The Influence of Reserpine and Ethylenediaminetetraacetic Acid (EDTA) on Serotonin Storage Organelles of Blood Platelets

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The present investigation has evaluated the influence of reserpine on the serotonin-rich organelles (dense bodies) in platelets from dogs, rabbits, and humans. Reserpine markedly depresses the levels of stored serotonin in human and animal platelets, accompanied by a small decrease in platelet ATP but no change in platelet ADP content. Thin sections of human platelets showed no change in the number or morphology of serotonin storage organelles during reserpine therapy, whereas a profound decrease in the size and number of dense bodies occurred in platelets from rabbits treated with reserpine. Dog platelets also showed a decrease in the number and density of serotonin storage organelles after reserpine therapy. The basis for the difference between rabbit and human platelets was explored by fixing platelets in glutaraldehyde and osmium in the presence or absence of the chelating agent ethylenediaminetetraacetic acid (EDTA). Most of the dense bodies in fixed human platelets were removed by EDTA while rabbit platelet dense bodies remained essentially intact. The results suggested that the opacity of rabbit platelet dense bodies following fixation with glutaraldehyde and osmium relate primarily to their serotonin content, while the electron density of human serotonin storage organelles in fixed cells is due primarily to their calcium content. Further confirmation of this concept came from studies of platelets using the whole mount technique. Rabbit platelet serotonin storage organelles were found to lack the inherent opacity of the human dense bodies, a finding consistent with the lower concentration of calcium in the rabbit organelles. (Am J Pathol 87:633–646, 1977)

NEARLY ALL OF THE SEROTONIN (5-hydroxytryptamine, 5-HT) transported in the circulation is concentrated in blood platelets.[1] In 1959, Baker, Blaschko, and Born [2] demonstrated that the amine was associated with ATP in platelet subcellular particles. The fine structural localization of 5-HT was more clearly defined in electron microscopic investigations. Utilizing techniques which had been successful in identifying catecholamine storage organelles in the adrenal gland, Wood reported the existence of specialized serotonin storage organelles in platelets.[3] The following year, Tranzer *et al.*[4] noted that platelets fixed in glutaraldehyde and osmic acid contained a small number of black storage organelles which were not present in platelets fixed in osmic acid alone. The frequency of electron-dense bodies in platelets from four different mamma-

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lian species correlated closely with their content of serotonin. Platelets from rabbits treated with reserpine contained almost no serotonin and only rare black bodies. The content of serotonin and black bodies was restored in reserpine-treated rabbit platelets following incubation with 5-HT *in vitro*.[4-8] The association of serotonin with the platelet dense body was confirmed by ultrastructural autoradiography [9] and by chemical determinations on platelet subcellular organelles isolated by density gradient sedimentation.[5]

The studies by Wood,[3] Tranzer *et al.*,[4] and others [10,11] emphasized the importance of dual fixation in preserving serotonin-rich organelles for study in the electron microscope. *In vitro* experiments demonstrated that addition of glutaraldehyde and osmic acid to pure serotonin caused formation of a black precipitate. It was postulated that glutaraldehyde reacted with free amino groups of 5-HT to form a Schiff base. Interaction of this reagent with either osmic acid or potassium dichromate at low pH would result in deposition of the opaque metallic oxidizing fixative at specific sites of serotonin concentration in glutaraldehyde-fixed platelets. Thus, deposition of osmium or dichromate in platelets fixed first in glutaraldehyde was considered a specific cytochemical test for serotonin and the biochemical basis for the blackness of dense bodies in the electron microscope.

Studies on human platelets in this laboratory raised serious questions about the general application of this hypothesis. Tranzer *et al.*[4] had observed that human platelets contained fewer serotonin storage organelles than rabbit cells after treatment with reserpine. May *et al.*[12] found only 16 dense bodies in thin sections of 1000 human platelets, and others had also suggested that dense bodies were rare in human cells. In contrast, studies of thin sections of human platelets in our laboratory revealed a frequency of over one dense body per cell,[13] a value more than sixty times higher than reported previously by others. The difference was not due to the fixatives but to the buffer which contained a low concentration of phosphate and a large amount of calcium. Subsequent studies by Spicer *et al.*[14] and ourselves [15] demonstrated that adjustment of the salt concentration and the nature of the salts in the buffer eliminated the need for glutaraldehyde and that dense bodies could be preserved in platelets fixed in osmic acid alone.

