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Incorporation of a circulating protein into megakaryocyte and platelet granules

(peroxidase/endocytosis/guinea pig)

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ABSTRACT **To determine whether or not proteins circulating in plasma can be incorporated into megakaryocytes and platelets, horseradish peroxidase (HRP) was injected intravenously into guinea pigs and these cells were examined for its uptake by electron microscopy and cytochemistry. Enriched samples of megakaryocytes enabled ultrastructural analysis of large numbers of these rare cells. In megakaryocytes, 50% of α granules contained HRP between 75 min and 7 hr after injection. At 24 hr, 25% of the megakaryocyte granules were peroxidase-positive, less were positive by 48 hr, and there were none at 4 days. Thus, the findings demonstrate that a circulating protein can be endocytosed by megakaryocytes and rapidly packaged into α granules. Platelet granules also contain HRP by 7 hr after injection, and they can secrete it in response to thrombin. Unfortunately, our present studies do not allow us to distinguish between direct endocytosis by the platelet and/or shedding of new platelets from recently labeled megakaryocytes. It is concluded that while some α granule proteins are synthesized by megakaryocytes, others may be acquired from plasma by endocytosis. In addition to providing evidence that some of the proteins of α granules may be of exogenous origin, this study has allowed the definition of a pathway whereby plasma proteins may be temporarily sequestered in megakaryocytes before reentering the circulation in platelets.**

One of the most striking characteristics of platelets is the large variety of proteins that are secreted during the release reaction. Platelets contain counterparts of several plasma proteins (albumin, fibrinogen, and others) in addition to platelet-specific proteins (platelet factor 4 and β-thromboglobulin). These proteins are localized in α granules, the primary storage organelle for platelet secretory proteins. Predictably, many of these proteins have been shown to be synthesized in megakaryocytes, the bone marrow precursor cell from which platelets are derived. Platelet-specific proteins appear to be synthesized solely by megakaryocytes, whereas proteins such as fibrinogen, coagulation factor V, and von Willebrand factor are synthesized by other cell types in addition to megakaryocytes (1, 2).

Recently, we discovered that immunoglobulin G is located in platelet α granules and is secreted after platelet activation (3). Since we knew of no studies of megakaryocyte synthesis of immunoglobulins, we hypothesized the existence of an endocytic pathway whereby megakaryocytes may take up circulating proteins and incorporate them into granules.

To test this hypothesis, in the present study we injected horseradish peroxidase (HRP), a protein tracer with a molecular weight of 40,000, intravenously into guinea pigs and determined (*i*) whether megakaryocytes could endocytose HRP; and if so (*ii*) whether the endocytosed tracer was incorporated into megakaryocyte granules. Here we present data on a pathway whereby circulating proteins can enter bone marrow megakaryocytes and get incorporated into storage granules.

MATERIALS AND METHODS

Reagents. HRP (type II), prostaglandin E_1, dibutyryl-cyclic AMP, adenosine, theophylline, bovine serum albumin, 3,3′-diaminobenzidine, Ficoll, and Percoll were all obtained from Sigma. Hanks' balanced salt solution (calcium- and magnesium-free) was obtained from GIBCO. Human α-thrombin was a gift from John W. Fenton II (Albany, NY).

***In Vivo* Studies.** Male Hartley guinea pigs (300–400 g) were anesthetized with ether, and 75 mg of HRP was dissolved in 1 ml of physiologic saline and injected into the femoral vein. The animals were sacrificed with ether at short (15, 45, and 75 min) and long (7 hr, 24 hr, 48 hr, and 4 days) intervals after the injection. Blood was collected into acid/citrate/dextrose by heart puncture immediately before the guinea pigs were killed. Platelets were counted by use of an automated analyzer (Coulter S + IV). Platelet-rich plasma was obtained by centrifugation of the blood at $100 \times g$ for 10 min at 22°C. Megakaryocytes were isolated from bone marrow according to a described technique (4), except that all procedures were carried out at 10°C and unit gravity sedimentation was used rather than centrifugation of the megakaryocyte-enriched fraction on a continuous Ficoll gradient.

Platelet Secretion. Platelets were harvested 7 hr after HRP injection. A suspension containing 10^8 platelets was stimulated with 1 unit of α-thrombin for 3 min. The reaction was stopped by adding fixative. Prostaglandin E_1 (10 μg/ml) and dibutyryl-cyclic AMP (1 mM) were added in some experiments to inhibit thrombin-induced secretion (3).

