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EFFECTS OF MEDIA OF LOW OSMOLARITY ON THE DENSE BODIES OF HUMAN PLATELETS

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Abstract—Exposure of human platelets to buffer at 30 milliosmoles (mOs) caused marked swelling of the cells (a volume increase of almost 300% as estimated from electronic sizing) without a statistically significant loss of dense bodies or a change in the measured diameter of the dense-body core (as examined electron-microscopically in air-dried whole mounts). Even biochemically isolated dense bodies maintained the integrity of their electron-opaque cores in 30 mOs buffer. However, exposure of platelets to 30 mOs buffer reduced the platelet content of cytoplasmic ^{14}C-labeled nucleotides and the vesicular content of [^{3}H]5-hydroxytryptamine (5-HT) by 90%. It thus seems likely that the dense-body membrane displays a sufficiently low permeability to water to prevent swelling and dissolution of the core, but that exposure to hypotonic media allows stored 5-HT to cross the vesicular membrane. Impermeability of this membrane to 5-HT under normal conditions may, therefore, play an important role in the maintenance of vesicular 5-HT stores, rather than the presence of an intact dense-body core. The data also suggest that an "osmotic lysis" or "chemiosmotic" model of secretion may not account for the exocytotic release of human platelet dense bodies.

Human platelets are known to increase in volume when exposed to media with osmolarities lower than physiologic levels [1–3]. The swelling observed at 200 milliosmoles (mOs), which is partly reversible in normal platelets, has been termed the "hypotonic stress test", and has been suggested to provide a useful indicator of platelet viability following storage [4–7]. To explore further the nature of the platelet response to osmotic stress, we have examined the effects of hypo-osmotic media on the number, size, and 5-hydroxytryptamine (5-HT) content of dense bodies in human platelets.

MATERIALS AND METHODS

Whole blood was collected in citrate-EDTA [8], and platelet-rich plasma (PRP) was prepared as described previously [9]. For evaluation of platelet 5-HT and nucleotides, PRP was incubated with 10^{-8} M [^{3}H]5-HT creatinine sulfate (New England Nuclear Corp., Boston, MA, 28.2 Ci/mmole) and 2×10^{-7} M [^{14}C]adenine (Amersham Corp., Arlington Heights, IL; 559 mCi/mmole) for 30 min at 37°. Platelets were pelleted, resuspended in buffers with osmolarities ranging from 300 to 30 mOs, held at room temperature, and examined as described below. Tris–citrate buffer at an osmotic strength of 300 mOs contained 116 mM NaCl, 4 mM KCl, 1.8 mM KH_2PO_4, 1.1 mM $MgSO_4$, 10.9 mM trisodium citrate, 29 mM Tris, 5.9 mM dextrose, and 0.35% bovine serum albumin (crystallized and lyophilized; Sigma Chemical Co., St. Louis, MO), and was adjusted to pH 7.35 with hydrochloric acid [10]. Buffers of lower osmolarity were prepared by dilution of the 300 mOs buffer with various amounts of distilled water. Osmolarities were checked on a freezing-point depression osmometer (Osmette, Precision Systems, Sudbury, MA).

For measurement of the disposition of platelet radioactivity, platelet aliquots were incubated for 2 min at 25° with no additions or with 2 units/ml of human thrombin (courtesy of Dr. David Aronson, Bureau of Biologics, Food and Drug Administration, Bethesda, MD) and then fixed with formaldehyde (1.5% final concentration) as described previously [11]. Platelets were pelleted; platelet label was mobilized with 0.4 N $HClO_4$ and counted on a Tracor Analytic Mark III liquid scintillation counter set to provide automated quench correction and calculation of carbon-14 and tritium (Tracor Analytic, Des Plaines, IL).

