AN IMPROVED METHOD FOR ISOLATING ALPHA
GRANULES AND MITOCHONDRIA FROM HUMAN PLATELETS

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INTRODUCTION

We describe a fractionation technique for human platelets which improves upon previous studies (1–3) in that we have obtained greater mitochondrial enrichment than heretofore shown, and provided evidence for heterogeneity of acid hydrolase-associated structures while distinguishing the latter from a rather pure α-granule fraction. We attribute our success to two departures: homogenization by cavitation forces developed by explosive decompression and the elimination of a preliminary centrifugation to remove debris before ultracentrifugation.

MATERIALS AND METHODS

Platelet Procurement

Washed platelets were derived by differential centrifugation of fresh acid citrate dextrose blood in plastic packs (Fenwal Laboratories, Morton Grove, Ill.) as recently described (4), except that the platelet pellet was washed once in 150 ml Tris/KCl/citrate solution (63 mM Tris, 100 mM KCl, 12 mM citrate, pH 6.2) before resuspension in 10–15 ml of the same medium.

Homogenization Procedure

The platelet suspension was homogenized by the nitrogen decompression technique at 1°C (Parr Cell

Disruption Bomb, Parr Instrument Co., Moline, Ill.)
(5, 6). In preliminary studies, pressures of 600-1,800
lb/in^2 (40-120 atm) and equilibration times of 10-30
min were tested.

Density Gradient Centrifugation

Linear, 30-60% sucrose gradients containing 5 mM
EDTA, pH 7.4, were prepared using a commercial
gradient former. Whole homogenate, 1.0-1.8 ml, was
layered on the gradient and centrifuged (120 min,
134,000 g_{max}) in a SW36 rotor (Beckman Instru-
ments, Inc., Palo Alto, Calif.). Gradients were sampled
through a bottom-piercing needle. Particulate zones
were identified by their light-scattering properties,
and after harvesting were diluted three- to fivefold
with 0.29 M sucrose and pelleted by ultracentrifuga-
tion (60 min, 130,000 g_{max}) before resuspension in
0.15 M NaCl containing 2 mM imidazole, pH 7.4.

For morphological studies, small pellets, 200-500
ng protein, were fixed for 1 h at 4°C with 2.5% glu-
taraldehyde in 0.1 M pH 7.2 sodium phosphate buffer,
and washed overnight at 4°C in phosphate buffer
before being postfixed, embedded, sectioned, and
stained using standard electron microscope tech-
niques.

Enzyme Assays

Glutamate dehydrogenase and succinate cyto-
chrome c reductase were inactive in unpelleted,
sucrose-containing fractions, presumably owing to
hyperosmolarity (7, 8). Hence, all mitochondrial frac-
tions were pelleted, resuspended in NaCl-imidazole
buffer, and then lysed with Triton X-100 (9) or by
hypotonic shock (10) to unmask full marker enzyme
activities. Acid hydrolases were assayed on whole frac-
tions in the presence and absence of 0.1% Triton X-
100 (11) using incubation times of 15 min for acid
p-nitrophenylphosphatase and 30 min for the re-
mainder. Osmotic buffering of reaction mixtures with
1.17 M sucrose augmented latency which was ex-
pressed as a percentage:

$$\frac{\text{activity with Triton} - \text{activity without Triton}}{\text{activity with Triton}} \times 100.$$

References for all enzyme assays (2, 9, 10, 12-17)
performed by us are listed with the individual
enzymes in Table I. Glycogen phosphorylase was
measured by Dr. Laurence M. Demers using a tech-
nique devised in his laboratory (18). Protein deter-
minations were by Miller's modification (19) of the
method of Lowry et al. (20) and proved to be accu-
rate and reproducible in all fractions of the gradient.

Enzyme distributions over the gradient were calcu-
lated as relative specific activities according to
Marcus et al. (1) and Day et al. (2), who, in turn,
followed the lead of de Duve et al. (10).

RESULTS

Homogenization Procedure

The data presented in this section were derived
from studies in which platelets were homogenized
by equilibration with nitrogen at 1,200 lb/in^2 (80
atm) for 15 min before explosive decompression.

Preliminary centrifugation at 500-1000 g sedi-
mented debris which contained moderate num-
bers of α-granules. Therefore, whole homogenate
was directly applied to the density gradient.
Optimal separations were achieved by layering
20-35 mg of platelet protein in 1.0-1.8 ml on 11
ml of the gradient.

