J. Physiol. (1976), **257**, *pp.* 495–502
With 2 plates and 2 text-figures
Printed in Great Britain

SUBCELLULAR LOCALIZATION OF THE HEPARIN-NEUTRALIZING FACTOR IN BLOOD PLATELETS

BY M. DA PRADA, M. JAKÁBOVÁ,* E. F. LÜSCHER,* A. PLETSCHER AND J. G. RICHARDS

*From the Research Division of F. Hoffmann–La Roche and Co. Ltd, Basel, Switzerland, and the *Theodor Kocher Institute, University of Berne, Switzerland*

(*Received 17 October 1975*)

SUMMARY

1. The distribution of the heparin-neutralizing factor (platelet factor 4, PF_4) in subcellular organelles of blood platelets of rabbits and man was investigated.

2. In both species the organelles storing 5-hydroxytryptamine (5-HT storage organelles) contained only trivial amounts of PF_4.

3. In contrast, the content of PF_4 was highest in the subcellular fractions rich in α-granules.

4. In conclusion, PF_4 is probably localized in the α-granules and therefore the platelets contain at least two types of organelles (5-HT organelles and α-granules) capable of releasing their contents in response to the same stimuli, such as exposure to collagen, thrombin, etc.

INTRODUCTION

Both rabbit and human blood platelets contain a heparin-neutralizing factor (platelet factor 4, PF_4) (Thomas, Niewiarowski & Ream, 1970). Human PF_4 has been characterized in some detail and found to consist of a basic protein of a molecular weight of about 30,000 which is attached to a proteoglycan carrier composed of a protein or polypeptide core and four chondroitin sulphate A residues (Barber, Käser-Glanzmann, Jakábová & Lüscher, 1972; Käser-Glanzmann, Jakábová & Lüscher, 1972, 1972/73). PF_4 is released from platelets whenever they are stimulated by agents such as collagen, thrombin, adenosine-5′-diphosphate (ADP) (in the presence of fibrinogen), or certain biogenic amines (Niewiarowski, Poplawski, Lipinski & Farbiszewski, 1968; Thomas *et al.* 1970; Youssef & Barkhan, 1968). This release reaction is a highly specific and fast process

which, under physiological conditions, accompanies the so-called second phase aggregation of the platelets (Mills, Robb & Roberts, 1968). There is general agreement that the released materials derive from storage organelles (Holmsen, Day & Stormorken, 1969); besides PF_4 they include 5-hydroxytryptamine (5-HT), adrenaline, histamine (from rabbit platelets only), adenosine nucleotides (ADP and adenosine-5′-triphosphate (ATP)), fibrinogen, albumin, another platelet-specific protein (Hagen, 1975), β-thromboglobulin (Moore, Pepper & Cash, 1975) as well as Ca^{2+} and K^+ ions. The 5-HT and adenine nucleotide storage organelles have been identified with the 'dense bodies' (Da Prada, Pletscher, Tranzer & Knuchel, 1967; Tranzer, Da Prada & Pletscher, 1966), a particularly prominent electron-dense particle. Since PF_4 is released simultaneously with 5-HT and nucleotides, it seemed reasonable to assume that it was also contained in the dense bodies. Support for this view seemed to come from observations made by Åborg & Uvnäs (1971) indicating the presence of mucopolysaccharides in the storage organelles of rat platelets, a statement which, however, is barely compatible with more recent results (Da Prada, von Berlepsch & Pletscher, 1972*a*).

In recent years, new evidence has become available which points strongly to the possibility that PF_4 was localized in a storage organelle different from the dense bodies (Lüscher & Käser-Glanzmann, 1975). Furthermore, PF_4 release was found normal in storage pool disease (Walsh & Gagnatelli, 1974; Weiss & Rogers, 1973), a condition which is characterized by a pronounced deficiency of the typical 5-HT storage organelle and, accordingly, an impaired release of 5-HT and adenine nucleotides.

In order to clarify the localization of PF_4, the distribution of this factor as well as that of 5-HT in electron microscopically characterized subcellular fractions of rabbit and human platelets has been investigated. Preliminary results have been communicated as a discussion remark by Lüscher (1976).

