactivation of some factor that activates the clotting mechanism must take place. In other experiments, where platelets from patients with lymphomata were studied, the only way found to prevent

clotting was to prepare platelet pellets and remove almost all of the supernatant citrated plasma before adding glutaraldehyde. It is of interest that Anderson (1965) relied on glutaraldehyde to solidify plasma in his method of preparing peripheral leukocytes for electron microscopy.

CONCLUSIONS

The experimental observations outlined above were made during a 5 year period that saw an almost explosive increase in our knowledge of platelet behaviour, morphology, biochemistry and function. They helped clarify our concepts of rabbit platelet behaviour *in vivo* and of rabbit platelet ultrastructure, in particular of cytoplasmic microtubules and very dense granules. The use of different aldehyde prefixatives indicated the best current method of preserving platelet ultrastructure and provided baseline descriptions of platelet ultrastructure when acetaldehyde or paraformaldehyde prefixation is used. The results of the third experiment suggest that acetaldehyde could be a useful prefixative when localizing platelet enzymes histochemically at the ultrastructural level.

Although rabbit platelets were studied the experiments have, by analogy, widened our knowledge of human platelet behaviour and fine structural morphology. The further application of this knowledge will permit a better understanding of the platelet both in normal and pathological conditions, and lead eventually to the goal in medicine, that is, the use of appropriate measures to prevent disease.

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The Very Dense Granule in Rabbit Platelets

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The morphology of the very dense granule in rabbit platelets is defined after fixation in glutaraldehyde, and acetaldehyde with or without OsO_4 postfixation. The granules are best preserved by glutaraldehyde and OsO_4. An unsubstituted amine has been localized within the granule by histochemical methods. Evidence is presented to support the viewpoint that the amine is serotonin.

When platelets from several mammalian species are fixed for electron microscopy glutaraldehyde, or in glutaraldehyde followed by osmium tetroxide solution (OsO they contain a very dense cytoplasmic granule (VDG) which differs morphologica from other granules. Recent studies indicate that 5-hydroxytryptamine (5HT) n be localized in the VDG of rabbit platelets (*24*). We have studied these granules rabbit platelets prepared for electron microscopy in a variety of fixatives and ha attempted, by histochemical methods, to localize amines in them.

MATERIALS AND METHODS

Siliconized hypodermic needles and glassware were used for all procedures. Heart blo was obtained from rabbits anesthetized with sodium Nembutal. In some instances it collected without an anticoagulant; in others 9 volumes of blood were mixed with 1 volu of an anticoagulant—either 3.8% trisodium citrate or a 2% solution of disodium diami ethanetetraacetic acid containing 5% dextran (Glaxo). Samples of platelet-rich plasma se rated by centrifugation (500 *g* for 5 minutes) were placed in an equal volume of fixat Table 1, read from left to right, indicates the fixatives or sequence of fixatives used for gro of experiments. The plasma—fixative mixture was centrifuged (2000 *g* for 10 minutes) obtain a platelet pellet. If more than one fixative was used, the pellets were washed in Sor sen's phosphate buffer before they were placed in the next fixative. After fixation, they w washed and dehydrated in graded ethanol. They were then transferred through mixtures propylene oxide which contained increasing amounts of embedding material and embed in an Epon-Araldite mixture. Sections were cut on an LKB ultramicrotome equipped w glass knives. They were stained with a saturated solution of uranyl acetate in 50% etha with lead solution (*11*, *14*) or with both heavy metals. Other sections were not stained. were studied with a Philips 200 electron microscope at 60 kV or with an RCA 3F mach at 50 kV.

TABLE I

FIXATIVES EMPLOYED IN EACH SERIES OF EXPERIMENTS

Fixative	Acetaldehyde, 10 % in Sorensen phosphate buffer containing 5.2 g % sucrose	Glutaraldehyde, 5 % in Sorensen phosphate buffer	Osmium tetroxide solution, 1 %, containing sucrose [Caulfield (*4*)]
pH	7.4	7.4	7.4
Osmolarity (mosmoles)	Not measured[a]	603	275
Duration of fixation	1 hour	1 hour	1 hour
Temperature of fixative	- 15°C	Room temperature	4°C
Series 1	+		
2	+	+	+
3	+		+
4		+	
5		+	+
6			+

[a] The extremely low temperature required to keep acetaldehyde in solution interfered with our measurements. Probably the solution was hypertonic.