Morphology

A typical density gradient separation showed
nine zones, five visible bands, and four clear areas
(Fig. 1). Zone 1 produced no pellet. Zone 2 con-
tained small vesicles. Most were single membraned
and empty; however, a few held amorphous
material. Zone 3 consisted mostly of larger vesicles
enclosing varying amounts of amorphous material.
There were also a few mitochondria as well as
some homogeneously electron-dense structures.
The latter were of two types: typically round or
oval α-granules, and anisometric bodies of the
same electron density as α-granules.

Zones 4-7 are described in the legends to Figs.
2-5. Zone 8 contained some whole platelets, but
mostly α-granules. Zone 9 macroscopically ap-
peared finely aggregated (21); however, on
electron microscopy, there was no evidence of
aggregation, and the pellet was found to consist
mostly of platelet remnants, numerous α-granules,
some mitochondria, and some bull's-eye struc-
tures.

Distribution of Protein and Marker Enzyme
Activities over the Gradient

These data appear in two illustrations. Fig. 6
depicts the relative specific activities of marker
enzymes in the nine zones. Table I shows total
recovery of enzymatic activities as well as their
enrichment in the nine zones as compared with
whole homogenate.

PROTEIN: Zone 1 contained 35% of the re-
covered protein. An additional 23% appeared in
zone 2. Of the latter approximately 8% was
particulate, so that, in effect, 50% of the protein
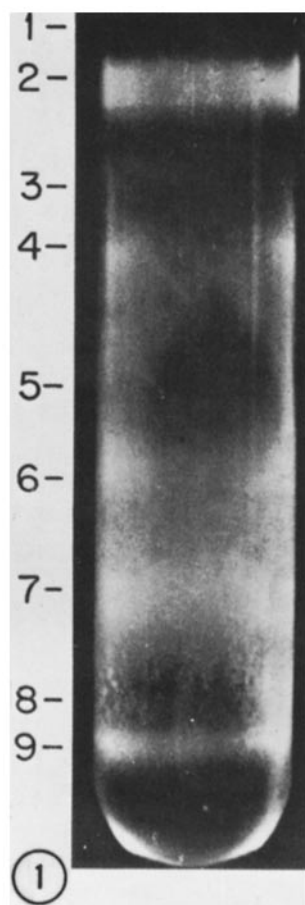was recovered from supernate. The remainder of

FIGURE 1 Photograph of a typical density gradient separation. See Materials and Methods for details of preparation of the platelet homogenate and loading and makeup of the gradient, as well as the conditions of ultracentrifugation. Nine zones, five bands and four clear areas, are labeled. Five of the bands (2, 4, 6, 7, 9) are quite distinct. The thin band of zone 3 was more readily discernible with the naked eye. For details of the morphology of zones 4–7, see Figs. 2–5.

the protein was distributed evenly over the gradient. The total protein recovery in the 9 zones was over 93% in all studies.

LACTATE DEHYDROGENASE: This enzyme was almost entirely confined to the supernate. Glycogen phosphorylase had an almost identical distribution.

MITOCHONDRIAL ENZYMES: The relative specific activities of glutamate dehydrogenase, succinate cytochrome c reductase, and monoamine oxidase peaked sharply in zone 4. By contrast, rotenone-insensitive NADH cytochrome c

reductase showed nearly as much activity in zone 3 as in zone 4.

ACID HYDROLASES: The acid hydrolases N-acetyl-β-glucosaminidase, β-galactosidase, β-glucuronidase, and β-glycerophosphatase had nearly superimposable broad peaks over zones 4–6. Acid p-nitrophenylphosphatase, although much used as a lysosomal marker, even in platelets (1, 2), peaked in zones 2 and 3. Glucose 6-phosphatase, in the absence of Triton, peaked in zone 3; in the presence of Triton, it peaked in zones 2 and 6.

## Inhibition Studies Relating to Glucose 6-Phosphatase (Table II)

Acid p-nitrophenylphosphatase was strongly inhibited by p-hydroxymercuribenzoate, but not at all by NaF. Glucose 6-phosphatase was much less inhibited by NaF than was β-glycerophosphatase, while both were similarly inhibited by p-hydroxymercuribenzoate.