The most telling argument against the hypothesis that dual fixation was required for preservation of serotonin storage organelles, however, was the observation that dense bodies were present in their usual number and distribution in human platelets studied by the whole mount technique which had not been fixed at all.[16] This finding, together with observations

on the importance of buffer salt composition for preservation of dense bodies in thin sections of fixed platelets, led to the conclusion that serotonin storage organelles in human cells were inherently electron opaque due to their high content of calcium or calcium salts. Subsequent investigations after employing combined x-ray spectrometry and electron microscopy have confirmed this suggestion and demonstrated that the serotonin-containing organelles in human platelets are rich in calcium.[17-19]

A cornerstone of the hypothesis put forth by Tranzer *et al.*[4-8] was the finding that reserpine treatment removed nearly all of the dense bodies from rabbit platelets and that incubation of cells from treated animals *in vitro* with serotonin restored dense bodies to the platelets. Recently, we have had the opportunity to carry out a similar study on human platelets after reserpine treatment and compare the results with the effects of reserpine on rabbit and canine cells. In addition, we have examined the influence of chelation during fixation on human and rabbit platelet dense bodies. The results suggest that some of the confusion generated in early studies may be due to fundamental differences in human and animal platelet dense bodies.

Materials and Methods

The procedures used in this laboratory to obtain blood from normal donors and patients and mix the samples immediately with 3.8% trisodium citrate or citrate-citric acid, pH 6.5, in a ratio of nine parts blood to one part anticoagulant; separate platelet-rich plasma by centrifugation at 100*g* for 20 minutes at room temperature; expose the samples of normal platelet-rich plasma to various drugs and experimental conditions; and prepare samples for electron microscopy by fixation in 0.1% glutaraldehyde, then 3% glutaraldehyde in White's saline, and subsequently in 1% osmic acid in Zetterquist's buffer before dehydration and embeddment in Epon have been described in previous publications.[20,21] In experiments in which samples were fixed in the presence of EDTA, this agent was added to both glutaraldehyde fixatives and to the osmic acid fixative in a final concentration of 0.01 M and the pH adjusted to 7.4. The time course of fixation in each of the three fixatives was identical to the usual procedure.[20] Thin sections of samples embedded in Epon were stained with lead citrate and uranyl acetate and examined in a Philips 301 electron microscope.

Unstained whole mounts of human and rabbit platelets were prepared by addition of a drop of citrate platelet-rich plasma to a carbon-stabilized, formvar-coated grid.[16,22] Excess plasma was removed from the edge with filter paper, and the platelets were examined under the electron microscope.

The patient studied was a 54-year-old female with essential hypertension who was treated with 0.25 mg reserpine daily for two separate periods. Samples were taken for electron microscopy prior to therapy, at intervals during therapy, and after termination of therapy. The 10 rabbits and 2 dogs used for this study were injected with 5 mg/kg reserpine intraperitoneally and were studied prior to and 1 to 7 days after the injection of reserpine. Platelet serotonin was measured as described by Rao *et al.*[23] Adenine nucleotides were measured using high pressure liquid chromatography as described previously.[24] Evaluation of serotonin uptake was performed according to the method of Rendu and Caen.[25] Values presented represent the mean of triplicate determinations. The number of dense bodies per platelet was determined by visual counting of platelets and dense bodies

under the electron microscope. For each value reported in Tables 1–4, at least 200 platelets were counted. Wherever a standard error is given, at least four series of 100 platelets were counted.

Results

The Influence of Reserpine on the Platelet Content of Serotonin and Adenine Nucleotides

Reserpine was found to markedly deplete the serotonin content of human, rabbit, and dog platelets (Tables 1–3). Measurements in dog, human, and rabbit platelets showed a small decrease in ATP content but no significant change in the ADP and AMP content of the platelets (Tables 1–3).

The Influence of Reserpine on the Platelet Dense Bodies

Human, dog, and rabbit platelets vary considerably in the number of dense bodies in thin sections of platelets. Rabbit platelets contained the most dense bodies, with an average of about two dense bodies per platelet thin section (Table 2); human platelets were of an intermediate value,

Table 1—The Influence of Reserpine on the Serotonin, Adenine Nucleotide, and Dense Body Content of Human Platelets