Some amines, for example, 5HT (*26*) and norepinephrine (*23*), when mixed with glutaraldehyde form a product that can reduce a metallic solution to form an electron opaque material. It is thought that the reaction involves the formation of a Schiff base and that a condensation product reduces the metallic solution (*23*, *26*). The reactions formed the basis of attempts to localize amines in platelets. All platelets subject to histochemical study (4 series of experiments) were fixed for an hour in 5% glutaraldehyde. They were then divided into two groups. One was exposed to ammoniacal silver hydroxide according to the method of Tramezzani *et al.* (*23*) and the other was incubated overnight in a potassium dichromate solution using the technique described by Wood (*26*). Platelets exposed to ammoniacal silver hydroxide were subsequently washed in a 1% solution of sodium thiosulfate to avoid nonspecific silver precipitation. Some platelets from both groups were postfixed for an hour in osmium tetroxide solution. All were dehydrated and prepared for electron microscopy in the manner outlined above. Stained and unstained sections from these preparations were examined.

OBSERVATIONS

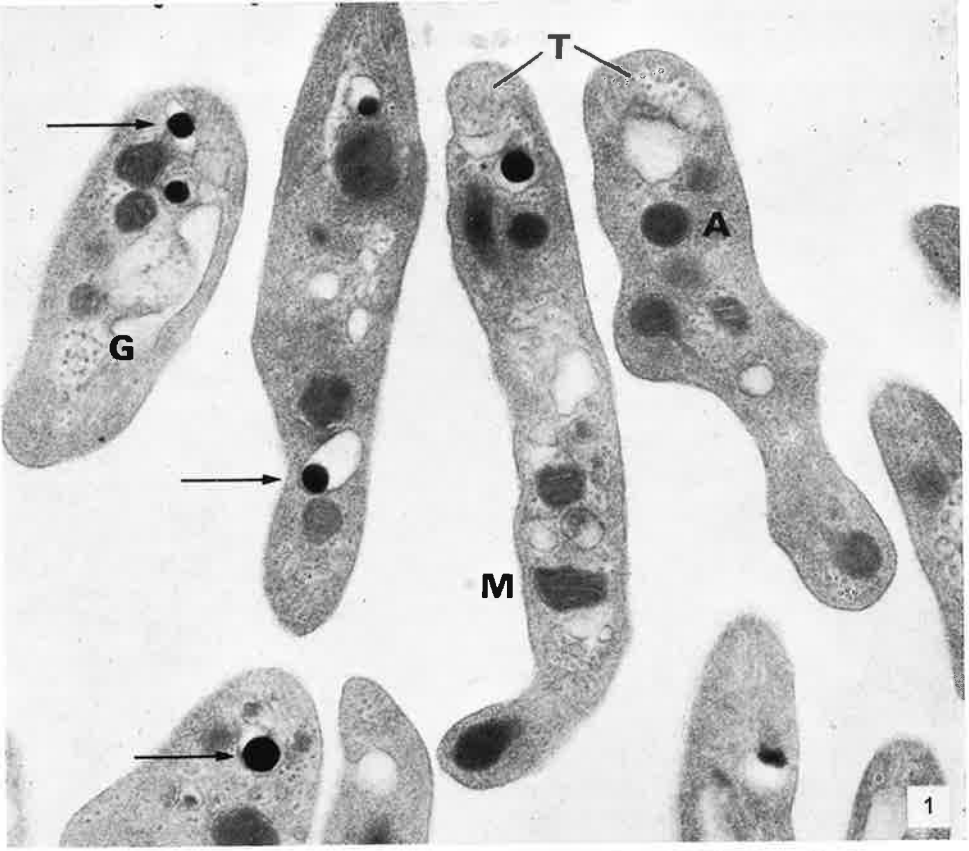
Many granules may be seen in the rabbit platelet cytoplasm after fixation in glutaraldehyde with or without postfixation in OsO_4 (Fig. 1). As in other species, (*6*, *15*) round to oval alpha granules (alpha granulomere, specific, azurophilic, or dense granules) 0.2–0.3 μ in diameter are the most numerous. The matrix of the alpha granules is granular and moderately electron dense. It may contain oval or rounded areas of increased density, usually located eccentrically in the granule. Generally, the matrix is separated from the organelle's unit membrane by a narrow halo-like electron translucent zone (Fig. 2). The small surface nodules that have been described on the granule (*6*) were not apparent in our preparations. Elongated "drumstick" granules,

