AN IMPROVED METHOD FOR THE ISOLATION OF DENSE STORAGE GRANULES FROM HUMAN PLATELETS

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ABSTRACT

Pretreatment of human platelets with the metabolic inhibitors rotenone and 2-deoxyglucose, before French press homogenization, has led to the isolation of dense storage granules in an overall yield of about 20%. The concentrations of serotonin, ATP and ADP were estimated in the dense granules. Serotonin was 40–60-fold enriched in the dense granules compared to the platelet homogenate. Stored ATP and ADP were also 40-fold enriched in the dense granules compared to the estimated storage nucleotide pool in intact platelets. The ATP to ADP ratio in the isolated dense granules was 0.68–0.70, the same as the ratio of the secreted ATP and ADP. In platelets prelabeled with [^{3}H]adenine, the specific radioactivities of the ATP and ADP in the isolated dense granules and of the secreted ATP and ADP were both negligible, whereas the estimated specific radioactivity of the metabolically active ATP and ADP was 2,000 cpm/nmol. These results confirm that the ATP and ADP in the isolated dense granules are the same as the secreted ATP and ADP in terms of metabolic inactivity and their ATP to ADP ratios.

KEY WORDS platelets · serotonin granules · storage ATP and ADP · subcellular fractionation

Blood platelets contain secretory granules which disappear when the cells are treated with appropriate stimuli such as ADP or thrombin (for review, see reference 13). At least two types of such granules are known to be present in platelets as shown by the selective release caused by certain of the inducing agents. ADP, for example, induces the release of serotonin, ATP, ADP, and calcium but not of lysosomal enzymes. It is considered that the former compounds are stored together in one type of granule which is different from the granules that contain lysosomal enzymes which can be released by thrombin. Radioautographic techniques have shown that serotonin is stored in the highly electron-dense granules (9), while electron microprobe X-ray spectroscopy revealed the presence of both calcium and phosphorus in them (21, 29). Further evidence for the common storage of these compounds in the dense granules is that platelets which lack dense granules contain very much less ATP, ADP, serotonin, and calcium than normal platelets (12, 18, 19).

Dense granules isolated by subcellular fractionation from the platelets of guinea pig, rabbit (6, 7), and human have been shown to contain serotonin and ATP (8) as well as ADP (14). However, generally, the preparation of dense granules from human platelets has been accompanied by such a

high degree of solubilization (3, 5, 7, 8, 10, 11, 14, 20, 27) that the evidence for the storage of serotonin, ATP, ADP, and calcium in the same granule fraction has been either equivocal or indirect as from selective secretion studies or from platelets of patients with storage pool deficiency (12, 18, 19).

Acting on the premise that the process of homogenization itself triggers the secretion process in human platelets, we sought to inhibit release before homogenization and fractionation. It has been shown that the use of a glycolytic inhibitor together with a respiratory blocker inhibits platelet secretion (23) by depleting the metabolically active ATP without affecting the intragranular ATP and ADP (16). This approach has made it possible to prepare a granule fraction that is 40–60-fold enriched in serotonin from human platelets with only about 20% solubilization of the amine. The ATP and ADP in this fraction have also been estimated and compared with the secreted amounts, which up to now have provided the main body of data about the granule contents.

MATERIALS AND METHODS

Fractionation

Platelet-rich plasma prepared from 140 ml of blood collected into 16 ml of ACD (1) from healthy donors was usually incubated for 15 min at 37°C with 1 μM [2-^{14}C]serotonin (5-hydroxytryptamine binoxalate, 48.5 Ci/mol from New England Nuclear Corp., Boston, Mass.). Then 0.1 M sodium EDTA, pH 7.4, was added to the platelet-rich plasma to make a final concentration of 5 mM, and the platelets were centrifuged at 3,000 g × 10 min at 4°C. The platelet pellet was suspended in an ice-cold, modified Tyrode's solution, pH 6.5, without calcium and with 1 mM EDTA, 0.1% bovine serum albumin (Calbiochem, San Diego, Calif., fraction V) and 2 mM $MgCl_2$. After centrifugation at 3,000 g × 10 min at 4°C, the platelets were resuspended in 15 ml of the same medium and incubated with the inhibitors 20 μM rotenone (Sigma Chemical Co., St. Louis, Mo.) and 5 mM 2-deoxyglucose at 37°C for 20 min. Then, 3 mg of Nagarse (Enzyme Development Corp., New York) and 10 mg of ATP were mixed into the suspension at room temperature.[1] 5 min later, 20 mg of soybean trypsin inhibitor (Sigma) was added to neutralize the nagarse, and after the suspension was stirred well, the platelets were centrifuged at 3,000 g × 10 min at 4°C. The platelet pellet was then resuspended in 20 ml of medium consisting of 0.25 M sucrose, 10 mM Tris chloride and 1 mM sodium EDTA, pH 7.4, 0°C (isolation medium). The platelet suspension was homogenized twice at 1,000 pounds per square inch in a French pressure cell as described previously (25) with one centrifugation in between the two breaks so that only the unbroken cells were subjected to the second homogenization. Differential centrifugation of the combined homogenate resulted in the isolation of the following fractions: F_1, 1,000 g × 22 min pellet (whole platelets and large fragments); F_2, 12,000 g × 20 min pellet (mitochondria and granules); F_3, 100,000 g × 60 min pellet (membranes); and F_4, the 100,000 g supernate (all soluble material).