For evaluation of platelet dense body size and numbers, platelets were resuspended in the buffer described above plus 5% Stractan (arabino-galactan polymer; courtesy of Dr. L. Corash, NIH Clinical Center, Bethesda, MD). Some aliquots were diluted 1:10 with distilled water containing 5% Stractan, and air-dried whole mounts were prepared and examined in the electron microscope as described elsewhere [12–14]. Immediately prior to sizing, platelet aliquots were further diluted 1:1000 either with Isoton II (Coulter Electronics, Hialeah, FL) ("unfixed platelets") or with Isoton II containing 0.1% glutaraldehyde (Fisher Scientific Co., Silver Spring, MD) ("fixed platelets") [15]. Platelet size measurements were made according to published methods [16, 17], utilizing an Electrozone Celloscope with a logarithmic amplifier and a 30 μm orifice (Particle Data Corp., Elmhurst, IL) coupled to a PDP 8 M computer (Digital Equipment Corp., Waltham, MA).

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Table 1. Comparison of the effects of normal and hypo-osmotic media on the average platelet size and on the number, distribution and diameter of dense bodies in human platelets

Composition of medium	Platelet size: Mean diameter of size distribution (μm, mean ± S.D.) Unfixed platelets	Platelet size: Mean diameter of size distribution (μm, mean ± S.D.) Fixed platelets	Platelet size: Percent of size distribution below 1.65 μm in diameter Unfixed platelets	Platelet size: Percent of size distribution below 1.65 μm in diameter Fixed platelets	No. of dense bodies per 100 platelets	Mean diameter of dense bodies (nm, mean ± S.E.M.)
Control (300 mOs)	2.33 ± 0.14	2.28 ± 0.16	6	10	631	172 ± 6
90% H_2O (30 mOs)	2.94 ± 0.24	3.15 ± 0.23	7	4	516*	165 ± 6†

* Distributions of dense bodies per platelet were not significantly different (non-parametric statistics, Smirnov T_1 value of 0.17).

† Not significantly different from control value (unpaired, Student's *t*-test, two-tailed).

Dense bodies were isolated by a modification of the procedure of Fukami *et al.* [18]. PRP was obtained by plateletphoresis on a Haemonetics model 30, using acid–citrate–dextrose (ACD; NIH Formula A) as the anticoagulant [19]. A final concentration of 1 mM disodium EDTA was added, and the platelets were pelleted and resuspended at a cell density of 1–2 × 10^8/ml in the Tris–citrate buffer described above. Cells were again pelleted, resuspended in 10 ml of 0.25 M sucrose containing 10 mM Tris–H_2SO_4, 5 mM EDTA, and 2 mM ATP at pH 7.4 (sucrose–Tris buffer), and kept at 0° while being sonicated at the "3" power setting for 25 sec (Branson model W-140 Sonifier with a 3 mm tip). The sonicate was spun at 11,500 *g* for 30 min, resuspended in 0.5 ml of 0.25 M sucrose–Tris, and layered over a continuous density gradient of Metrizamide (Accurate Chemical Co., Hicksville, NY). Gradients were formed in nitrocellulose tubes from iso-osmotic 16% Metrizamide Tris–citrate buffer at pH 7.4, and iso-osmotic 35% Metrizamide in 10 mM TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; Sigma Chemical Co.) at pH 7.4. Gradients were centrifuged for 60 min at 100,000 *g* in a swinging-bucket rotor (Beckman SW-40), and the appropriate bands were aspirated with a Pasteur pipette whose tip had been bent at a 90° angle. The dense-body fraction was diluted to 10 ml with 0.25 M sucrose–Tris, centrifuged at 11,500 *g* for 20 min, and resuspended in 1 ml of 0.25 M sucrose–Tris. Aliquots of the resuspended dense bodies were diluted 1:100 (vv) with Tris–citrate buffer containing 5% Stractan at osmolarities ranging from 30 to 300 mOs. Dense-body whole mounts were made by placing carbon-coated grids (E. F. Fullman Co., Schnectaday, NY) in the EM-90 rotor of an Airfuge (Beckman Instrument Co., Palo Alto, CA), adding 100 μl of the resuspended dense bodies, and centrifuging for 1 min with a driving force of 30 lb/in² (approximately 95,000 *g*). Grids were blotted dry (Whatman No. 4