Fixation, Peroxidase Cytochemistry, and Electron Microscopy. Cells were fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hr at 22°C. Peroxidase was localized by the cytochemical method of Graham and Karnovsky (5). The cells were then postfixed with reduced osmium, dehydrated in a graded series of ethanol, and embedded in Epon as described (6). Subcellular localization of peroxidase in megakaryocytes and platelets was assessed on lead-stained sections at each time interval after injection of HRP.

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Abbreviations: DMS, demarcation membrane system; HRP, horseradish peroxidase.

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RESULTS

The isolation procedure yielded a megakaryocyte-enriched fraction with a purity of ≈50%, facilitating analysis of large numbers of these cells.

Uptake of HRP by Megakaryocytes. At the earliest time interval studied, 15 min after HRP injection, the megakaryocyte plasma membrane was negative but the demarcation membrane system (DMS), which is in continuity with the extracellular space, showed dense reaction product for peroxidase. The granules were devoid of peroxidase activity. At 15–45 min, numerous peroxidase-positive vesicles, occasionally coated, were seen in the cytoplasm (Fig. 1*a*). As early as 45 min after injection, up to 10% of granules were peroxidase-positive. By 75 min, 40–50% of the granules were peroxidase-positive, and moderate amounts of enzyme were still present in the DMS (Fig. 1*b*). This pattern was observed in 95% of the megakaryocytes examined and persisted up to 7 hr. These peroxidase-positive organelles most closely resemble α granules in number and morphology (7). Although it was common to see many positive megakaryocyte granules, the Golgi complex was consistently negative (Fig. 1*c*). Peroxidase activity in the DMS progressively decreased in megakaryocytes isolated 24 and 48 hr after HRP injection, but there were still 20–30% peroxidase-positive granules at 24 hr. At 48 hr, there were <10% positive granules. At 4 days, no peroxidase activity was detected in megakaryocytes.

Appearance of HRP in Platelet Granules. To determine whether megakaryocytes with HRP-containing granules can produce platelets capable of circulating, cytochemical analysis was performed on platelets obtained at different time intervals after HRP injection. The platelet count decreased to 5–10% of the preinjection value within 45 min of HRP injection. The count began to increase slowly, reached 30–40% of the preinjection value at 7 hr, and was normal by 48 hr. Due to severe thrombocytopenia that followed HRP injection, it was very difficult to obtain adequate numbers of platelets for analysis at time intervals prior to 7 hr. At 7 hr after HRP injection, 60–80% of the granules were labeled in all platelets (Fig. 2*a*). The number of positive platelets as well as the number of positive granules per platelet progressively decreased over the next 48 hr. At this time, there appeared to be two populations of platelets. The first population contained peroxidase-positive granules and constituted 70–80% of the total platelets. The other population did not have peroxidase-positive granules. At 4 days, there were no positive granules. These results are summarized in Table 1.

Platelet Secretion. Thrombin caused secretion of platelet α granules, as evidenced by the almost complete disappearance of HRP positive granules (Fig. 2*b*). In the presence of inhibitors, thrombin caused only minimal secretion (data not shown).

Other Cells. Macrophages with endocytosed HRP were occasionally observed. Basophils and lymphocytes were not examined, as they were eliminated by the isolation procedure. It was impossible to assess incorporation of HRP into neutrophils and eosinophils because of the presence of endogenous peroxidase.

Control Experiments. Megakaryocytes and platelets from guinea pigs not injected with HRP showed no peroxidase activity in granules or other intracellular organelles (data not shown).

DISCUSSION

We have demonstrated that the tracer protein HRP, when given intravenously to guinea pigs, is rapidly taken up from circulation and incorporated into granules by bone marrow megakaryocytes. Our findings demonstrate a circuitous pathway by which circulating plasma proteins may enter megakaryocytes, become stored in cytoplasmic granules, and return via platelets to be subsequently secreted back into the circulation during the platelet release reaction.