The granule fraction, F_2, was resuspended in 2.5 ml of the sucrose isolation medium, and 1.5 ml of this suspension was further fractionated on a sucrose step gradient that increased from 0.8 to 2.0 M in 0.2-M increments as described previously (26).

In some of the experiments, platelets were prelabeled for 30 min at room temperature in platelet-rich plasma with 0.2 μM [G-^{3}H]adenine (6,000 Ci/mol from New England Nuclear Corp. [15]); part of the washed platelet suspension was reserved for secretion studies, and the rest was fractionated as described above.

Secretion Studies

0.2 ml portions of the suspension of washed platelets that were not treated with rotenone and 2-deoxyglucose were incubated at 37°C for 5 min in the presence and absence of 5 U of thrombin. The platelets were removed from the medium by centrifugation for analysis of the secreted substances in the supernatant solution and compared to total content.

Assays

β-*N*-Acetylglucosaminidase, acid phosphatase, and α-glycerophosphate oxidase were assayed by standard methods (10, 24) as described previously (26). [^{14}C]Serotonin was monitored by adding 20–50-μl aliquots of sample to 15 ml of a toluene/Triton X-100 (2:1) cocktail with 6 g/liter of 2,5-diphenyloxazole (PPO) and 75 mg/liter of 1,4-bis[2(5-phenyloxazole)] (POPOP) for counting in a Nuclear-Chicago liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). The absolute amounts of serotonin were estimated in a few experiments by the butanol extraction-HCl fluorescence method described by Weissbach et al. (30) in the platelet homogenate, F_2 and in the pellet at the bottom of the 2.0 M sucrose gradient and was found to be proportional to the counts per minute. ATP and ADP were assayed by the firefly method (17) or fluorimetrically (33) in extracts prepared by the addition of one portion of sample to two portions 10 mM EDTA in

[1] Granule preparations in which the nagarse pretreatment was omitted could not be resuspended well and did not separate on the sucrose gradient to give a pellet. The use of ATP with the nagarse was arrived at empirically; for some reason ATP seems to prevent platelet clumping that often occurs with nagarse addition (26).

86% ethanol at 0°C. Lactate dehydrogenase was assayed by following NADH oxidation at 340 nm after the addition of pyruvate. Proteins were determined by Miller's modification (22) of Lowry's method; albumin standards were run at each sucrose concentration of the gradient.

Electron Microscopy

Fractions F_1, F_2, and F_3 and subfractions A-P (from the sucrose density gradient) were fixed in 2.5% glutaraldehyde in 0.067 M cacodylate buffer, pH 7.4, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol and embedded in Epon 812. Sections were stained with uranyl-acetate followed by lead citrate. Addition of sucrose to the glutaraldehyde to increase the osmolality had no effect on the appearance of the samples.

RESULTS

Initial attempts at the isolation of human platelet dense granules were made by the direct application of the method developed in this laboratory for pig platelet fractionation (26). Unlike pig platelets, human platelets aggregated and released more than 90% of their serotonin content to the extracellular medium during the centrifugation after the nagarse pretreatment. Addition of trypsin inhibitor after the nagarse pretreatment resulted in occasional preparations that did not aggregate and release, but the yield of serotonin in the granule fraction was extremely variable (see Table I). In experiments 1 and 2 (Table I), about half of the serotonin was found in the soluble phase, but the amount in the granules differed sharply. In experiments 3–6, the homogenization pressure was decreased to 800 pounds per square inch to try to reduce serotonin release, with the result that fewer platelets were disrupted than at 1,000 pounds per square inch, and the granule yields remained low.

At this point, it was decided to try metabolic inhibitors in combination with a homogenization pressure of 1,000 pounds per square inch to determine whether the high degree of serotonin solubilization was due to secretion or to organelle damage. The optimal conditions for inhibition of platelet secretion were first established by incubating platelets with rotenone, a mitochondrial inhibitor, and 2-deoxyglucose, an inhibitor of glycolysis; at various times, thrombin was added to aliquots of platelet suspension to induce secretion. Table II shows that thrombin-induced secretion in washed platelets was maximally inhibited by rotenone and 2-deoxyglucose in about 15 min.