METHODS

Human and rabbit blood platelets were isolated as previously described from blood containing 1/10 vol. ethylene diamine-tetra-acetate. After ultrasonication, the homogenate was submitted to ultracentrifugation in a continuous density gradient of Urografin® (Schering, Berlin) (Da Prada & Pletscher, 1968; Da Prada *et al.* 1972*a*). The gradient was then divided into eight fractions (fractions I, II, 20 ml.; III–VII, 5 ml.; VIII, 25 ml.) plus a thin layer localized at the bottom of the centrifuge tube (bottom layer, fraction IX). Fractions I–VIII were diluted with cold 0·9% NaCl (1:1, v/v) and centrifuged at 130,000 *g* for 30 min. Factor 4, 5-HT (rabbits) and proteins were determined in the sedimented material as well as in the bottom layer according to previously described methods (Bogdanski, Pletscher, Brodie & Udenfriend, 1956; Käser-Glanzmann *et al.* 1972/73; Lowry, Rosebrough, Farr & Randall, 1951). In addition, the particulate matter of all the platelet fractions

(including fraction IX) was double-fixed with glutaraldehyde and osmium tetroxide, embedded in Epon, and ultrathin sections, counterstained with uranyl acetate and lead citrate, were examined in a Philips electron microscope (EM 300).

RESULTS

Electron microscopy

Rabbit platelets. Whole rabbit platelets showed the typical ultrastructural features described by Tranzer *et al.* (1966), namely (*a*) highly electron-dense organelles (5-HT organelles) limited by a single membrane with an average diameter of 180 nm; (*b*) less electron-dense spherical or somewhat elongated organelles with an average diameter of 300 nm (previously identified as α-granules by Schulz, 1960) which were more numerous, and (*c*) mitochondria as well as glycogen particles (Pl. 1*a*).

The platelet subcellular fractions I and II were mainly composed of microsomal elements while fraction III was richest in mitochondria. None of these three fractions contained α-granules or 5-HT organelles. Fractions IV and V consisted mostly of unidentified, moderately electron-dense organelles (possibly lysosomes) and some α-granules (fraction V); fractions VI, VII (Pl. 1*b*) and VIII were rich in α-granules, but contaminated with a few mitochondria. Finally, the bottom layer (fraction IX) was very rich in highly dense, osmiophilic 5-HT organelles, but also contained some electron-lucent vesicles (empty 5-HT organelles?) (Pl. 1*c*). In fractions IV–VIII, 5-HT organelles were only rarely observed.

Human platelets. These showed ultrastructural features similar to those of rabbits, although there were fewer 5-HT organelles containing highly electron-dense cores (Pl. 2*a*) (Da Prada, Tranzer & Pletscher, 1972*b*). The subcellular fractions revealed the following composition. Fractions I and II mainly consisted of microsomal elements. Fractions III and IV also contained microsomes, but in addition α-granules. Fractions V (Pl. 2*b*), VI and VII consisted mostly of α-granules, but were slightly contaminated with mitochondria and glycogen particles. Fraction VIII was richest in mitochondria, but also showed unidentified, moderately electron-dense organelles and some α-granules. The bottom layer (fraction IX) was less homogeneous than that of rabbit platelets. However, in agreement with earlier findings it contained numerous organelles with a highly electron-dense osmiophilic core surrounded by a single membrane. They had a spherical or ellipsoidal shape and their diameter was similar to that of the 5-HT organelles of rabbit platelets (Pl. 2*c*). These organelles have been previously identified as the storage sites for 5-HT (Da Prada *et al.* 1972*b*). Fractions I–VIII rarely contained 5-HT organelles.

Distribution studies

5-Hydroxytryptamine. As found earlier (Da Prada *et al.* 1967; Da Prada & Pletscher, 1968), the fraction of rabbit platelets showing the highest concentration (per mg protein) and total content of 5-HT was that of the 5-HT organelles (fraction IX). All the other fractions contained considerably less of the amine (Text-fig. 1). Human platelets also exhibited a 10–100 times higher 5-HT concentration in the bottom layer than in the other subcellular fractions (Da Prada *et al.* 1972*b*). However, owing to a poor recovery of the 5-HT organelles, the total amount of 5-HT found in the bottom fraction was approximately 15% only of that present in all particulate fractions together (Da Prada *et al.* 1972*b*).