	Serotonin (ng/10^9 platelets)	Uptake of ^{14}C-serotinin by platelets	Adenine nucleotides (μmoles/10^{11} platelets) Total	ATP	ADP	AMP	Ratio ATP/ADP	No. of dense bodies/100 platelets (mean±SE)
Normal individuals (N = 10)	940 ± 60	90–100%	12.56 ±0.41	7.54 ±0.2	3.92 ±0.2	0.70 ±0.06	1.87 ±0.06	109 ± 6
Patient BH								
Prior to reserpine	601	—	—	—	—	—	—	96.7 ± 3.8
After beginning first course of reserpine								
7 Days	50	0%	8.90	—	—	—	—	95.3 ± 8.6
13 Days	0	30%	11.07	—	—	—	—	94.3 ± 5.3
16 Days	21	33%	9.34	—	—	—	—	—
29 Days	50	33%	11.81	5.37	4.40	2.04	1.22	95.0 ± 1.8
During the second course of reserpine	64	0%	—	—	—	—	—	101.3 ± 6.6
After discontinuation of reserpine	769	100%	11.47	6.97	4.50	1.34	1.55	103.0 ± 6.9

with an average of about one dense body per platelet thin section (Table 1); dog platelets contained the fewest dense bodies, with an average of about one per two platelets (Table 3). In addition, the ultrastructural appearance of rabbit dense bodies was noticeably different from human dense bodies. Almost all rabbit dense bodies were round, solid, uniformly black organelles. In contrast, human platelet dense bodies varied widely in size, shape, and structure, and some were noticeably granular. Human platelet dense bodies occasionally had elongated tails, whereas such tails were not present in rabbit dense bodies. Following treatment with reserpine, human platelets showed no significant changes in the platelet content or morphology of these organelles (Figure 1). In contrast, dogs treated with reserpine showed about a 30% reduction in the platelet content of dense bodies, while rabbits showed a 79 to 96% reduction in dense bodies (Figure 2). In addition to the reduction of serotonin storage organelles in the rabbit and dog platelets, there was a noticeable decrease in the size and density of the organelles remaining in the cells. The dog and rabbit dense bodies were noticeably smaller and less dense, with fewer solidly black organelles.

The Influence of Fixation by Glutaraldehyde and Osmium Solution Containing EDTA on the Appearance of Platelet Dense Bodies in Humans and Rabbits

It has been suggested that the concentration of calcium in the buffer vehicle for the glutaraldehyde and osmium used to fix the platelets is

Table 2—The Influence of Reserpine on the Serotonin, Adenine Nucleotide, and Dense Body Content of Rabbit Platelets*

	Serotonin (ng/10^9 platelets)	Adenine nucleotides (μmoles/10^{11} platelets)					No. of dense bodies/ 100 platelets
		Total	ATP	ADP	AMP	Ratio ATP/ ADP	
Rabbit 1							
Before reserpine	15,940	11.2	7.5	2.2	1.47	3.4	228
After reserpine							
24 Hours	770	7.38	4.9	1.5	0.98	3.2	49 (−79%)
96 Hours	80	—	—	—	—	—	8 (−96%)
7 Days	350	—	—	—	—	—	11 (−95%)
Rabbit 2							
Before reserpine	10,310	15.1	10.5	2.44	2.15	4.30	145
After reserpine							
24 Hours	180	11.17	7.26	2.33	1.58	3.11	27 (−81%)
96 Hours	50	—	—	—	—	—	24 (−83%)
7 Days	50	—	—	—	—	—	17 (−88%)

* A total of 10 rabbits were treated with reserpine, and the serotonin levels and number of dense bodies/100 platelets were analyzed before and after reserpine. The results in the 2 rabbits shown here are representative of the studies on all 10 rabbits.

critical to the visualization of human dense bodies.[15,19] Therefore, samples of human and rabbit platelets were incubated in fixatives with buffer containing calcium and in fixatives dissolved in buffer without calcium containing 0.01 M EDTA (Table 4). Thin sections of human platelets that had been fixed in the presence of EDTA lost 81 to 96% of their dense bodies (Figure 3). In contrast, rabbit platelets fixed in the presence of EDTA were not significantly different from cells fixed without EDTA (Figure 4). The results of these studies suggested that metal ions, most likely calcium,[17-19] were critical to the opaque appearance of human dense bodies but played only a minor role in the black appearance of the organelles in thin sections of rabbit platelets.