Platelets pretreated with inhibitors for 20 min as described in Table II before the nagarse treatment did not aggregate or release upon centrifugation. In addition, some 30% of the total platelet serotonin was found in the crude granule fraction (F_2) after homogenization (Table I). Attempts to improve this yield by increasing the homogenization pressure and rupturing more of the platelets that appeared in the low-speed pellet (F_1) resulted only in greater serotonin solubilization. Although the membrane fraction (F_3) was not separated

TABLE I

Effect of Metabolic Inhibitors on [^{14}C]Serotonin Distribution in Primary Fractions

	Exp no.	F_1 (Cells & Debris)	F_2 (Granules)	F_{3+4} (Membranes + Cytosol)
		%	%	%
No Inhibitors	1	12.4	23.9	63.7
	2	21.9	5.2	46.8
	3	55.0	11.4	21.8
	4	68.8	11.2	20.0
	5	70.2	4.6	25.1
	6	72.4	7.8	19.7
Rotenone 2-Deoxyglucose Mean (n = 9) ± SEM		31.4 ±1.83	34.9 ±2.10	32.7 ±2.44

Platelets were homogenized as described in Materials and Methods with or without preincubation with rotenone 20 μM and 2-deoxyglucose 5 mM at 37°C for 20 min. The first two experiments without inhibitors were carried out at homogenization pressures of 1,000 pounds per square inch and the next four at 800 pounds per square inch. The nine experiments with inhibitors were carried out at 1,000 pounds per square inch.

TABLE II

Inhibition of [^{14}C]Serotonin Secretion by Rotenone and 2-Deoxyglucose

Incubation time (*min*) before thrombin addition	0	5	10	20	30
	%	%	%	%	%
Control	59.0	61.23	60.20	64.15	48.00
SEM	±7.86	±8.26	±7.78	±8.02	±8.62
Rotenone + 2-deoxy-glucose	19.68	12.03	6.08	5.08	2.93
SEM	±9.12	±6.54	±3.08	±1.98	±0.62

Rotenone 20 μM and 2-deoxyglucose 5 mM (final concentrations) were added to one of two identical portions of platelet suspensions (about 1.5–2.0 mg of protein/ml) that had been preincubated at 37°C for 10 min. Aliquots of 0.5 ml were taken from the suspension to which the inhibitors were added as well as from the control suspension at the times noted and challenged with 0.5 unit of thrombin. The aliquots were centrifuged after 10 min at 37°C and the amounts of [^{14}C]serotonin present in the supernates were compared to those in the whole suspension. The results are presented as means ± SEM (n = 4).

TABLE III

Distribution of [^{14}C]Serotonin

	F_1	F_2	F_3	F_4	F_{3+4}
n	9	9	5	5	4
			% of Total homogenate		
Mean	31.44	34.89	9.84	20.60	32.66
SEM	1.83	2.10	1.49	2.66	2.44

	A	B	C	D	E	F	E + F	P
n	9	9	9	9	7	7	2	9
				% of F_2 on sucrose density gradient				
Mean	11.89	3.22	4.56	5.56	5.14	8.43	11.50	61.44
SEM	0.92	0.28	0.63	0.44	0.83	1.11	2.50	1.49

The mean ± S.E.M. is given for the percent of [^{14}C]serotonin recovered in each fraction and subfraction in nine preparations. In four fractionations, F_3 and F_4 were not separated, and in two preparations, subfractions E and F were not collected separately.

from the soluble fraction (F_4) in these preliminary studies, this separation was carried out after the optimal fractionation conditions were established, and only 20% of the serotonin was found to be solubilized (see Table III).

Table IV shows the distribution of [^{14}C]serotonin, acid phosphatase, β-*N*-acetylglucosaminidase and α-glycerophosphate oxidase in a typical fractionation. Fraction 2 contained the most protein of the particulate fractions, the largest amounts of the assayed components and less than 1% of the lactate dehydrogenase (not shown). The specific activities of the marker enzymes in Table IV also peaked in fraction 2, although the concentration of serotonin in cpm/mg of protein was greater in fraction 1 which consisted of whole platelets and large fragments.[2] Electron micrographs showed fraction 2 to consist primarily of platelet mitochondria, granules, and membrane vesicles (Fig. 1*a*).

Fraction 2 was centrifuged on the sucrose density gradient described in Materials and Methods, and five bands of material plus a small pellet were visible (Fig. 2). Seven subfractions, A–F and P, were collected as indicated, and the assayed components were found to be distributed as shown in

[2] The distribution of particle size as determined with a Coulter Counter Z_BI in washed platelets showed the same profile as fraction 1 and indicated that the homogenization procedure was not selective with respect to platelet size.