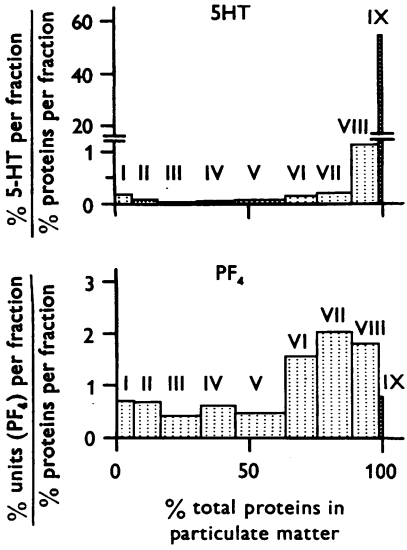


Fig. 1

Text-fig. 1. Subcellular distribution of 5-hydroxytryptamine (5-HT) and heparin-neutralizing factor (PF_4) in blood platelets of rabbits. The roman numbers indicate the various subcellular fractions; fraction IX is the bottom layer consisting of virtually pure 5-HT organelles.

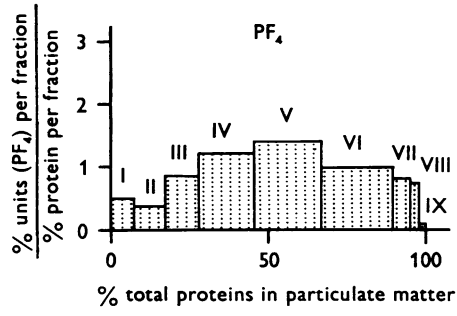


Fig. 2

Text-fig. 2. Subcellular distribution of heparin-neutralizing factor (PF_4) in blood platelets of man. The roman numbers indicate the various subcellular fractions.

Platelet factor 4. In platelets of rabbits, the concentration as well as the total amount of PF_4 was highest in the fractions richest in α-granules (fractions VI–VIII). The other fractions, especially the bottom layer, consisting of the pure 5-HT organelles, contained relatively little of this

factor (Text-fig. 1). In human platelets the concentration of PF_4, in contrast to that of 5-HT, was also very low in the bottom layer. Regarding the other fractions, PF_4 showed a more equal distribution than in rabbits, although its concentration appeared to be higher in the fractions containing α-granules (III–VII) (Text-fig. 2).

DISCUSSION

Because of the subcellular distribution studies in rabbit platelets, a major localization of PF_4 in the 5-HT storage organelles is unlikely. Thus, these organelles which were isolated in a virtually pure form with a fair percentage of recovery (about 50%) (Da Prada *et al.* 1972*b*) contained at most very small amounts of PF_4. However, the preferential accumulation of the latter in the subcellular fractions (VI–VIII) richest in α-granules indicates that PF_4 is mainly located in these organelles.

The findings in human platelets are less clear since the recovery of the 5-HT organelles was low and since the subcellular distribution of PF_4 was more uniform than in rabbit platelets. Nevertheless, in human platelets the situation seemed to be essentially similar to that in rabbit platelets. In fact, the concentration (per mg protein) of PF_4 in the fraction with the highest concentration of 5-HT organelles (bottom layer) was virtually zero, whereas it appeared to be greatest in those fractions containing a relatively high number of α-granules (fractions III–VII). Therefore, these results together with those obtained in rabbits indicate that in human platelets, contrary to previous opinion (Day, Stormorken & Holmsen, 1973), PF_4 is probably not located in the 5-HT organelles, but rather in a distinct, second type of storage organelle, i.e. the α-granules.

A partial redistribution of PF_4 during the homogenization and fractionation procedure cannot be excluded. However, the virtually complete absence of PF_4 in the bottom layer containing the 5-HT organelles (which showed a well preserved ultrastructure) is unlikely to be due to a leakage of PF_4 during the experimental procedure. On the other hand, the small amount of PF_4 activity in the lightest fractions of the gradient (Text-figs. 1 and 2) may be due to non-specific adsorption to microsomes of PF_4 originating from broken storage organelles. Since these fractions include fragments of the plasma membrane, the possibility must also be considered that this activity corresponds to the membrane-bound PF_4-like material described by O'Brien, Finch & Clark (1970). However, this represents only a small part of the total heparin-neutralizing activity.