with a matrix like that of the alpha granule, are occasionally present but are never numerous. Several mitochondria (beta granulomere) are usually found in each section. Ribosomes and groups of glycogen granules which together form the epsilon granulomere are also present. Vesicular structures which have a clear interior with dense granules the size of ferritin particles lining their membrane (delta granulomere) are most uncommon. We found that they were best seen when OsO_4 is used alone for fixation.

In addition to all these, very dense granules (VDG) are also present in platelets fixed in glutaraldehyde $\pm OsO_4$. Two or three examples are seen in each section (Fig. 1). Generally, these granules are smaller than alpha granules but may be of comparable size (0.25–0.5 μ in diameter). They are defined by a unit membrane (Fig. 2) and they have a homogeneous matrix which is extremely electron dense, much denser than any part of an alpha granule. The matrix may extend to the organelle's limiting membrane, but much more frequently it forms a rounded or oval body which is eccentrically placed in the vesicle (vesicular form) and is surrounded by an electron translucent zone in which granular material may be scattered or may line the vesicular membrane. The VDG occur at random throughout the cytoplasm. Their morphology is not altered by the anticoagulants used. They can be distinguished in unstained sections and in platelets collected from rabbits that have not been anesthetized. We have seen them in platelets embedded in water-soluble material (Durcupan). They occur in both isolated platelets and in those preserved *in vivo* in bone marrow. Exactly similar structures are seen occasionally in magakaryocytes in the outer third of the cytoplasm of mature cells (Fig. 3). We have observed far more VDG in rabbit platelets than in those from humans, guinea pigs, or mice fixed in glutaraldehyde with or without OsO_4.

When platelets are fixed in glutaraldehyde alone (Fig. 4), the opaque body within the vesicle of the VDG is smaller than when both glutaraldehyde and OsO_4 are used. It has a uniform density in most cases but may be granular. If so, the matrix has a greater density than that of an alpha granule. However the difference is not enough to distinguish an alpha granule from a VDG positively, unless the VDG matrix is eccentrically placed. Acetaldehyde fixation causes a marked change in platelet shape and morphology (*8*). Only the vesicular forms of VDG can be identified after such fixation, and these are not frequent. Often the body in the vesicle is small (Fig. 6). As all granules have an increased density after acetaldehyde OsO_4 fixation it is almost im-

FIG. 1. Rabbit platelets fixed in glutaraldehyde and OsO_4 showing alpha granules (*A*), mitochondria (*M*), glycogen granules (*G*) and VDG (arrows). Microtubules (*T*) are seen in some platelets. × 25,000.
FIG. 2. Electron translucent halo surrounding matrix of an alpha granule (*A*) between it and the membrane. Trilaminar membrane of VDG (arrow). Platelet fixed in glutaraldehyde and OsO_4. × 116,000.