TABLE IV

Distribution of Marker Components in Primary Fractions

No. 98-817 Fraction	Protein	Serotonin	Acid phosphatase*	β-*N*-acetyl-glucosaminidase*	α-Glycerophosphate oxidase‡
	mg/ml	*cpm × 10⁻³/mg*	*nmol/min/mg*	*nmol/min/mg*	*natoms/min/mg*
Homogenate	1.89	11.17	64.92	13.72	40.74
F_1	0.86 (11.6%)	28.82 (37.2%)	82.09 (15.7%)	22.40 (22.1%)	89.53 (31.0%)
F_2	2.25 (15.2%)	24.66 (41.5%)	135.38 (33.9%)	42.20 (54.5%)	146.7 (66.4%)
F_3	0.61 (8.2%)	4.30 (4.2%)	128.03 (17.4%)	23.00 (16.1%)	10.8 (2.7%)
F_4	0.32 (64.9%)	2.39 (17.2%)	29.75 (33.0%)	1.12 (7.3%)	Not measurable
Total recovery	88.9%	71.9%	83.1%	76.3%	73.4%

Activities are expressed as cpm, *p*-nitrophenol* production or oxygen‡ consumption per milligram of protein. The percentage of the total recovered activity found in each fraction is given in parentheses. All enzymes were assayed in the presence of 0.5% Triton X-100.

Table V and Fig. 2. Acid phosphatase activity was highest in the upper part of the sucrose gradient, A and B. β-*N*-Acetylglucosaminidase activity peaked in B and C, although a small amount (2.9%) peaked in the pellet. The mitochondrial marker enzyme, α-glycerophosphate oxidase, was concentrated 10-fold over the original homogenate in subfraction B. Serotonin, which was found mostly in the pellet, was enriched 49 times compared to the homogenate. The actual amount of serotonin in the pellet was 92.2 nmol/mg of protein. Since the [^{14}C]serotonin incorporation into platelets varied somewhat from preparation to preparation, the cpm/mg of protein could not be compared advantageously, but the normalized distribution of labeled serotonin in nine fractionations was very reproducible and is shown in Table III.

Electron micrographs showed that subfraction A consisted primarily of membrane and membrane vesicles, which was consistent with acid phosphatase activity, and some entrapped granules (not shown). Subfraction B was greatly enriched in mitochondria (Fig. 1*b*), which agrees with the localization of the mitochondrial marker enzyme, α-glycerophosphate oxidase (Table V, Fig. 2). Subfractions D and E consisted of α-granules that appeared to be ultrastructurally homogeneous (Fig. 3*a*); these fractions appear to contain more protein than would be accounted for by the enzyme markers used in this study and are probably enriched in some of the other proteins known to be secreted by platelets. Subfraction P, in which the greatest concentration of serotonin was found, contained several forms of granules (Fig. 3*b*). One type was the typical bull's eye, and the others were more completely filled and varied in shape from round to elongated.

Determination of the subcellular localization of the storage or secretable ATP and ADP is complicated by the presence of the metabolically active adenine nucleotides of the cell that are not secreted (1). These two pools can be distinguished by the fact that the metabolically active adenine nucleotides can be labeled in vitro by preincubation of an appropriate precursor such as [^{3}H]- or [^{14}C]adenine with platelets in plasma; under these conditions, the stored nucleotides are not labeled (15). Therefore, a series of fractionation experiments were performed with platelets in which the metabolically active adenine nucleotides were prelabeled with radioactive adenine instead of [^{14}C]serotonin.

The amounts and the specific radioactivity of the ATP and ADP in various fractions of platelets prelabeled with [^{3}H]adenine are shown in Table VI. The ratio of ATP and ADP in whole platelets was 1.64, and the specific radioactivity of the ATP + ADP was 866 cpm/nmol. When the platelets were treated with thrombin with thrombin and centrifuged out of the medium, the supernatant solution contained about 55% of the platelet ATP and ADP in a ratio of 0.68. Most of the ADP in the platelets was secreted, so that the ratio of nonsecretable ATP to ADP (the metabolically active pool) in the resting platelet was 9.63. The