Platelets (and megakaryocytes) contain four types of distinguishable granules or vesicles: dense granules, lysosomes, peroxisomes, and the primary secretory protein storage organelle, the α granule (7). Although platelet α-granule formation occurs in the megakaryocyte, the origin of most proteins stored in these granules is unclear. Some α-granule proteins, such as platelet factor 4, appear to be synthesized only by megakaryocytes (1, 2, 8). Other α-granule proteins, such as fibrinogen (9, 10), coagulation factor V (11, 12), and von Willebrand factor (13), are synthesized by megakaryocytes in addition to other cell types. Serotonin, an amine synthesized mainly by enterochromaffin cells, is taken up and incorporated into dense granules by platelets and to a lesser extent by megakaryocytes (14, 15). This mechanism of serotonin uptake differs from the endocytosis of proteins in that it involves active membrane transport across plasma and dense granule membranes (16). Although uptake and incorporation of this exogenous amine is well known, the incorporation of a circulating exogenous protein into platelet and megakaryocyte α granules has not been demonstrated. In a study similar to ours, Behnke (17) investigated megakaryocyte uptake of HRP after intravenous injection into rats but had different results. The tracer was observed inside the DMS and an occasional cytoplasmic vesicle but never in granules or phagosomes. Although the marked differences from our findings might be due to differences in the species studied, they might alternatively reflect technical limitations in Behnke's study. The use of small pieces of bone marrow for cytochemical processing might have resulted in examination of few megakaryocytes and poor penetration of the cytochemical reagents into the bone marrow cells. Incorporation of HRP into megakaryocyte granules might therefore have escaped detection. In previous *in vitro* studies in which HRP was added to megakaryocytes (15, 18), the enzyme was not incorporated into storage granules. *In vitro* studies on platelets have mainly demonstrated their ability to sequester certain substances in the surface-connected canalicular system, but one such study showed thorium dioxide was incorporated into platelet granules (19). Uptake and incorporation of exogenous protein into granules have been demonstrated in other hematopoietic cell types. Thus, HRP injected into guinea pigs *in vivo* was shown to be transported in pinocytic vesicles to the granules of basophils (20). *In vitro* experiments have demonstrated that basophils, mast cells, and granule-containing lymphoid cells can internalize eosinophil peroxidase and incorporate it into granules in a similar manner (21).

At 7 hr after HRP injection, the platelet count had returned to 30–40% of the normal value, and most of the granules in each platelet were peroxidase-positive; this observation could have at least two explanations. The first explanation is that circulating platelets can directly endocytose and incorporate HRP into their granules. In this case, the transient thrombocytopenia may have been due to reversible platelet aggregation. Previous *in vitro* studies (22, 23), however, have failed to demonstrate the incorporation of HRP into platelet granules. *In vivo* HRP uptake by platelets has not been studied. The second possibility is that the thrombocytopenia was caused by accelerated destruction of platelets, and that platelets with peroxidase-positive granules represent newly shed platelets from megakaryocytes with HRP incorporated granules in response to thrombocytopenia. Further studies are needed to distinguish between these two possible mechanisms. These HRP-labeled platelets disappear from circulation by 4 days, suggesting that their life span in the guinea pig is similar to rabbit platelets, which have a mean life span of 3–4 days (24). Furthermore, the appearance of unlabeled