T
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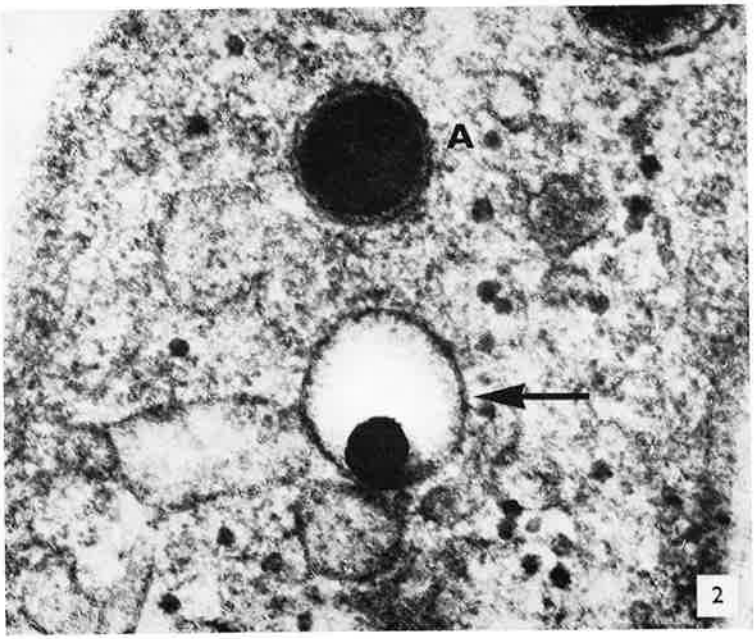
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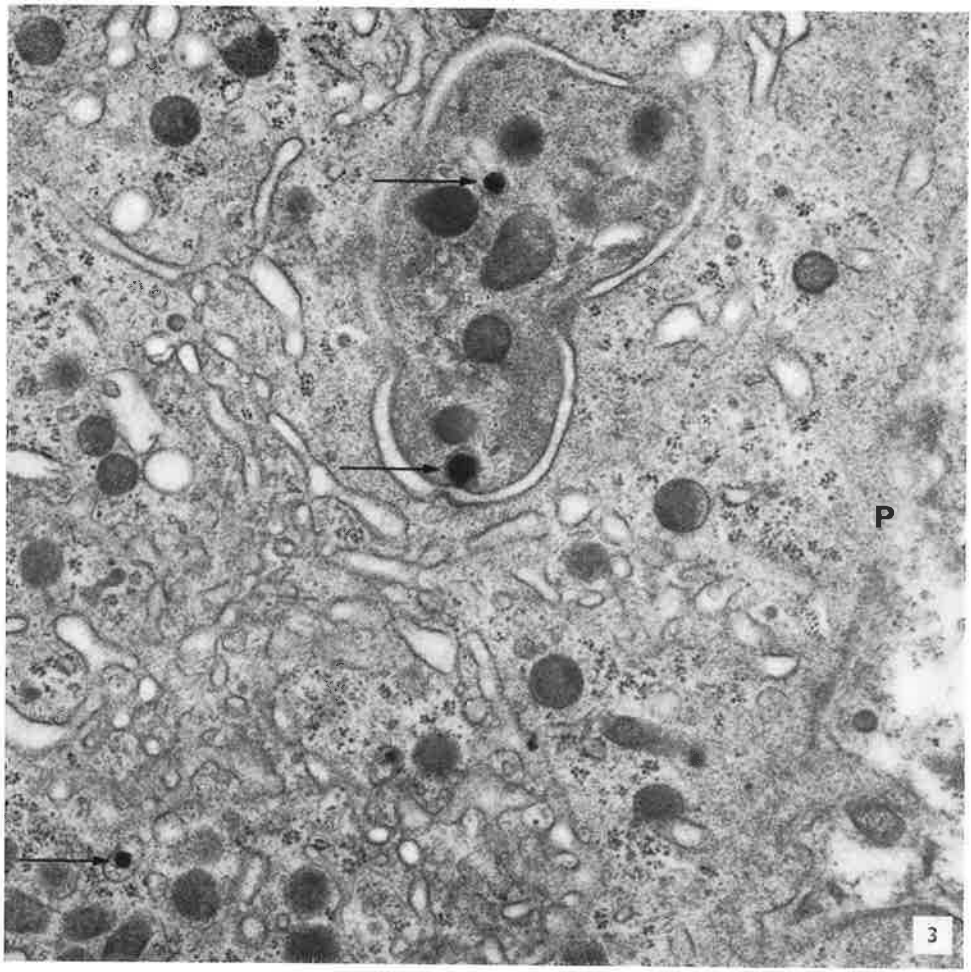


FIG. 3. Outer part of mature megakaryocyte defined by plasma membrane (*P*). VDG (arrows) may be seen in cytoplasm. Glutaraldehyde and OsO_4 fixation. ×38,000.

possible to distinguish between alpha and VDG granules. (Fig. 5.) Acetaldehyde–glutaraldehyde–OsO_4 fixation gives the same results as those seen after glutaraldehyde and OsO_4.

When glutaraldehyde-fixed platelets are treated with dichromate the electron density of the VDG matrix seems to increase (Fig. 7), but no electron dense particles are apparent. After treatment with ammoniacal silver hydroxide, platelets show electron dense particles within the matrix of the VDG, but not within alpha granules. The

size and opacity of the particles makes them easy to distinguish from the granular material found within the vesicular form of VDG. The particles are difficult to define after glutaraldehyde and OsO_4 fixation because the VDG is so dense; they are better seen if glutaraldehyde alone is used (Figs. 8 and 9).