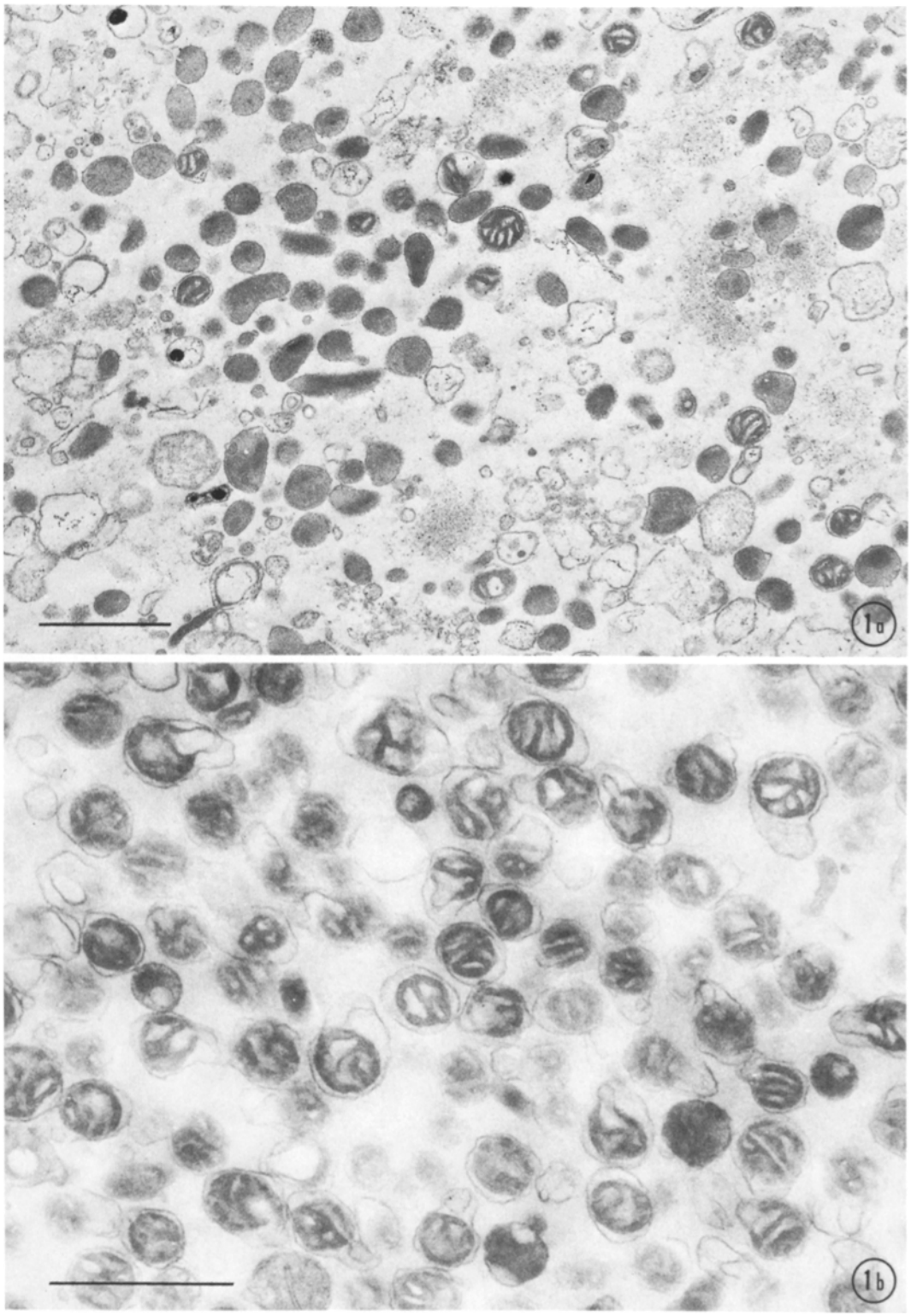

FIGURE 1 (*a*) Fraction 2 from differential centrifugation. Membrane fragments, α-granules, mitochondria, and dense granules, including bull's eyes, were all present in this fraction. Bar, 1 μm. × 20,500. (*b*) Subfraction B from sucrose density gradient. This band consisted mostly of mitochondria in the condensed form with a little contamination from α-granules. Bar, 0.5 μm. × 41,600.