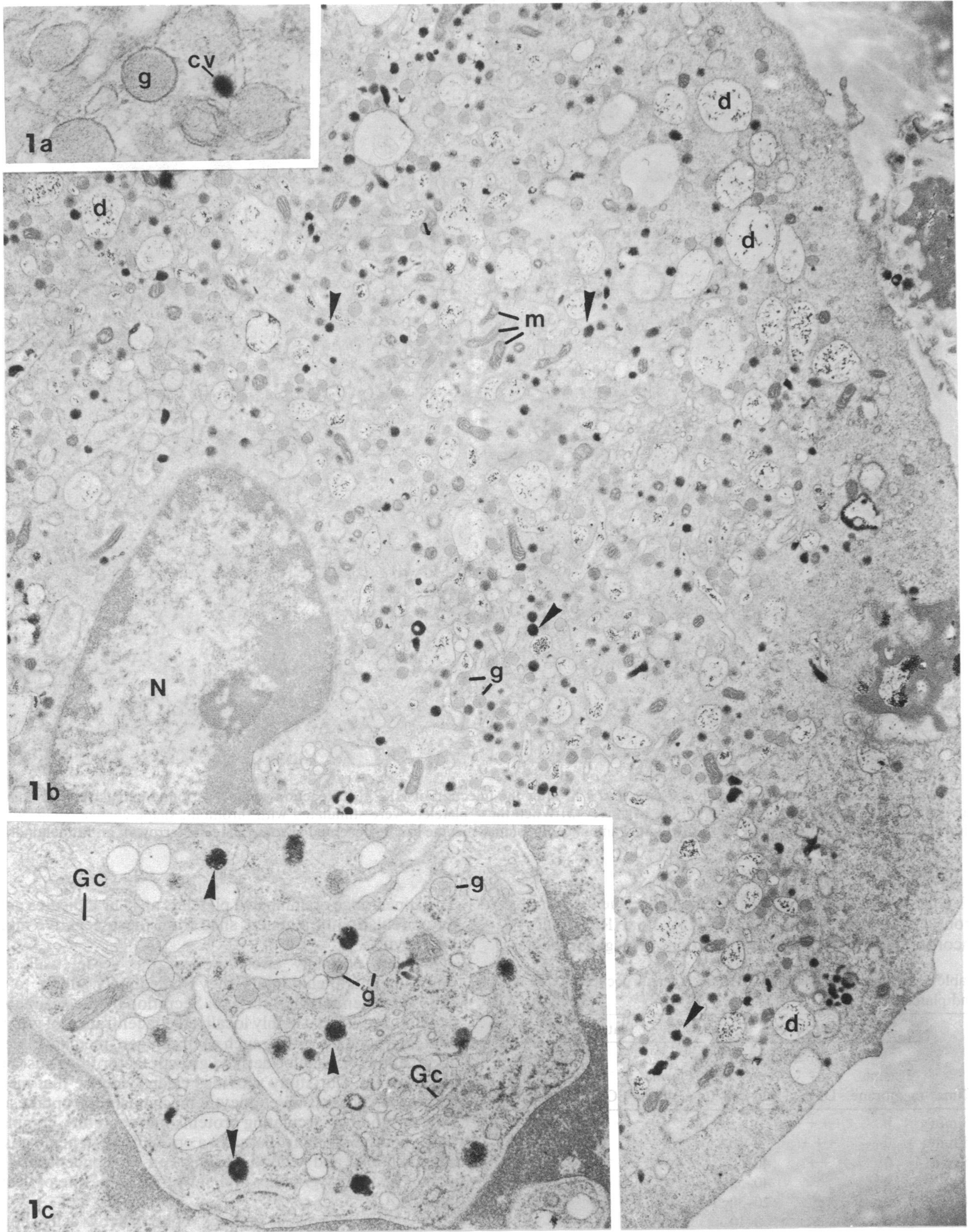

FIG. 1. Maturing megakaryocytes were isolated at various times after HRP injection and assayed for peroxidase. (*a*) At 45 min, coated vesicles (cv) contain dense peroxidase reaction product. The adjacent α granules (g) are peroxidase negative. (*b*) At 75 min, moderate amounts of enzyme reaction product are present within the DMS (d). Many of the granules are peroxidase-positive (arrowheads), although some are peroxidase-negative (g). N, nucleus; m, mitochondrion. This point is better appreciated at higher magnification, as shown in *c*. The Golgi complex (Gc) was never peroxidase-positive, whereas some of the adjacent granules (arrowheads) contain HRP. (*a*, ×63,400; *b*, ×12,480; *c*, ×23,000.)