DISCUSSION

Schulz and Wedell (*16*) described large and small fat droplets in platelets from patients who had hyperlipemia as a result of fat infusion or pathological conditions. The large droplets, like those observed in the cytoplasm of platelets from persons who had thrombopathies (*10*) were not membrane bound and so may be distinguished from VDG. The small droplets, on the other hand, closely resemble VDG morphologically. Most of the blood used in our experiments was collected in the morning. To exclude the possibility that the animals may have had a postprandial hyperlipemia and that VDG represent phagocytosed lipid, platelets from 4 rabbits starved for 18–24 hours were examined. These were found to contain as many VDG as those from the other animals. Furthermore platelets from rabbits made hyperlipemic as a result of eating a cholesterol-rich diet for many weeks do not contain more VDG than do those from normal rabbits (*18*). Thus while the matrix of the VDG may contain a lipid component, to explain its dense osmophilia, we do not believe that the VDG itself is the result of the phagocytosis of fat.

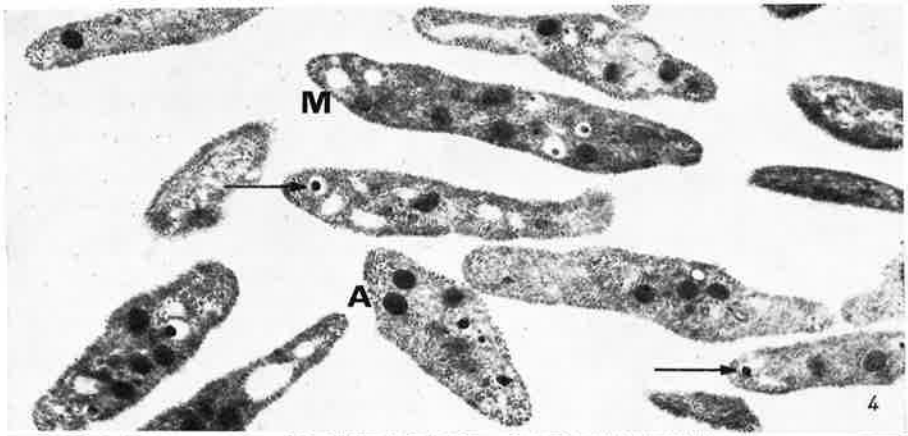
Reserpine releases 5HT from platelets both *in vitro* (*17*) and *in vivo* (*3*). Tranzer *et al.* (*24*) observed a diminution in the number of VDG and of the 5HT content of the platelets when rabbits were treated with this agent, and an increase in both the number of VDG and of 5HT content when platelets from reserpinized rabbits were incubated with 5HT. As a result of these observations Tranzer and his colleagues concluded that 5HT was probably stored in the VDG of rabbit platelets. Telkkä *et al.* (*22*) also examined the fine structure of platelets from reserpinized rabbits and reported that the only structural alteration was a swelling of the platelets. They used 1.2% potassium permanganate as a fixative. In our hands this solution has proved to be a poor preservative of platelet fine structure. All organelles are partially destroyed, and VDG are not apparent. Therefore, it is probable that Telkkä *et al.* could not detect VDG.

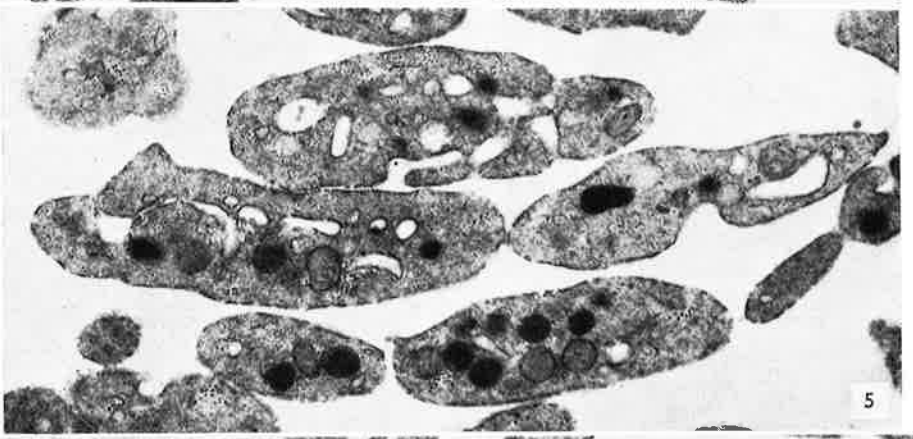
Ultracentrifuge studies with rabbit platelets indicate that in the bound form 5HT is qualitatively localized in the granule fraction (*27*). Solatunturi and Paasonen (*19*) subfractionated an 18,500 *g* sediment which contained granules and vacuoles, and found that 22% of the recoverable 5HT and 25% of the histamine was present in one of the subfractions. Morphological examination of this fraction, after fixation in glutaraldehyde and OsO_4, showed that it contained almost all of the most electron