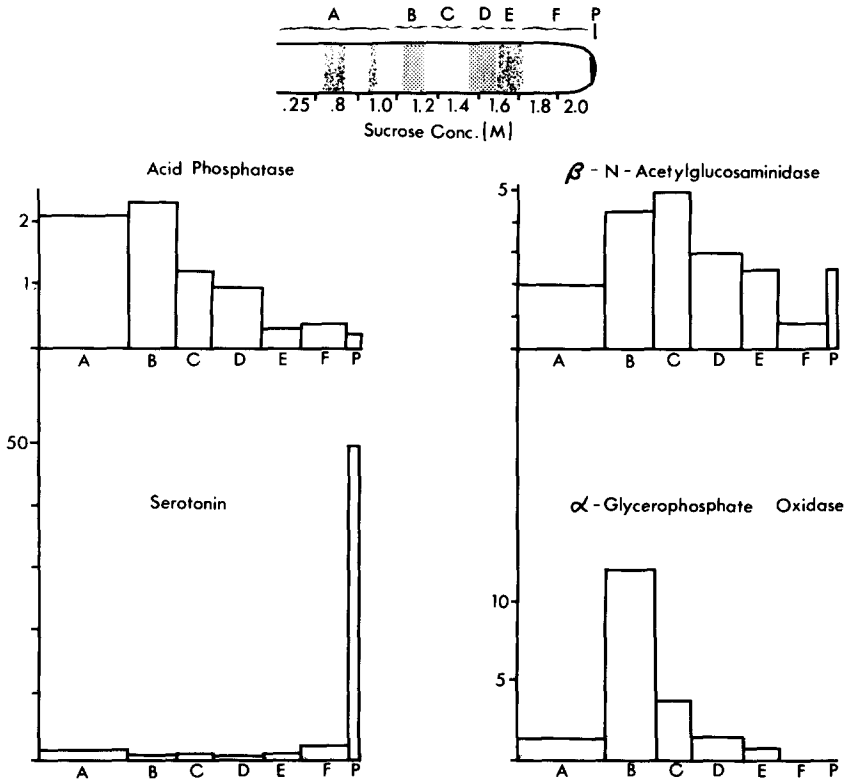

FIGURE 2 Relative specific activities of marker components in sucrose density gradient subfractions. A diagram of the sucrose density gradient and the subfractions into which it was divided is shown (top center). The relative specific activities of the components were calculated as the quotient of the specific activity in a subfraction divided by the specific activity of the homogenate as given in Tables I and II.

TABLE V

Distribution of Marker Components in Subfractions of Fraction 2

No. 98-817 Fraction	Protein	Serotonin	Acid phosphatase	β-*N*-acetylglucosaminidase	α-Glycerophosphate oxidase
	mg/ml	*cpm* × 10^{-3}*/mg*	*nmol/min/mg*	*nmol/min/mg*	*natoms/min/mg*
F_2	2.25	24.66	135.38	42.20	146.7
A	0.22 (27.3%)	17.52 (15.4%)	134.78 (43.8%)	28.30 (20.3%)	49.1 (11.4%)
B	0.39 (15.5%)	7.50 (3.7%)	144.94 (26.8%)	58.41 (23.8%)	488.5 (64.6%)
C	0.40 (11.3%)	8.61 (3.1%)	78.48 (10.6%)	68.45 (20.4%)	137.5 (13.3%)
D	0.56 (15.8%)	7.98 (4.1%)	59.93 (11.3%)	40.95 (17.0%)	59.2 (8.0%)
E	0.41 (11.6%)	9.21 (3.4%)	18.68 (2.6%)	34.02 (10.4%)	26.8 (2.7%)
F	0.21 (15.3%)	26.90 (13.3%)	24.62 (4.5%)	11.83 (5.1%)	Not measurable
P	0.11 (3.2%)	548.50 (57.0%)	12.98 (0.5%)	34.39 (2.9%)	Not measurable
Total recovery	104.6%	131.8%	64.9%	94.2%	83.6%

Activities and recoveries are expressed as in Table I.