The question arises whether still other types of secretory organelles are present in platelets. Preliminary experiments indicate that the organelle-bound fibrinogen of human platelets follows the same distribution as

does PF_4. Thus, it appears most likely that fibrinogen is also contained in α-granules. Other releasable components, such as the thrombin-sensitive protein (Hagen, 1975), β-thromboglobulin (Moore *et al.* 1975), and albumin, are possibly present in these organelles. Thus, the α-granule may turn out to be the storage site of the platelet for releasable high molecular weight substances, whereas the 5-HT organelle (which contains very little soluble protein; Da Prada & Pletscher, 1975) stores primarily low molecular weight materials such as biogenic amines, adenine nucleotides, and bivalent metal ions.

Very recent findings with human platelets (Broekman, Handin & Cohen, 1975) are in partial agreement with the present results. Thus PF_4 and fibrinogen were concentrated in the subcellular fractions previously shown to contain α-granules. However, no pure fraction of 5-HT organelles has been obtained.

REFERENCES

Åborg, C. H. & Uvnäs, B. (1971). A mucopolysaccharide-protein complex with amine binding properties in rat thrombocytes. *Acta physiol. scand.* **81**, 568–570.

Barber, A. J., Käser-Glanzmann, R., Jakábová, M. & Lüscher, E. F. (1972). Characterization of a chondroitin-4-sulfate proteoglycan carrier for heparin neutralizing activity (platelet factor 4) released from human blood platelets. *Biochim. biophys. Acta* **286**, 312–329.

Bogdanski, D. F., Pletscher, A., Brodie, B. B. & Udenfriend, S. (1956). Identification and assay of serotonin in brain. *J. Pharmac. exp. Ther.* **117**, 82–88.

Broekman, M. J., Handin, R. I. & Cohen, P. (1975). Distribution of fibrinogen, and platelet factors 4 and XIII in subcellular fractions of human platelets. *Br. J. Haemat.* **31**, 51–55.

Da Prada, M. & Pletscher, A. (1968). Isolated 5-hydroxytryptamine organelles of rabbit blood platelets: physiological properties and drug-induced changes. *Br. J. Pharmac. Chemother.* **34**, 591–597.

Da Prada, M. & Pletscher, A. (1975). Mechanisms of 5-hydroxytryptamine storage in subcellular organelles of blood platelets. In *Advances in Biochemical Psychopharmacology*, vol. 10, ed. Costa, E., Gessa, G. L. & Sandler, M. pp. 311–320, New York: Raven Press.

Da Prada, M., von Berlepsch, K. & Pletscher, A. (1972*a*). Storage of biogenic amines in blood platelets and adrenal medulla. *Naunyn-Schmiedebergs Arch. Pharmacol.* **275**, 315–322.

Da Prada, M., Tranzer, J. P. & Pletscher, A. (1972*b*). Storage of 5-hydroxytryptamine in human blood platelets. *Experientia* **28**, 1328–1329.

Da Prada, M., Pletscher, A., Tranzer, J. P. & Knuchel, H. (1967). Subcellular localization of 5-hydroxytryptamine and histamine in blood platelets. *Nature, Lond.* **216**, 1315–1317.

Day, H. J., Stormorken, H. & Holmsen, H. (1973). Subcellular localization of platelet factor 3 and platelet factor 4. *Scand. J. Haematol.* **10**, 254–260.

Hagen, I. (1975). Effects of thrombin on washed human platelets. Changes in the subcellular fractions. *Biochim. biophys. Acta* **393**, 242–254.

Holmsen, H., Day, H. J. & Stormorken, H. (1969). The blood platelet release reaction. *Scand. J. Haematol.* suppl. 8, 1–26.