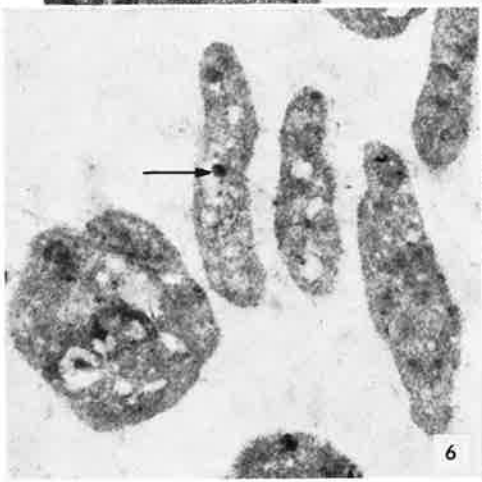
dense particles. An illlustration of the granules shows that they closely resemble the VDG in intact platelets. Smaller quantities of 5HT and of histamine were also present in other subfractions. These, with a larger amount of amines in a final fraction that contained particles from all of the preceding fractions may have been due to contamination or to membrane rupture. da Prada *et al.* (*13*) have isolated dense bodies (equivalent to VDG) by centrifugation in a continuous density gradient. The organelles, with vesicle-like structures, formed a layer which contained the greatest amount of 5HT and of histamine. The high concentration of the latter suggested to the authors that the two amines might be localized in the same or in similar subcellular structures.

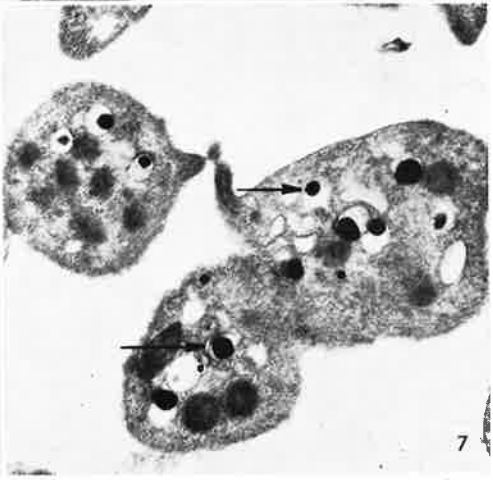
Aldehyde fixation may stabilize some component of the VDG matrix. This would explain the better preservation of the VDG following aldehyde fixation than osmium tetroxide fixation. In this respect glutaraldehyde would seem to have a greater action than acetaldehyde—perhaps because of its two aldehyde groups. It is possible that some component of the matrix is rendered insoluble. For example, the admixture in a test tube of seroronin ($6 \times 10^{-3} M$) and 5% glutaraldehyde made up in Sorensen's buffer produces a yellowish precipitate. Addition of ammoniacal silver hydroxide to the precipitate, after it has been washed thoroughly several times in buffer, changes the color to a darker red brown. Examination of this material in an electron microscope shows that it is composed of granules that have the same size as those seen in our platelet micrographs (a mixture of Sorensen's buffer and silver solution produces a clear liquid). A rust brown precipitate is the final result when norepinephrine ($6 \times 10^{-3} M$) is treated in this manner. It too is composed of granules having a similar size. On the other hand, when glutaraldehyde and histamine ($6 \times 10^{-3} M$) are mixed the solution remains clear and colorless. The particulate deposit obtained in electron micrographs of platelets after silver treatment is distinct and is consistently localized to the VDG. Therefore, we conclude that the matrix of the VDG contains an unsubstituted amine. Rabbit platelets contain norepinephrine, histamine, and serotonin. Norepinephrine might cause the observed result, but it is not certain whether the small quantity present in platelets would trigger the reaction. Our observations *in vitro* suggest that histamine may not be stabilized during aldehyde fixation. Thus this amine could be leached from the platelets during fixation and/or washing. These considera-