Studies of Human and Rabbit Platelets by the Whole Mount Technique

Our results suggested that calcium ions did not make a major contribution to the black appearance of rabbit platelet serotonin storage organelles. Therefore, we examined platelets using the whole mount technique, since it has been suggested that calcium or metal ions make the major contribution to the inherent resistence of the organelles to transmission of the electron beam when visualized by this procedure.[16-19] Whole mounts of unfixed, unstained rabbit platelets were found to lack electron-dense granules (Figures 5–7). Instead of the typical opaque, electron-dense granules visible in whole mounts of human platelets (Figure 8), there were only gray appearing granules present which were much lighter than the usual human dense bodies. Our initial impression was that these gray organelles might be the serotonin storage organelles. However, treatment of rabbits with reserpine failed to remove the gray granules, as would be expected if they were indeed the serotonin storage organelles.

Table 3—The Influence of Reserpine on the Serotonin, Adenine Nucleotide, and Dense Body Content of Dog Platelets

	Serotonin (ng/10^9 platelets)	Adenine nuclotides (μM/10^{11} platelets)					No. of dense bodies/ 100 platelets (mean ± SE)
		Total	ATP	ADP	AMP	Ratio ATP/ ADP	
Dog A							
Prior to reserpine	1,245	12.3	7.2	4.4	0.75	1.63	45.6 ± 6.4
Following reserpine	31	11.8	6.9	4.2	0.72	1.63	31.4 ± 6.9
Dog B							
Prior to reserpine	2,073						47.8 ± 4.5
Following reserpine	246						34.9 ± 5.2

Discussion

The present investigation began with a study of the influence of reserpine therapy on human platelet serotonin and dense body content. We found that reserpine depleted the serotonin content of the platelets with little effect on the platelet content of ADP. However, when we studied the dense bodies in platelets from our patient on five occasions during two different courses of reserpine therapy, there was no change in either morphology or number of the opaque organelles. The result suggested that the electron opacity and preservation for ultrastructural study of human dense bodies as visualized in platelet thin sections is not primarily related to the serotonin content of these organelles.

The lack of effect of reserpine on human dense bodies was at variance with earlier studies which had demonstrated that depletion of rabbit dense bodies occurred following treatment with reserpine.[4,6,11] Therefore, we repeated the experiments on rabbits. Our studies confirmed the earlier studies showing that reserpine treatment did, indeed, decrease the dense body content of rabbit platelets. Reserpine also decreased the dense body content of dog platelets, though the effect was less marked than in rabbits. The results supported the thesis that serotonin makes an important contribution to the opacity of rabbit platelets fixed in glutaraldehyde and osmium, and also demonstrated a fundamental difference between rabbit and human platelets.

To explore the apparent difference between rabbit and human platelets, human and rabbit platelets were fixed in glutaraldehyde and osmium solutions containing EDTA to remove the calcium from the dense bodies. The EDTA largely removed dense bodies from human platelets, but left the rabbit platelets unaffected. The result is consistent with the hypothesis that the black staining of dense bodies in thin sections of rabbit

Table 4—The Influence of Fixation in Glutaraldehyde and Osmium in the Presence of 0.01 M EDTA

	Dense bodies/100 platelets		Percent change in dense bodies due to EDTA
	No EDTA	EDTA	
Rabbit platelets			
Rabbit 1	204	214	+ 5%
Rabbit 2	143	129	−10%
Rabbit 3	220	211	− 4%
Rabbit 4	132	143	+ 8%
Human platelets			
Donor 1	93	18	− 81%
Donor 2	89	9	− 90%
Donor 3	112	4	− 96%

Table 5—Biochemical Studies of the Content of Human and Rabbit Platelets*

	Human platelets (μmoles/10^{11} platelets)	Isolated rabbit platelet serotonin storage organelles (μmoles/μg total protein)
Serotonin	0.464 ± 0.079†	21.0 ± 1.7§
Calcium	27.2 ± 1.44‡	2.1§
Magnesium	5.7 ± 0.46‡	8.4§
ATP	5.4 ± 0.32‡	9.1 ± 0.1§
ADP	3.2 ± 0.38‡	1.9 ± 0.1§
Histamine	Traces§	9.0 ± 0.1§

* The values for human platelets and rabbit organelles shown here are not exactly comparable since the human results are based on whole platelets and the rabbit data is based on isolated organelles, and since the human values are expressed per 10^{11} platelets, while the rabbit results are expressed per microgram protein. Nevertheless, most platelet serotonin and calcium is believed to be found in dense bodies. Thus, the relative molar concentrations of calcium and serotonin in human platelets is 1:59, while the same ratio in the rabbit organelles is 10:1.