KÄSER-GLANZMANN, R., JAKÁBOVÁ, M. & LÜSCHER, E. F. (1972). Isolation and some properties of the heparin-neutralizing factor (PF_4) released from human blood platelets. *Experientia* **28**, 1221–1223.

KÄSER-GLANZMANN, R., JAKÁBOVÁ, M. & LÜSCHER, E. F. (1972/73). Heparin-neutralizing factor (platelet factor 4) from human blood platelets and its reactivity with fibrinogen and soluble fibrin-monomer complexes. *Haemostasis* **1**, 136–147.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

LÜSCHER, E. F. (1976). Discussion remark. In *Report of Task Force on Platelet Nomenclature*. Proc. Int. Committee on Thrombosis and Hemostasis, Paris. Stuttgart and New York: Schattauer (in the Press).

LÜSCHER, E. F. & KÄSER-GLANZMANN, R. (1975). Platelet heparin-neutralizing factor (platelet factor 4). *Thromb. Diath. haemorrh.* **33**, 66–72.

MILLS, D. C. B., ROBB, I. A. & ROBERTS, G. C. K. (1968). The release of nucleotides, 5-hydroxytryptamine and enzymes from human blood platelets during aggregation. *J. Physiol.* **195**, 715–729.

MOORE, S., PEPPER, D. S. & CASH, J. D. (1975). The isolation and characterization of a platelet-specific β-globulin (β-thromboglobulin) and the detection of anti-urokinase and antiplasmin released from thrombin aggregated washed human platelets. *Biochim. biophys. Acta* **379**, 360–369.

NIEWIAROWSKI, S. & THOMAS, D. P. (1969). Platelet factor 4 and adenosine diphosphate release during platelet aggregation. *Nature, Lond.* **222**, 1269.

NIEWIAROWSKI, S., POPLAWSKI, A., LIPINSKI, B. & FARBISZEWSKI, R. (1968). The release of platelet clotting factors during aggregation and viscous metamorphosis. *Exp. Biol. Med.* **3**, 121–128.

O'BRIEN, J. R., FINCH, W. & CLARK, E. (1970). Platelet-bound and soluble platelet factor 4: Effects of aggregating agents, of aggregation, and of aspirin. *Proc. Soc. exp. Biol. Med.* **134**, 1128–1131.

SCHULZ, H. (1960). Submikroskopische Beiträge zur Orthologie und Pathologie der Thrombocyten. *Verh. dt. Ges. inn. Med.* **66**, 832–848.

THOMAS, D. P., NIEWIAROWSKI, S. & REAM, V. J. (1970). Release of adenine nucleotides and platelet factor 4 from platelets of man and four other species. *J. Lab. clin. Med.* **75**, 607–618.

TRANZER, J. P., DA PRADA, M. & PLETSCHER, A. (1966). Ultrastructural localization of 5-hydroxytryptamine in blood platelets. *Nature, Lond.* **212**, 1574–1575.

WALSH, P. N. & GAGNATELLI, G. (1974). Platelet antiheparin activity: storage site and release mechanism. *Blood* **44**, 157–168.

WEISS, H. J. & ROGERS, J. (1973). Platelet factor 4 in platelet disorders. Storage location and the requirement of endogenous ADP for its release. *Proc. Soc. exp. Biol. Med.* **142**, 30–35.

YOUSSEF, A. & BARKHAN, P. (1968). Release of platelet factor 4 by adenosine diphosphate and other platelet aggregating agents. *Br. med. J.* **1**, 746.