Latency Studies Relating to Acid Hydrolases

Detergents have gained ascendancy as the means for revealing enzyme latency (11). The data in Table III were obtained using Triton X-100. Several observations are noteworthy. First, three acid hydrolases (β-glucuronidase, N-acetyl-β-glucosaminidase and β-glycerophosphatase) showed 54–86% latency in all particulate zones except zone 9. By contrast, the latency of β-galactosidase was lower in all zones except zone 1. Second, acid p-nitrophenylphosphatase showed 15–35% latency, with gradually decreasing values from zones 1 to 9. Third, in zones 3 and 4 glucose 6-phosphatase was strongly inhibited by Triton X-100 (22). Fourth, no enzyme had a specific activity peak in zone 7, the purest fraction of α-granules.

DISCUSSION

We draw attention to four aspects of our results: (*a*) the unique features of our homogenization and fractionation techniques, (*b*) the higher degree of purity of our mitochondrial and α-granule fractions as compared with those reported elsewhere, (*c*) the apparent heterogeneity of platelet acid hydrolase-associated structures, (*d*) the evidence for a microsomal glucose 6-phosphatase in platelets.
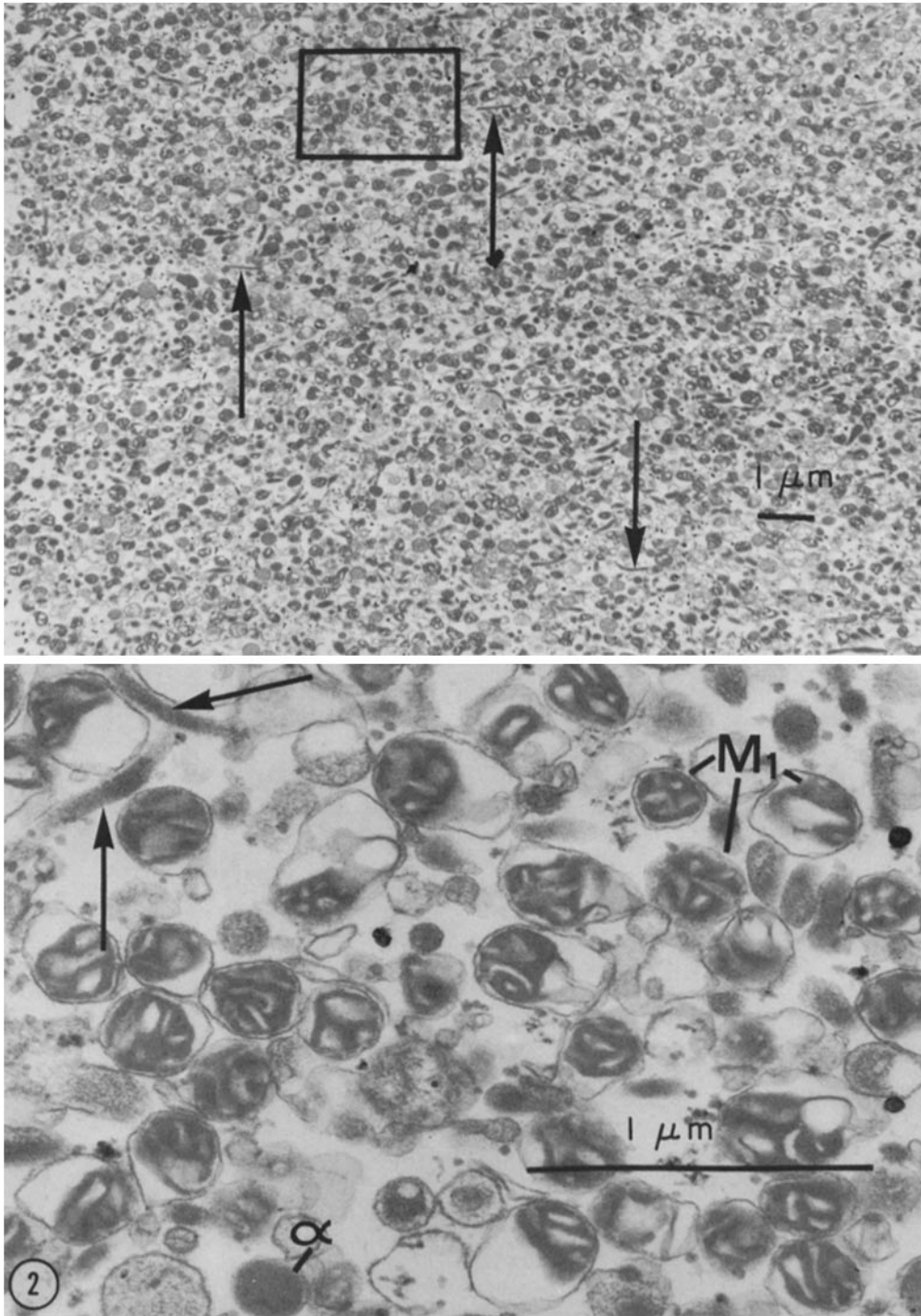
FIGURES 2–5 Electron micrographs of the pellets harvested from zones 4–7 of Fig. 1. See Materials and Methods for details of preparing material for electron microscopy. Two areas of each pellet are shown: the upper, a representative view at × 8,000; the lower, a × 48,000 view of a rectangular inset in the upper photograph. Each of the legends begins with a parenthesis in which are named the zone, and, where applicable, the enzyme(s) showing peak relative specific activity in that zone.