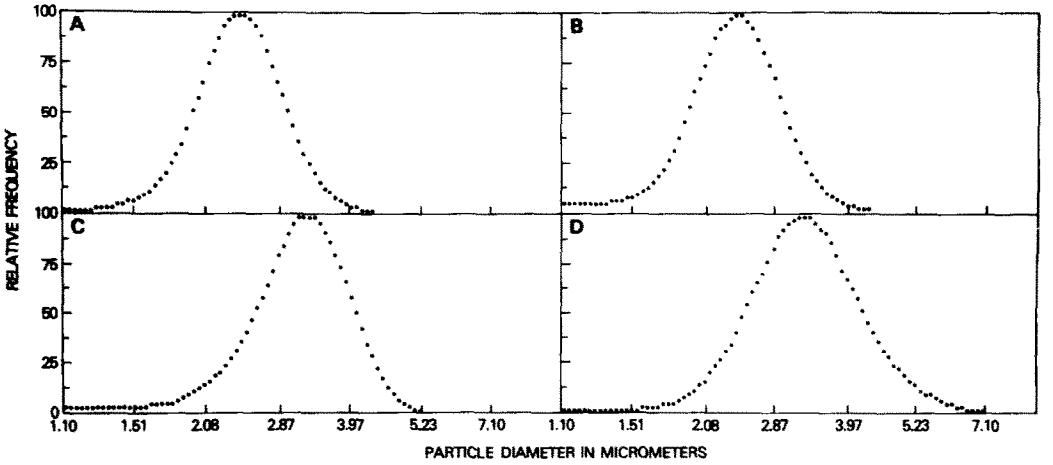

Fig. 1. Size distributions of human platelets suspended in buffers of various osmolarities and then diluted in Isoton or Isoton plus glutaraldehyde (see text for details). Distributions A and B are of platelets in 300 mOs medium diluted with either Isoton (A) or Isoton plus 0.1% glutaraldehyde (B) immediately prior to sizing. Distributions C and D are of platelets in 30 mOs medium diluted with either Isoton (C) or Isoton + 0.1% glutaraldehyde (D) before sizing.

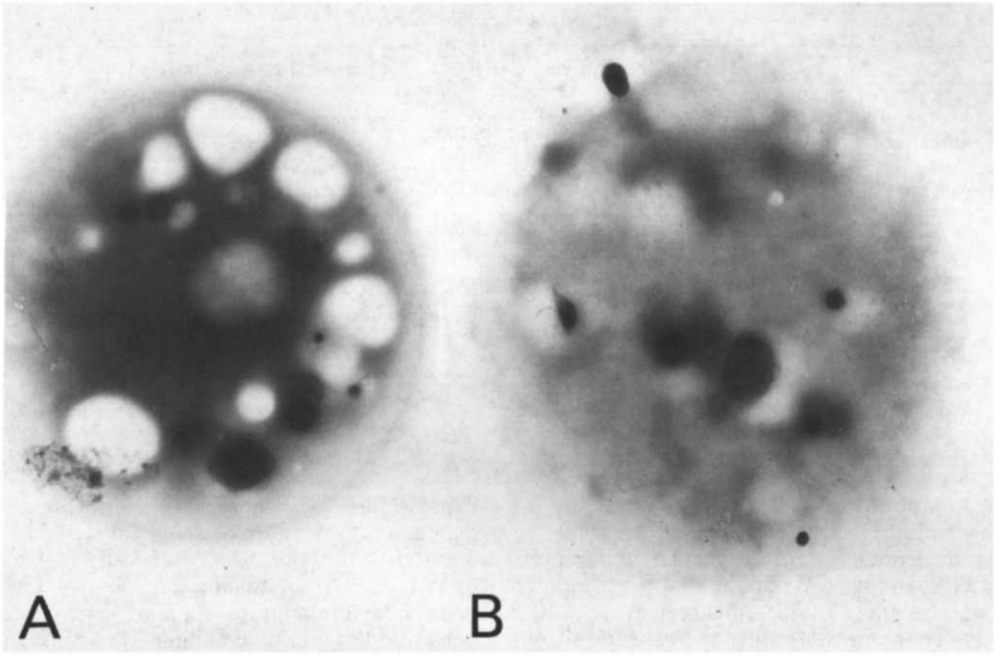

Fig. 2. Air-dried whole mounts of human platelets suspended in 300 mOs buffer plus 5% Stractan (A) or in 30 mOs buffer plus 5% Stractan (B). Both micrographs ×14,670.

filter paper) and examined in the electron microscope (Philips EM-400). Randomly selected areas were photographed at a screen magnification of 12,500 and enlarged photographically 3.1 times.