† Gerrard *et al.*, 1974.[26]

‡ Holmsen *et al.*, 1975.[27]

§ Da Prada *et al.*, 1975.[28]

platelets is related primarily to their serotonin content, whereas the opacity of dense bodies in thin sections of human platelets is related primarily to calcium content. The observations agree with present biochemical information (Table 5),[26-28] which reveals that human serotonin containing dense bodies are rich in calcium, whereas rabbit dense bodies are rich in serotonin but have a lesser calcium content.

As pointed out in an earlier study using the whole mount technique, human platelet dense bodies are inherently electron dense, as would be expected from organelles rich in calcium. In contrast, rabbit dense bodies examined by the whole mount technique in the present study are either not inherently electron dense or are much less opaque than human organelles. There are several possible explanations for the gray granules seen on whole mounts of rabbit platelets. Reserpine, while removing the serotonin, may leave behind the calcium and magnesium which confer the gray appearance to the organelles seen on whole mount. However, it is also possible that the gray granules represent the α-granules or a subpopulation of these organelles. Further experiments will be necessary to distinguish among these possibilities.

Our findings may resolve a conflict that has existed in the literature for some time as to whether the black appearance of dense bodies in platelets fixed with glutaraldehyde and osmium is due to calcium or serotonin. The problem appears to be related to a species variation and to the ionic composition of the buffer used to fix platelets. In human platelets which contain substantial quantities of calcium and a relatively low amount of serotonin the opacity of the dense body is due primarily to

calcium. In rabbit platelets which contain a large amount of serotonin, the black dense body is due primarily to serotonin. It is probable that the nature of the molecular interactions resulting in serotonin and calcium binding are fundamentally different in the rabbit and human platelet dense body. For instance, some evidence has been presented to suggest that serotonin is bound together with ATP,[28] while calcium is bound with ADP.[27] Our observations that reserpine partially depletes platelet ATP concomitantly with serotonin but has no effect on dense body calcium or ADP content are consistent with these hypotheses. However, additional factors may be important in determining the species variability of dense body composition, and analytical electron microscopic studies currently in progress may resolve the remaining questions.

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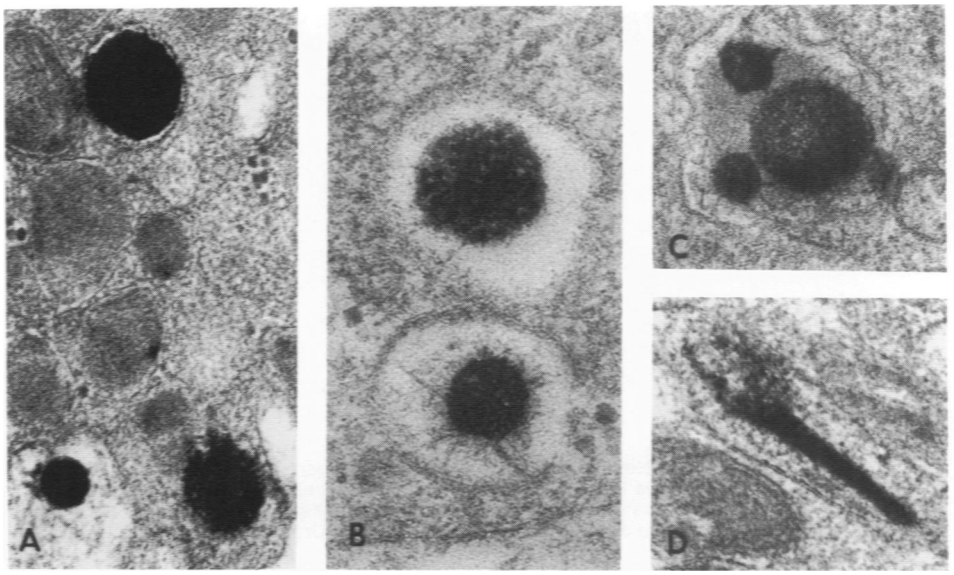