FIGURE 2 (Zone 4, mitochondrial markers and acid hydrolases.) At low power, this zone is obviously heavily enriched with mitochondria. There are also electron-dense, eellike, anisometric bodies (arrows). At high power, the abundance of mitochondria, mostly in the condensed (M_1) conformation, is confirmed. There is moderate contamination of this layer with two types of homogeneous electron-dense structures: typically round or oval α-granules (α), and anisometric bodies of the same electron density as α-granules.
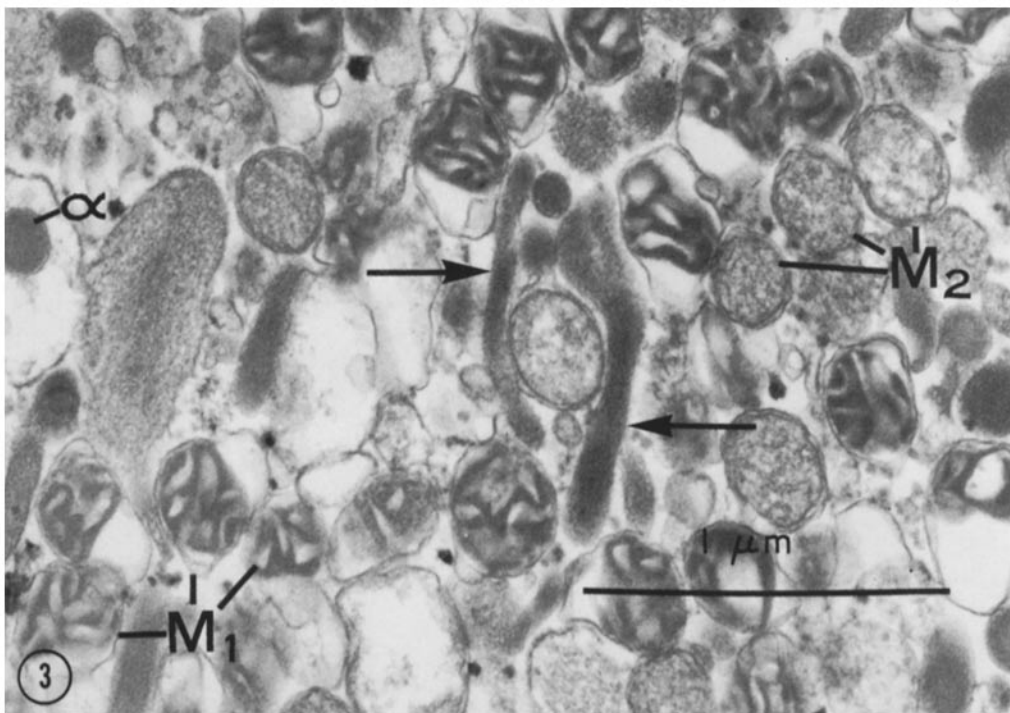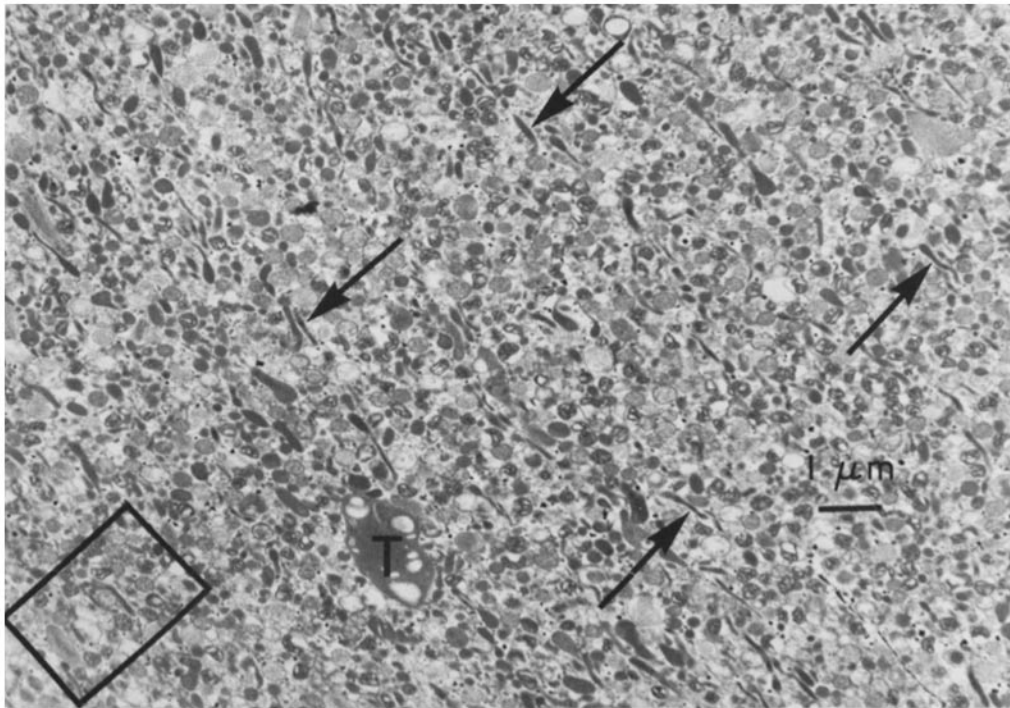