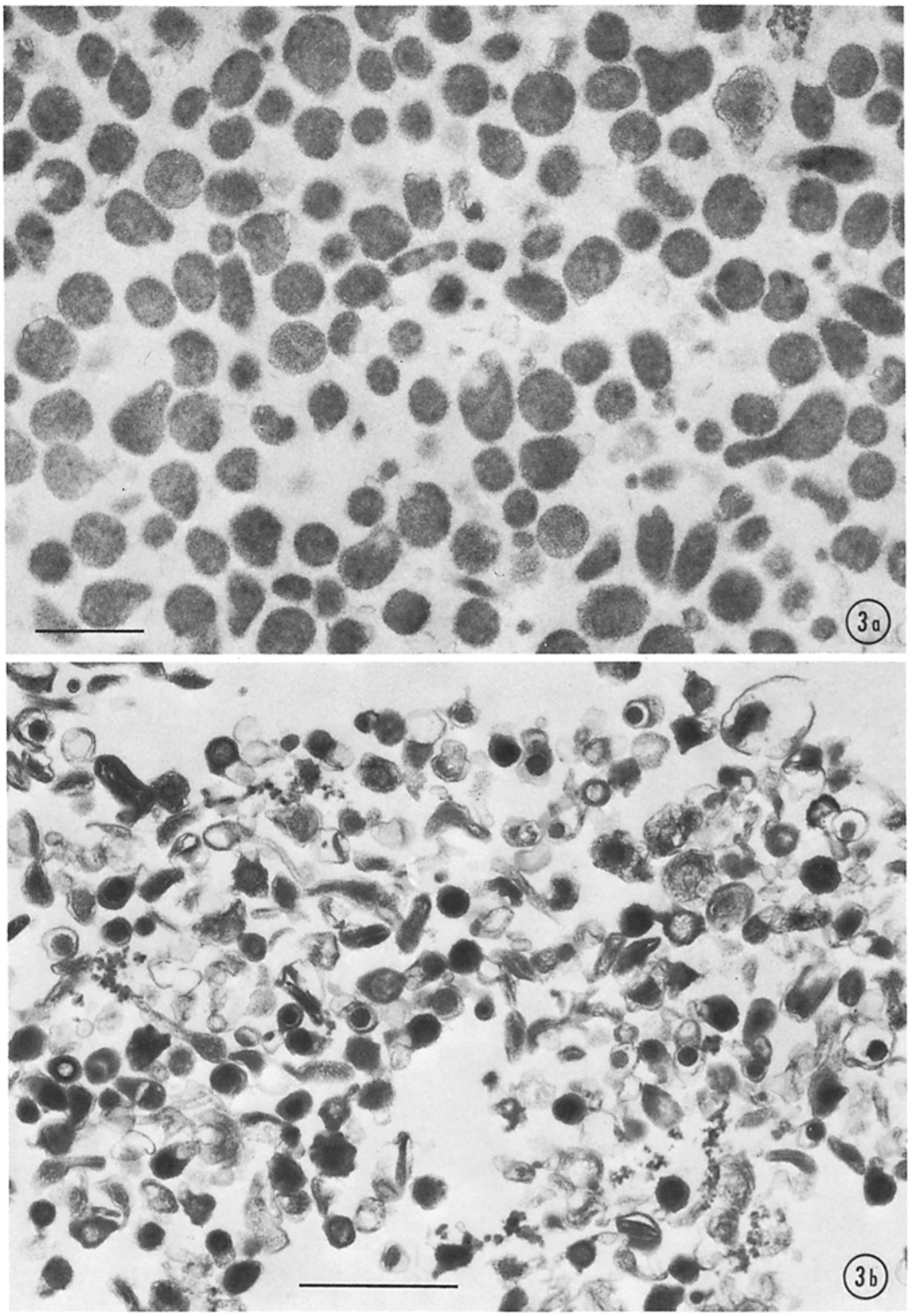

FIGURE 3 (*a*) Subfraction D from sucrose density gradient. This band consisted of α-granules with very little contamination with other organelles or membranes. The granules were round, elongated, or racket shaped. Bar, 0.5 μm. × 32,500. (*b*) Pellet (Subfraction P) from sucrose density gradient. This fraction was greatly enriched in dense granules of the bull's-eye type compared to the starting material, Fig. 1*a*. However, there were also a few membrane fragments and numerous other granules (see Discussion section about their possible identity). Bar, 1 μm. × 25,000.

TABLE VI

Localization of Storage Pool ATP and ADP

	ATP	ADP	$\frac{ATP}{ADP}$	ATP + ADP
	nmol/mg of protein	*nmol/mg of protein*		*cpm/nmol*
Whole platelets	23.8 ± 3.45	14.5 ± 2.37	1.64	866
Platelet supernate after thrombin*	9.71 ± 1.31	14.1 ± 1.76	0.68	40
Whole platelets minus platelet supernate after thrombin	14.17 ± 3.01	1.47 ± 0.47	9.63	2,236
Crude granule fraction (F_2)	16.15 ± 5.42	26.69 ± 7.09	0.60	174
Dense granules (P)	441 ± 95.9	633 ± 142	0.70	28

Secretion experiments were performed on aliquots that were reserved before treatment of platelets with rotenone and 2-deoxyglucose for homogenization. The secreted ATP and ADP which appeared in the platelet supernate after 5 units of thrombin was added had a low specific radioactivity as did the dense granule material. The ATP and ADP concentrations are given as the means ±SEM., $n = 5$. The ATP/ADP ratios are derived from the means. The specific radioactivities are the average of two determinations. (ATP and ADP were separated by high voltage paper electrophoresis [16].)

* The concentrations of ATP and ADP in the platelet supernate are expressed in terms of whole platelet protein rather than secreted protein.

secreted ATP and ADP did not exchange rapidly with the labeled pool and had a specific radioactivity of 40 cpm/nmol compared to 2,236 cpm/nmol for the metabolically active pool. Fraction 2, the granule preparation obtained by differential centrifugation, appeared to contain mostly storage nucleotides, from the similarities of the ATP/ADP ratio of 0.60 and specific radioactivity of 174 cpm/nmol compared to those of the secreted material. The ATP and ADP in subfraction P from sucrose density gradient fractionation were about 40 times more concentrated per milligram of protein than the secreted material per milligram of platelet protein; the ATP to ADP ratio and specific radioactivity were similar to those of the secreted ATP and ADP. The recovery of ATP and ADP in the dense granule pellet in five experiments was 35–49% of that measured in fraction 2 which averaged 16% of the total platelet ATP and ADP.