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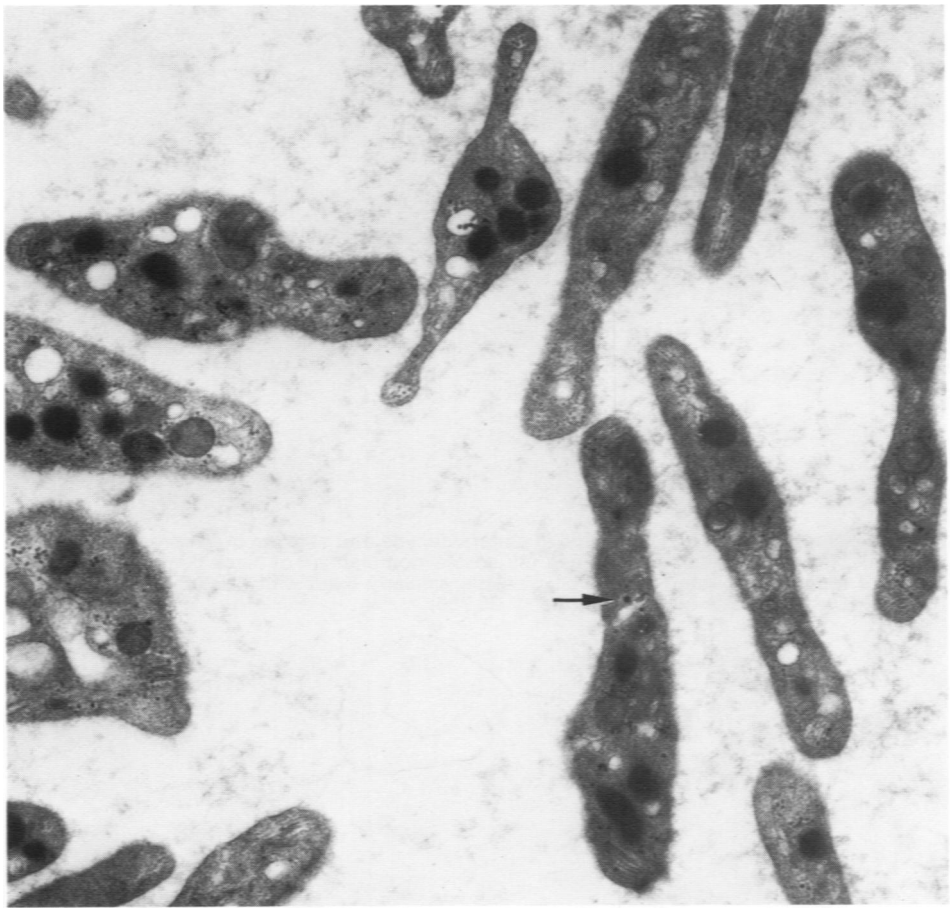