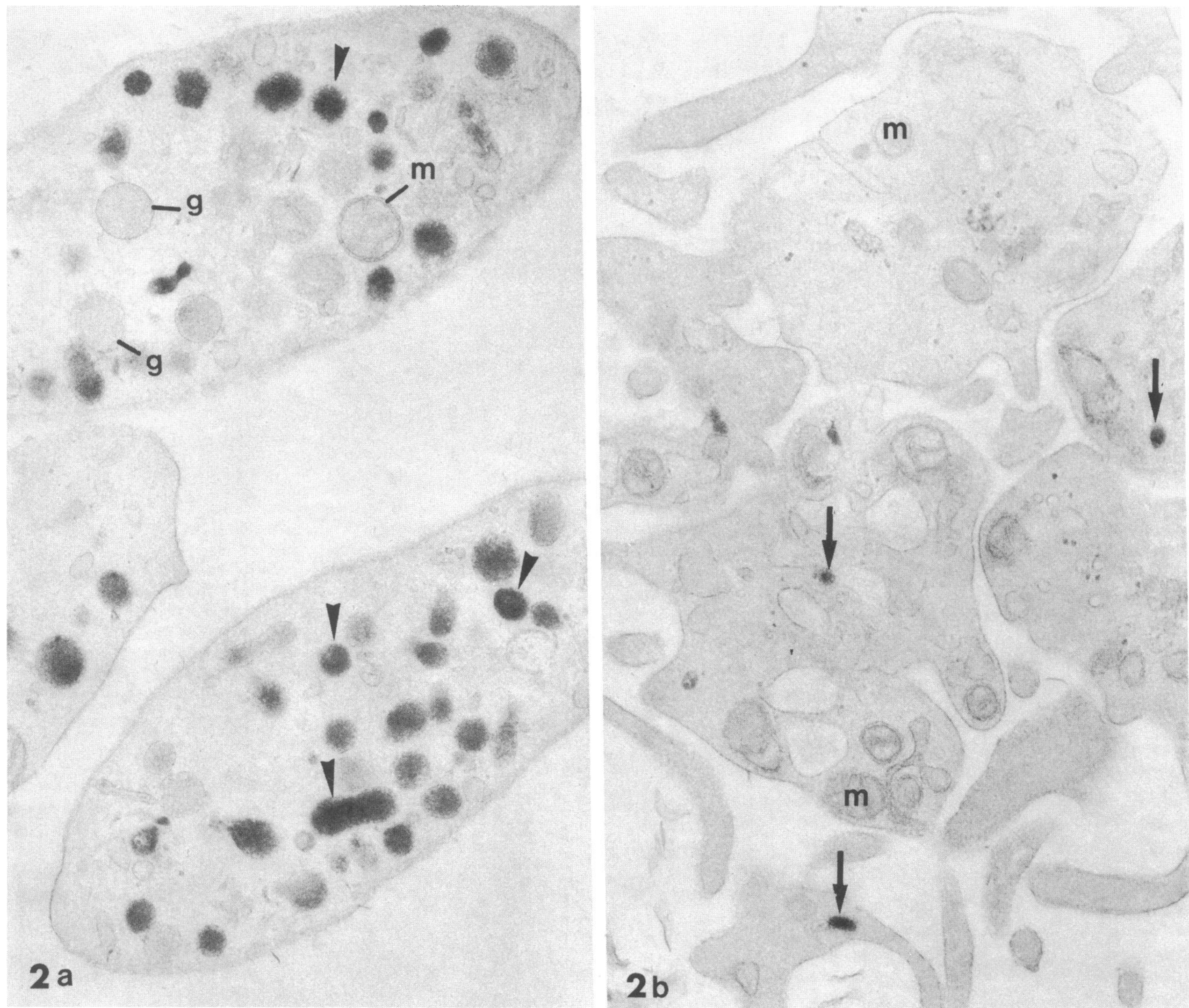

FIG. 2. Guinea pig platelets from blood collected 7 hr after the injection of HRP. (*a*) Reaction product is found exclusively in granules (arrowheads), although some granules (g) are negative. (*b*) When these platelets were stimulated with thrombin, shape change occurred and almost all the granules were secreted. Only limited amounts of residual enzyme can be observed in some platelets (arrows). m, Mitochondrion. (*a*, ×32,600; *b*, ×35,500.)

platelets at 48 hr deserves further comment. Due to clearance from circulation within minutes (25), no HRP would be incorporated into successive populations of megakaryocytes.

Table 1. Organelle location of HRP in megakaryocytes and platelets

	Megakaryocytes			Platelets		
Time	Plasma membrane	DMS	Granules, % positive per cell	Plasma membrane	SCCS	Granules, % positive per cell
15 min	0	++++	0			
45 min	0	+++	0–10			
75 min	0	++	40–50			
7 hr	0	±	40–55	0	0	60–80
24 hr	0	±	20–30	0	0	25–50
48 hr	0	±	0–10	0	0	0–30*
96 hr	0	0	0	0	0	0

Time is the interval between infusion of HRP into guinea pigs and sacrifice for examination of megakaryocytes and platelets. Staining intensity is graded from 0 to ++++. SCCS, surface-connected canalicular system. The patterns described above were observed in <90% of the megakaryocytes at any one time interval.
*Two populations of platelets were observed (see *Results*).

The appearance of unlabeled platelets at 48 hr indicates entry into circulation of platelets shed from these negative megakaryocytes.

Platelets normally circulate near the vessel wall, and following endothelial injury they immediately adhere to the exposed subendothelial surface. Secretion by these adherent platelets produces a highly localized concentration of granule contents, some of which, such as fibrinogen, mediate platelet aggregation, or platelet-derived growth factor fibroblast proliferation (1). Our study provides clear evidence that megakaryocytes possess an endocytic mechanism for uptake and concentration of circulating proteins into secretory granules. Thus, plasma proteins that play a critical role in hemostasis, inflammation, and wound healing can be concentrated in platelet secretory granules and delivered to the site of vessel injury.