DISCUSSION

Results from secretion (13) and ultrastructural studies (21, 29) have suggested that the dense granules in human platelets contain serotonin, ATP, ADP, and calcium. However, isolation of these granules from human platelets has been difficult since a large percentage of the granule constituents was released to the soluble phase during fractionation in most previous studies (3, 5, 8, 10, 11, 14, 20, 27). One notable exception is the report by Buckingham and Maynert in which only 20% of the serotonin appeared in the supernate, but other granule constituents were not measured (4). DaPrada et al. isolated granules that were greatly enriched in serotonin and ATP from several animal species (6, 7) and from human platelets (5, 8), but the yield of serotonin granules in the latter preparations was apparently rather poor (5). Holmsen et al. described a fraction obtained from human platelets that was several-fold enriched in serotonin, ATP and ADP, but over 90% of these substances was found in the soluble fractions (14). Dense bodies from human platelets isolated by Broekman et al. were only three- to fourfold enriched in serotonin, and much of it was solubilized (3).

In our preparation of human platelet storage granules, the serotonin was 40–60 times enriched compared to the platelet homogenate, and only 20% was in the soluble phase after homogenization. We attribute the minimization of solubilization to the use of inhibitors of energy metabolism to block platelet secretion (23) induced by homogenization. Aspirin was used in our initial studies, but solubilization occurred in about 50% of the preparations. The combination of mitochondrial and glycolytic inhibitors, which is known to deplete metabolic ATP without affecting the stored ATP and ADP (16), was effective in blocking secretion (Table II). In addition, it

has been brought to our attention that rotenone, besides being a respiratory inhibitor, also inhibits microtubule assembly (2) in a manner similar to colchicine which has been reported to dissolve platelet microtubules and results in loss of the platelet's ability to maintain a discoid form in plasma (31); this effect of rotenone on microtubules may also contribute to our final results.

The recovery of ATP and ADP in the storage granules compared to intact platelets is more difficult to evaluate than that of serotonin because the metabolically active portion of the platelet ATP and ADP is not secreted. Furthermore, when platelets undergo secretion or are treated with inhibitors of energy metabolism, the metabolically active ATP is degraded rapidly through the sequence ADP, AMP, IMP, to inosine and hypoxanthine (13). Therefore, calculations of the metabolically active pool must be made by subtraction of the secreted materials from the total contents of platelets in the resting state as in Table IV. If one considers that all of the serotonin and about half of the platelet ATP and ADP is stored in subfraction P, a yield of ATP and ADP of 10% would be comparable to a recovery of 20% for serotonin. We found somewhat less, about 8%, and there are several possible explanations for this. The possibility that the isolated granules were accumulating solubilized serotonin was checked by adding labeled serotonin to a platelet homogenate from which all intact platelets had been removed. After fractionation, about 5% of the label was found to be incorporated into subfraction P at the bottom of the sucrose gradient, which indicated that the dense granules were indeed able to accumulate some serotonin after removal from the intact platelet. A second possibility is that some solubilization of granule contents can occur with normal handling (centrifugation and resuspension), and that solubilization may be greater for some compounds than others, depending on their storage mechanisms, which are unknown. A third and more unlikely possibility is that some of the nonmetabolic ATP and ADP is located in other compartments.

Electron micrographs of subfraction P show that the dense bodies are not uniform in appearance. Da Prada et al. also isolated a human platelet dense granule fraction (5) which was more heterogeneous in appearance than rabbit dense granules which seemed to consist mainly of the bull's eye type (7). However, it has been suggested by other workers that serotonin storage organelles in human platelets are heterogeneous in appearance. White has characterized dense bodies in human platelets as being of several types, bull's eye as well as signet ring in shape, with some that fill the enclosing membrane completely while others have long shaftlike tails (32). Skaer et al. have also observed these different shapes of dense bodies *in situ*; they found that the P:Ca atomic ratio was the same for the various shapes (29).

Preliminary work indicated that high concentrations of calcium are also present in these dense granules, and the atomic ratio of P:Ca as calculated from the ATP and ADP content was comparable to the value of 1.76 which was found by electron microprobe analysis (29).

Serotonin storage granules have previously been isolated from the platelets of guinea pig, rabbit, and pig (6, 7, 26) without resorting to extraordinary procedures, but human platelet dense bodies have always seemed to be more labile and their contents more easily solubilized. Our fractionation results with human platelets in which secretion was inhibited indicate that the granules *per se* are not more fragile than those in platelets of other species, but that secretion is easily induced in human platelets by the usual homogenization procedures, with the possible exception of ultrasonication (4).

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