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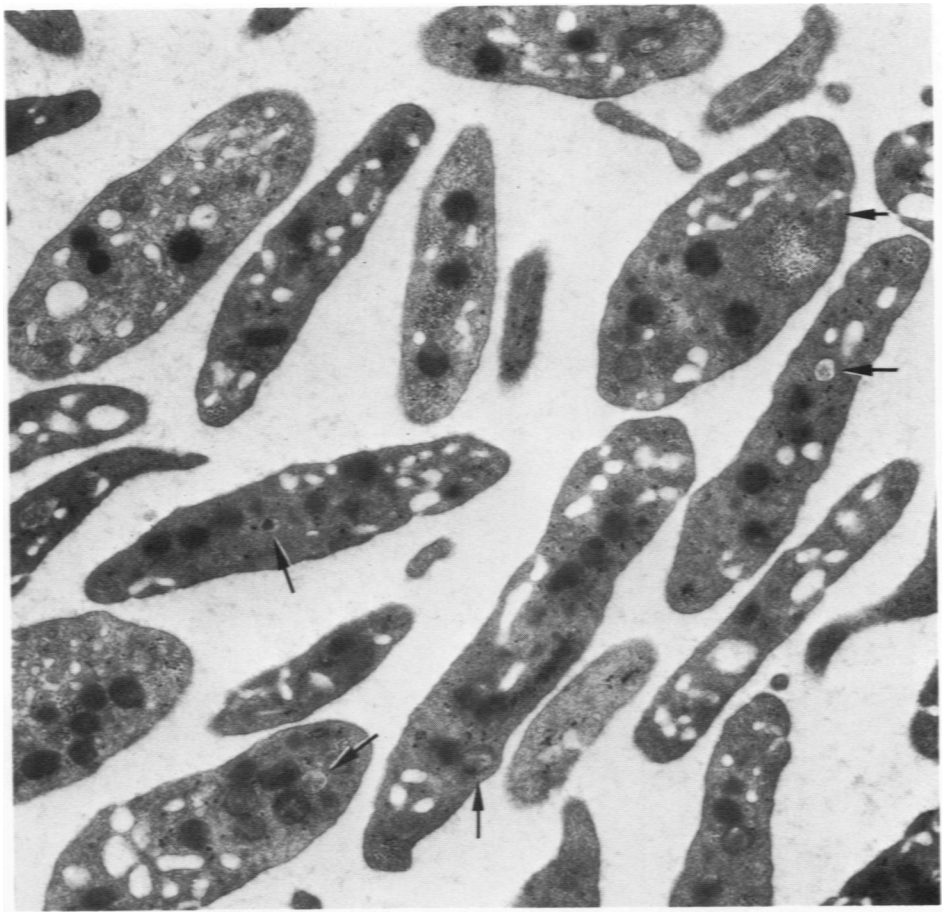
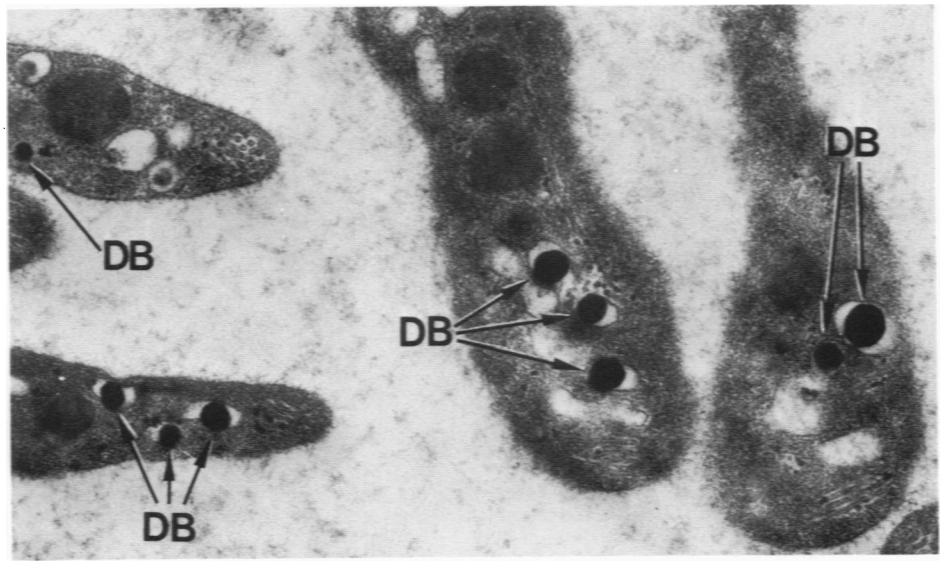
Figures 1A-D—Human platelet dense bodies from a patient receiving reserpine therapy. Reserpine treatment had no effect on the number or morphology of the dense bodies. The granular structure of human dense bodies is particularly apparent in Band C. In D, a dense body tail is visible. Such dense body tails are seen frequently in human platelets. (Uranyl acetate and lead citrate, A, × 58,100; B, × 72,000; C, × 85,000; D, × 74,500) **Figure 2**—Platelets from a rabbit treated with reserpine. Dense bodies are missing from most platelets. One small shrunken remnant dense body can be seen (*arrow*). (Uranyl acetate and lead citrate, × 20,800)

Figure 3—Normal human platelets fixed in glutaraldehyde and osmium in the presence of 0.01 M EDTA. These platelets have lost almost all their dense bodies. In several places (*arrows*) granules containing fibrillar material can be seen . These are likely the remnants of the dense bodies left after removal of the opaque calcium by the EDTA. (Uranyl acetate and lead citrate, × 17,500)

Figure 4—Normal rabbit platelets fixed in glutaraldehyde and osmium in the presence of 0.01 M EDTA. These platelets have retained the normal complement of black dense bodies (*DB*) and are indistinguishable from rabbit platelets similarly fixed with calcium present in the fixative. (Uranyl acetate and lead citrate, × 35,750)

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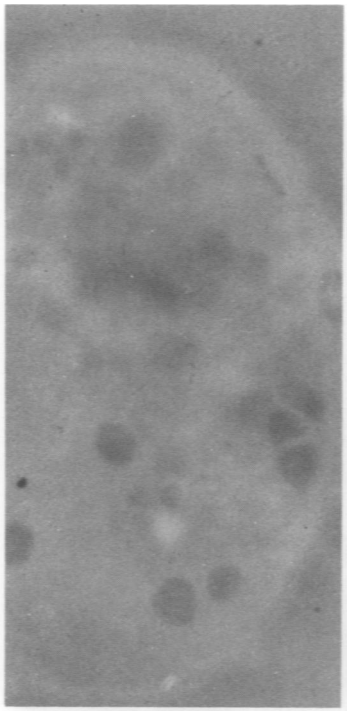