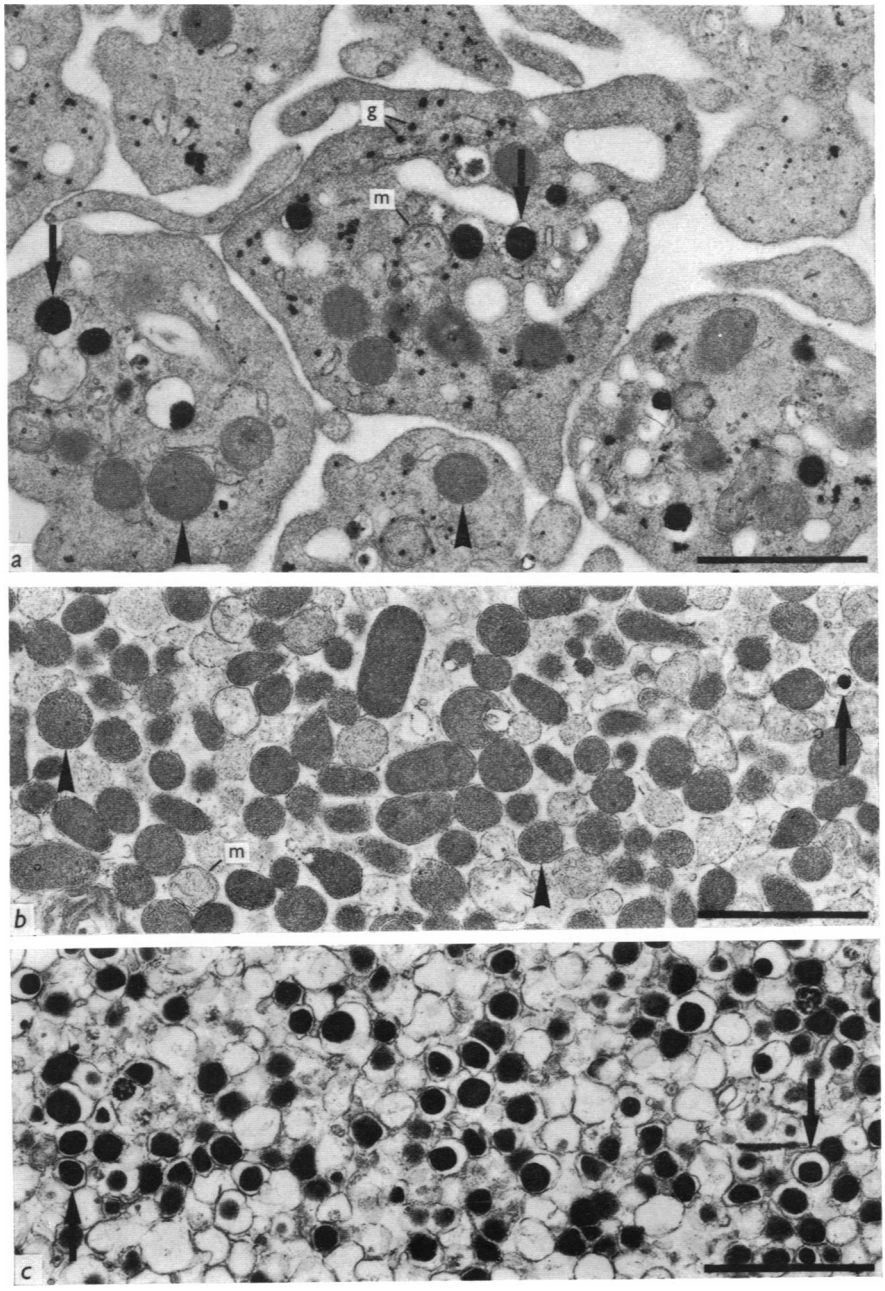
EXPLANATION OF PLATES

PLATE 1

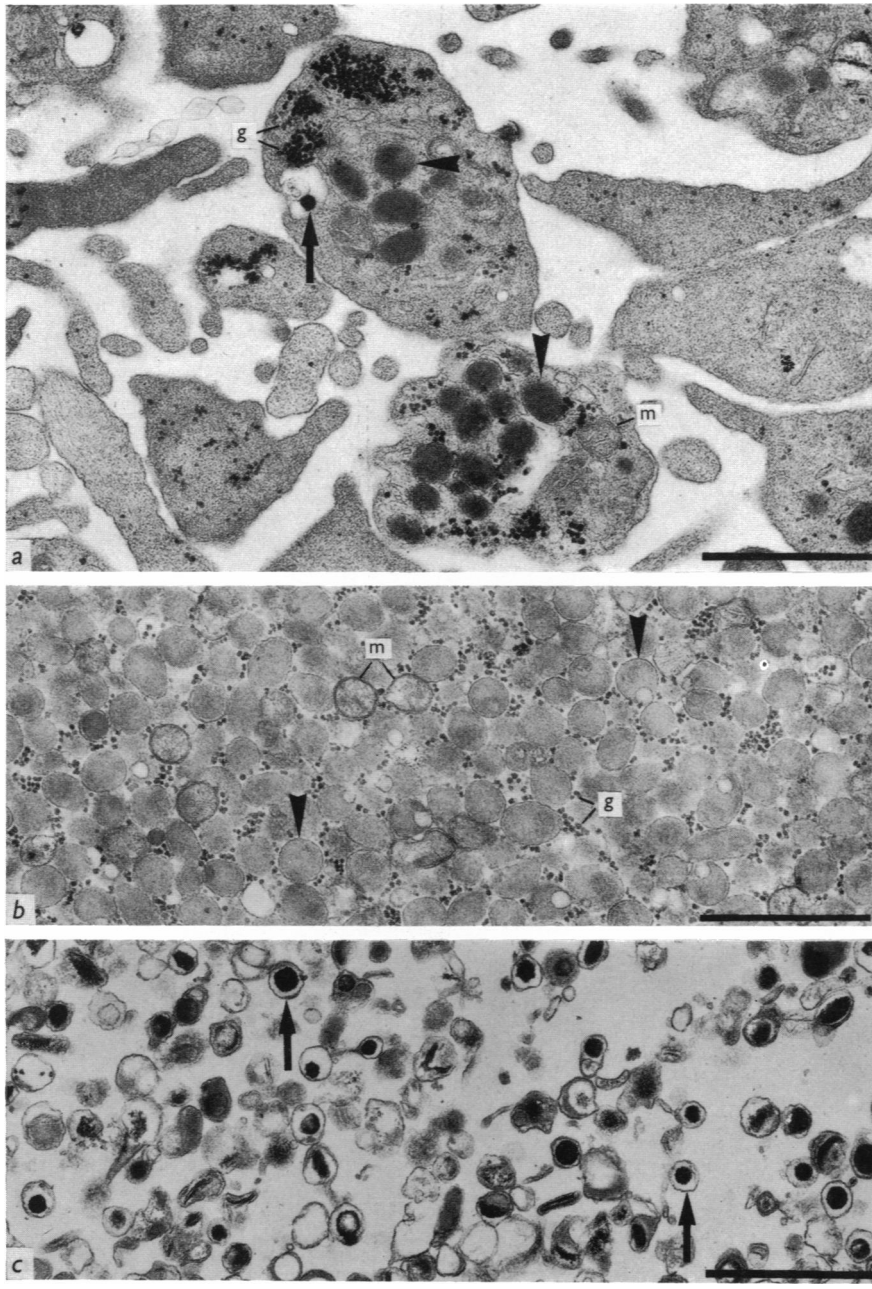
Ultrastructural features of rabbit whole platelets (*a*) and of subcellular fractions rich in α-granules (*b*) and 5-HT organelles (*c*). *a*: note the presence of highly electron-dense 5-HT organelles (→), α granules (▶), some mitochondria (m) and numerous glycogen particles (g). *b*: ultrastructural aspect of fraction VII. Numerous α-granules (▶), some mitochondria (m) and rare 5-HT organelles (→) are observed. *c*: ultrastructural aspect of fraction IX. This is a virtually pure fraction of 5-HT organelles containing highly electron-dense cores (→) and some electron translucent vesicles. Bar = 1 μm; magnification: × 22,000.

PLATE 2

Ultrastructural features of human whole platelets (*a*) and of subcellular fractions rich in α-granules (*b*) and 5HT organelles (*c*). *a*: note the presence of a single highly electron dense 5-HT organelle (→) in one platelet profile, several α-granules (▶), some mitochondria (m) and numerous glycogen particles (g). *b*: ultrastructural aspect of fraction V. Numerous α-granules (▶) are observed together with glycogen particles (g) and occasional mitochondria (m). *c*: ultrastructural aspect of fraction IX. This fraction contains several 5-HT organelles (→), but is highly contaminated with α-granules and other unidentified structures. Bar = 1 μm; magnification: × 22,000.



(*Facing p.* 502)


g
m
a
m
g
b
c