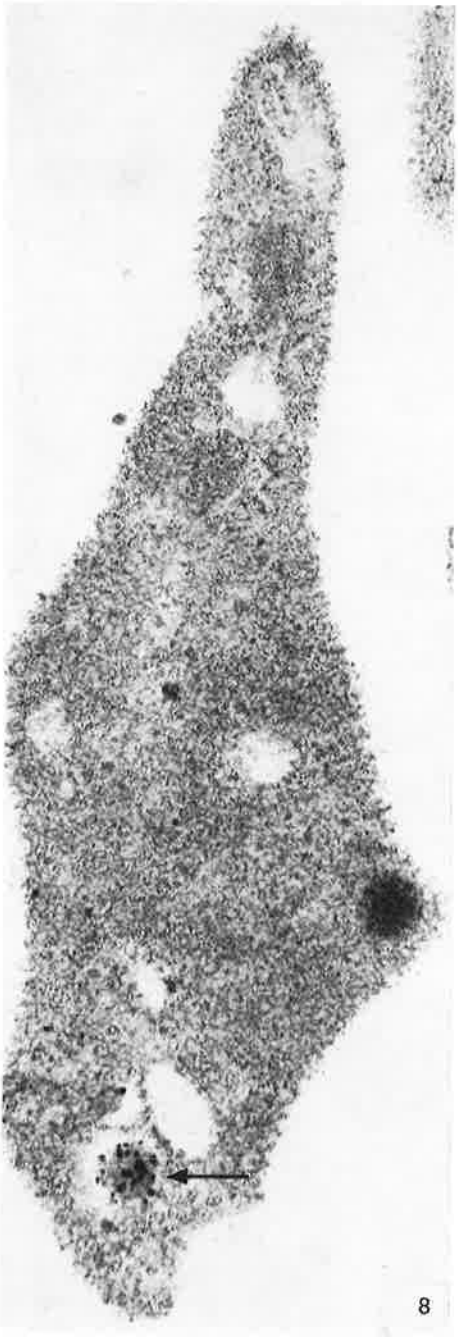
FIG. 4. Platelets fixed in glutaraldehyde. Membranes not well preserved. VDG (arrows) small but may be distinguished from alpha granules (*A*) and mitochondria (*M*). × 13,000.
FIG. 5. Platelets after fixation in acetaldehyde and OsO_4. VDG cannot be distinguished with certainty. Microtubules not apparent, probably because of low temperature of fixation (*2*). × 13,000.
FIG. 6. Poor platelet preservation after fixation in acetaldehyde. One VDG (arrow) can be seen. × 13,000.
FIG. 7. VDG (arrows) after treatment with dichromate. Glutaraldehyde and OsO_4 fixation. × 13,000.