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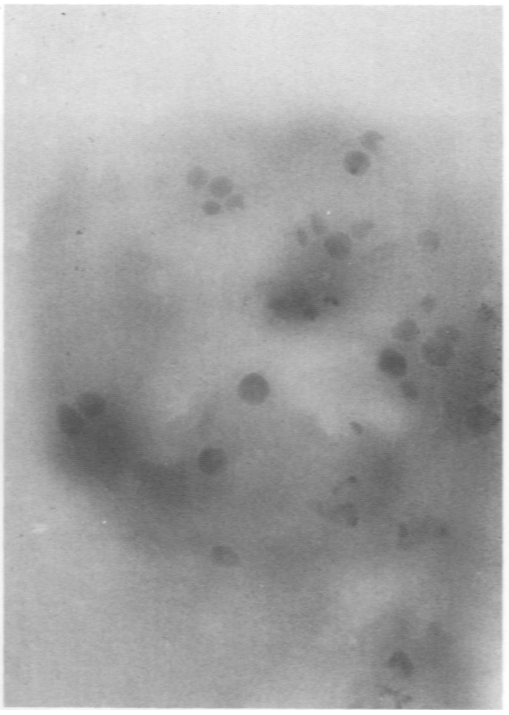

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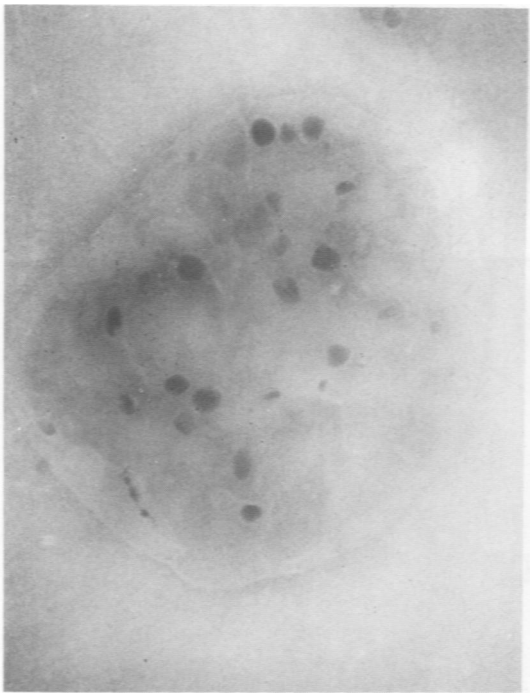

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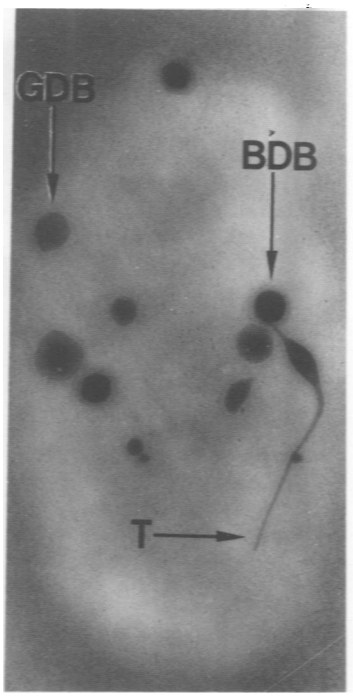

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Figures 5 and 6—Unstained whole mounts of normal rabbit platelets. Gray organelles are visible in these platelets. These organelles varied somewhat in their relative electron opacity but never achieved the deep black characteristic of most human dense bodies. (**5**, × 36,000; **6**, × 22,000) **Figure 7**—An unstained whole mount of rabbit platelet following reserpine treatment. The gray granules present in normal rabbit platelets are still present and appear unchanged in size, shape, or number. Thin sections of platelets from this rabbit taken at the same time showed a picture identical to that seen in Figure 3. Whole mounts prepared from 3 other rabbits similarly treated with reserpine showed a similar appearance. (× 22,000) **Figure 8**—An unstained whole mount of a normal human platelet. Most normal human dense bodies appear solidly black (*BDB*). The opacity of these dense bodies is, however, somewhat variable, and several of the dense bodies seen in this platelet are somewhat lighter in appearance and appear similar to the gray granules visible in whole mounts of rabbit platelets (*GDB*). One dense body with a tail (*T*) can be seen. (× 22,000)