In light of the present findings, bone marrow cells should also be investigated in kinetic studies as a possible repository for normal plasma proteins such as albumin, which is known to be a carrier of hormones, metals, ions, fatty acids, amino acids, enzymes, bilirubin, and drugs (26). In studies of injected ^{131}I-labeled serum albumin (27), the major sites of distribution were identified as plasma, skin, muscle, heart, lungs, liver, kidney, and spleen. Bone marrow was not

tested. Further studies are needed to determine to what extent megakaryocytes can acquire proteins, such as albumin and IgG, from plasma and package them into granules for subsequent reentry into circulation in platelets.

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1. George, J. N., Nurden, A. T. & Phillips, D. R. (1984) *N. Engl. J. Med.* **311,** 1084–1098.
2. Holmsen, H. (1985) *Semin. Hematol.* **22,** 219–240.
3. George, J. N., Saucerman, S., Levine, S. P., Knieriem, L. K. & Bainton, D. F. (1985) *J. Clin. Invest.* **76,** 2020–2025.
4. Rabellino, E. M., Nachman, R. L., Williams, N., Winchester, R. J. & Ross, G. D. (1979) *J. Exp. Med.* **149,** 1273–1287.
5. Graham, R. C., Jr., & Karnovsky, M. J. (1966) *Histochem. Cytochem.* **14,** 291–302.
6. Stenberg, P. E., Shuman, M. A., Levine, S. P. & Bainton, D. F. (1984) *J. Cell Biol.* **98,** 748–760.
7. Bentfeld-Barker, M. E. & Bainton, D. F. (1982) *Blood* **59,** 472–481.
8. Ryo, R., Nakeff, A., Huang, S. S., Ginsberg, M. & Deuel, T. F. (1983) *J. Cell Biol.* **96,** 515–520.
9. Leven, R. M., Schick, P. K. & Budzynski, A. Z. (1985) *Blood* **65,** 501–504.
10. Belloc, F., Hourdille, P., Fialon, P., Boisseau, M. R. & Soria, J. (1985) *Thromb. Res.* **38,** 341–351.
11. Chiu, H. C., Schick, P. K. & Colman, R. W. (1985) *J. Clin. Invest.* **75,** 339–346.
12. Gerwirtz, A. M., Keefer, M., Doshi, K., Annamalai, A. E., Chiu, H. C. & Coleman, R. W. (1986) *Blood* **67,** 1639–1648.
13. Nachman, R., Levine, R. & Jaffe, E. A. (1977) *J. Clin. Invest.* **60,** 914–921.
14. Tranzer, J. P., Da Prada, M. & Pletscher, A. (1972) *J. Cell Biol.* **52,** 191–197.
15. Fedorko, M. E. (1977) *Lab. Invest.* **36,** 321–328.
16. Rudnick, G., Humphreys, C. J. & Dean, G. E. (1985) *Ann. N.Y. Acad. Sci.* **456,** 277–278.
17. Behnke, O. (1968) *J. Ultrastruct. Res.* **24,** 412–433.
18. Zucker-Franklin, D. & Petursson, S. (1984) *J. Cell Biol.* **99,** 390–402.
19. White, J. G. (1968) *Am. J. Pathol.* **53,** 567–575.
20. Dvorak, A. M., Dvorak, H. F. & Karnovsky, M. J. (1972) *Lab. Invest.* **26,** 27–39.
21. Dvorak, A. M., Klebanoff, S. J., Henderson, W. R., Monahan, R. A., Pyne, K. & Galli, S. J. (1985) *Am. J. Pathol.* **118,** 425–438.
22. Zucker-Franklin, D. (1970) *J. Cell Biol.* **47,** 293–299.
23. Zucker-Franklin, D. (1981) *J. Cell Biol.* **91,** 706–715.
24. Ebbe, S., Baldini, M. & Donovan, J. (1965) *Blood* **25,** 548–565.
25. Rodman, J. S., Schlesinger, P. & Stahl, P. (1978) *FEBS Lett.* **85,** 345–348.
26. Ganong, W. F. (1979) *Review of Medical Physiology* (Lange Medical Publications, Los Altos, CA), p. 410.
27. Rothschild, M. A., Bauman, A., Yalow, R. S. & Berson, S. A. (1955) *J. Clin. Invest.* **34,** 1354–1358.