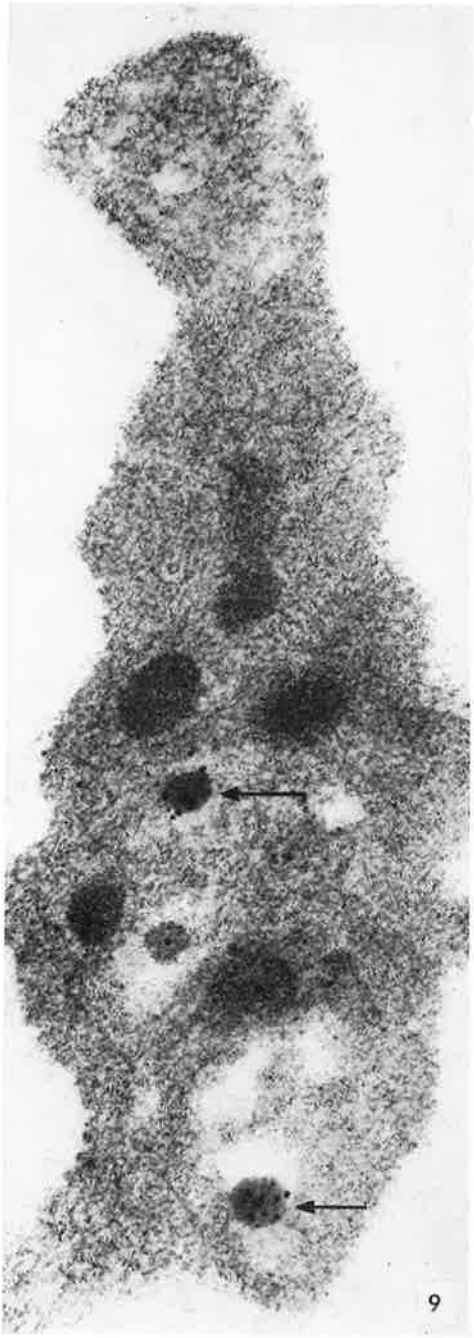

M
A
4


5


6


7





FIGS. 8 and 9. Deposits of electron dense material within VDG (arrows) after treatment with ammoniacal silver hydroxide. Glutaraldehyde fixation. × 52,000.

tions and those discussed above make it highly likely that the unsubstituted amine demonstrated is 5HT. However, we cannot state this categorically, because other amines could cause the reaction.

We did not obtain a particulate deposit over VDG when platelets were treated with dichromate but had the impression that the density of the VDG matrix increased. Wood (*25*) reported a similar finding. He presented one electron micrograph of a platelet in tissue from a cat's central nervous system that had been fixed in glutaraldehyde and treated with dichromate. The platelet contained two organelles that could be vesicular forms of VDG, although VDG do not appear to be particularly numerous in cat platelets (*20*). The matrix of both organelles was very electron opaque but no distinct particles are obvious within it.

Davis and Kay (*7*) attempted to localize 5HT in rabbit platelets using tritiated 5HT. Their results—the platelets were fixed in glutaraldehyde and OsO_4—showed a significant concentration of radioactivity diffusely over the platelet, but little selective localization over granules.

Like Tranzer *et al.* (*24*), we found that rabbit platelets contain far more VDG than do those from some other species. This may be because rabbit platelets contain much more serotonin (*9*). However, VDG may not be the only site of 5HT storage in platelets. For example, ultracentrifuge studies suggest that there may be unbound 5HT within rabbit platelets. These results must be interpreted with caution, because organelle membranes are ruptured during such preparation. Furthermore, it seems likely that any free 5HT within the platelet cytoplasm would be metabolized by monoamine oxidase localized there (*12*). Baker *et al.* (*1*) showed that granules from human platelets contained 5HT and adenosine triphosphate. Crawford *et al.* (*5*) studied patelets from 6 patients with the carcinoid syndrome. The platelets contained far more serotonin than did those from 35 normal subjects or those from 75 patients who did not have the carcinoid syndrome. Platelets from the 6 patients and from 3 control subjects were fixed for electron microscopy in glutaraldehyde and OsO_4. The authors did not describe VDG in platelets from either group. In platelets from the patients, they noticed an increased degree of vacuolation and a dilatation of both a granular "microtubule" system and of vesicles. Cytoplasmic granules and mitochondria were not changed. The authors concluded that serotonin in human platelets when present in high concentration, and when the normal storage compartments have reached capacity, might be localized within an extra granular system. Thus there may be species differences in the localization of 5HT within platelets.

Recently, with the aid of a histochemical fluorescence method, Tanaka *et al.* (*21*) demonstrated 5HT in peripheral blood platelets and in mature bone marrow megakaryocytes of rabbits and humans. We have seen organelles like VDG in the outer third of the cytoplasm of mature megakaryocytes. Possibly they are the site of 5HT

storage in the megakaryocyte. If this is so one is faced with the problem of deciding which of the granules in less mature megakaryocytes is a precursor of the VDG. Furthermore one must question the source of the 5HT. Either it is brought to the megakaryocytes, or it is synthesized *in situ*. At the moment a local synthesis seems unlikely because the enzyme said to decarboxylate 5-hydroxytryptophan has not been demonstrated in bone marrow cells.

Although the VDG could be one of the recognized cytoplasmic granules altered by preparative methods, we favor the view that it represents a specific species of granule which can be distinguished from the alpha granule by its morphology and by its histochemical characteristics.

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