RESULTS

Platelet aliquots were exposed to a medium of a given osmolarity for 2 min, diluted 1:1000 with 300 mOs Isoton II (with or without 0.1% glutaraldehyde) and sized (Table 1 and Fig. 1). Platelets at 300 mOs had diameters which were log-normally distributed [16, 17] with a mean diameter of 2.33 μm without, and 2.28 μm with, 0.1% glutaraldehyde present in the Isoton solution. Without glutaraldehyde, only 6% of the total platelets sized had average diameters less than 1.65 μm, as compared to 10% with average diameters less than 1.65 μm with glutaraldehyde present. Platelets in buffer at 30 mOs, diluted and sized in Isoton, also had diameters which were log-normally distributed, but the distributions had a mean diameter of 2.94 μm without, and 3.15 μm with, glutaraldehyde present. Seven percent of the unfixed cells and only 4% of the fixed cells had diameters smaller than 1.65 μm. The platelet counts of cells in either the 300 mOs or 30 mOs buffer, obtained using the same instrument as for sizing, were identical (they averaged 2.6×10^8 cells/ml for the experiment presented in Fig. 1), and there was no evidence of debris in the size range from 1.0 to 1.7 μm in either sizing distribution.

We concluded that platelets exposed briefly to a 30 mOs medium swelled to at least 2.9 times their original volume, but did not lyse. Since our measurements were made when cells were returned to physiological osmolarity (that of the Isoton), the platelets may have had a larger volume at 30 mOs but have decreased somewhat in volume prior to sizing. This is suggested by the fact that cells placed in Isoton plus glutaraldehyde had significantly larger volumes than those placed in Isoton alone.

As seen in the electron microscope, whole mounts of cells suspended in 5% Stractan plus 30 mOs buffer appeared larger than cells in 300 mOs buffer plus 5% Stractan (Fig. 2). In a random sample of 100 platelets from the 30 mOs medium, the total number of dense bodies, the distribution of dense bodies per platelet, and the mean diameter of the dense-body electron-opaque core were all not significantly different from those measured in 100 platelets from the 300 mOs medium (Table 1).

Platelet aliquots were also labeled with [^{3}H]5-HT and [^{14}C]adenine, resuspended, incubated in buffers of progressively lower osmolarities for 2 min at room temperature, and then fixed with formaldehyde (Table 2). Under the labeling conditions employed, essentially 100% of the [^{3}H]5-HT enters the vesicular (dense-body) compartment, which is released to a variable amount (typically ranging from 70 to 90%) by brief thrombin treatment [10, 20]. Platelets incubated with [^{14}C]adenine are known to incorporate the label into a pool of metabolic (extra-vesicular) adenine nucleotides, and to lose 10–20% from the cell by the formation of hypoxanthine or inosine following brief thrombin treatment [21, 22]. Platelets in buffers as low as 150 mOs maintained control levels of both [^{3}H]5-HT and metabolic ^{14}C-labeled nucleotides. Since the percent loss of both components following brief thrombin treatment was also unchanged, the cells apparently retained both their metabolic responsivity to thrombin and their content

Table 2. Effect of media of various osmolarities on vesicular [^{3}H]5-HT and metabolic ^{14}C-labeled nucleotides in human platelets

Osmolarity of extracellular medium (mOs)	Disposition of vesicular [^{3}H]5-HT		Disposition of metabolic ^{14}C-labeled nucleotides	
	Amount present (moles/platelet × 10^{21}, mean ± S.E.M.)	Percent loss after thrombin	Amount present (moles/platelet × 10^{20}, mean ± S.E.M.)	Percent loss after thrombin
300	9.75 ± 0.14	81	7.47 ± 0.08	34
270	9.49 ± 0.37	80	7.27 ± 0.24	29
225	9.33 ± 0.25	82	7.56 ± 0.11	33
150	9.86 ± 0.27	81	7.23 ± 0.16	37
75	6.50 ± 0.46*	28	4.75 ± 0.33*	12
30	1.31 ± 0.03*	<5	1.04 ± 0.01*	<5

* Significantly different from value for 300 mOs, $P < 0.001$ (unpaired Student's t-test, two-tailed).

of 5-HT inside the vesicular compartment. At 75 mOs, both the total [^{3}H]5-HT and the total ^{14}C-labeled nucleotides were decreased by approximately 33%, and the loss of both following thrombin treatment was much less than that seen in the control platelets. Thus, the cells appeared capable of only a blunted metabolic response to thrombin. In addition, either they secreted a smaller total percentage of their vesicular pool, or they allowed some of their vesicular 5-HT to move into an extra-vesicular, non-releasable (cytoplasmic) compartment [20]. At 30 mOs, both the [^{3}H]5-HT and the ^{14}C-labeled nucleotides were decreased by almost 90%, and no measurable loss was induced by thrombin. The remaining [^{3}H]5-HT may have been non-releasable because it was all cytoplasmic (extra-vesicular) in location, or simply because the cells failed to release any dense bodies in response to the thrombin treatment.

It seemed probable that the dense-body membrane was not subjected to the same osmotic gradients as the plasma membrane when the cells were first exposed to 30 mOs buffer. To explore in a more direct fashion the osmotic sensitvity of the dense bodies, we isolated dense bodies and exposed them to media of various osmolarities (Table 3). Isolated dense bodies in isotonic buffer had a mean diameter of 155 nm, a value not significantly different from that seen in intact platelets and reported for isolated dense bodies elsewhere [14]. None of the mean diameters at lower osmolarities was significantly different from the control values. Electron micrographs of randomly selected areas of control (300 mOs) dense-body preparations contained an average of 16 dense bodies per micrograph. A similar average number per micrograph was found at 200 and 150 mOs; at 100–30 mOs, the mean number ranged from 7 to 11 per micrograph.

Table 3. Effects of media of various osmolarities on the electron-microscopic appearance of dense bodies isolated from human platelets

Osmolarity of medium (mOs)	Average diameter of dense bodies (nm, mean ± S.E.M.)	Average number of dense bodies per micrograph
300	155 ± 10	16
200	158 ± 8	14
150	157 ± 7	17
100	159 ± 10	8
75	186 ± 13	10
50	167 ± 13	11
30	148 ± 9	7

DISCUSSION

Our sizing data, as well as direct observation of platelet whole mounts in the electron microscope, indicate that human platelets do not lyse when suspended in extremely hypotonic solutions, and that the dense-body cores of calcium, pyrophosphate, and nucleotides [23] do not change in volume or appearance and are not physically lost from the cells. Even isolated dense bodies are relatively resistant to swelling or lysis in a hypotonic medium. When solid analogues of the dense-body core are prepared as aqueous slurries containing precipitates with a composition identical to that of dense bodies, the solids begin to dissolve when the slurry is diluted 1:10 with iso-osmotic saline, and dissolve completely at a 1:100 dilution [24]. One would therefore expect the entry of appreciable quantities of water into the interior of the dense body to lead to swelling and dissolution of the core material. Since dense bodies retain their physical integrity in hypotonic media, it seems probable that the vesicle membrane has a very limited permeability to water.

Nevertheless, at or below 75 mOs the vesicular and plasma membranes appear to become sufficiently permeable to permit the efflux of stored 5-HT (which is lost from the cell along with cytoplasmic nucleotides). The maintenance of 5-HT stores inside dense bodies may, therefore, under normal circumstances depend more on the relative impermeability of the membrane to 5-HT than on the binding of 5-HT to core constituents.

Given the apparent limited permeability of the dense-body membrane to water despite its ability to pass 5-HT, it seems unlikely that exocytotic secretion of human platelet dense bodies occurs by an "osmotic lysis" type of mechanism similar to that described

for isolated chromaffin vesicles [25, 26], unless the secretory stimulus leads to a dramatic increase in the permeability of the dense-body membrane to water. Our data thus appear to agree with those of Bennett *et al.* [27] in arguing against a "chemiosmotic" model for platelet secretion.

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