FIGURE 3 (Zone 5, mitochondrial markers and acid hydrolases, both with lower relative specific activities than in zone 4.) At low power, two types of electron-dense particles predominate: mitochondria and anisometric bodies. The latter show much pleomorphism with eellike forms predominating. There is also some contamination with α-granules and vesicular material. In addition, there is one platelet-like (thrombocytoid [T]) structure which appears in greater abundance in zone 6. At high power, the similar electron density of the anisometric bodies (arrows) and α-granules (α) is underscored. There are many mitochondria, with condensed (M_1) predominating over orthodox (M_2) conformations.
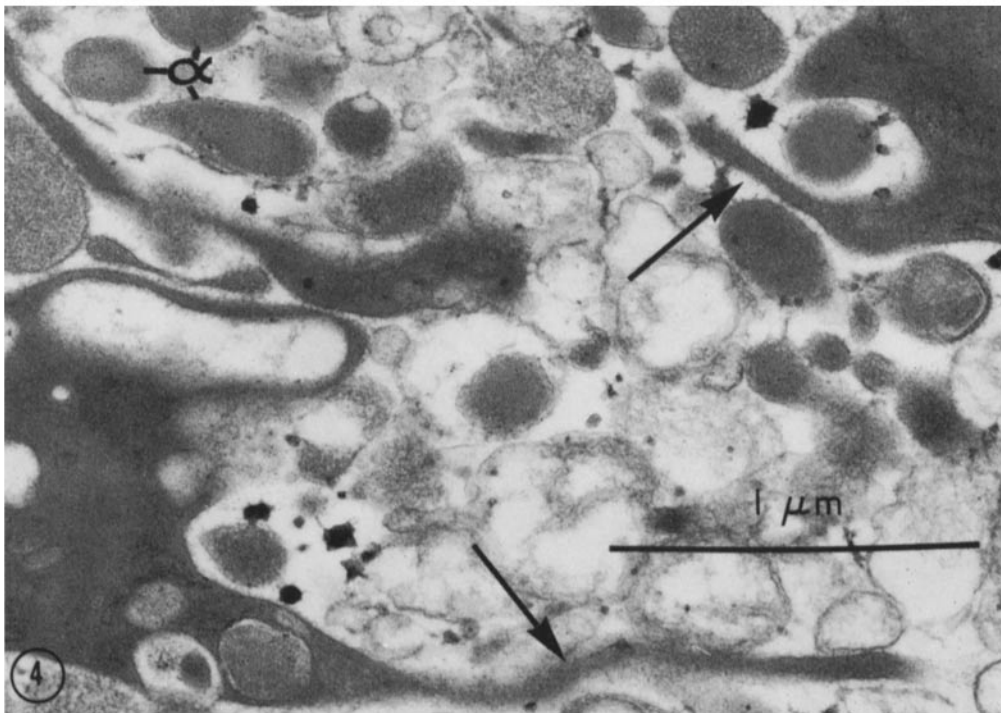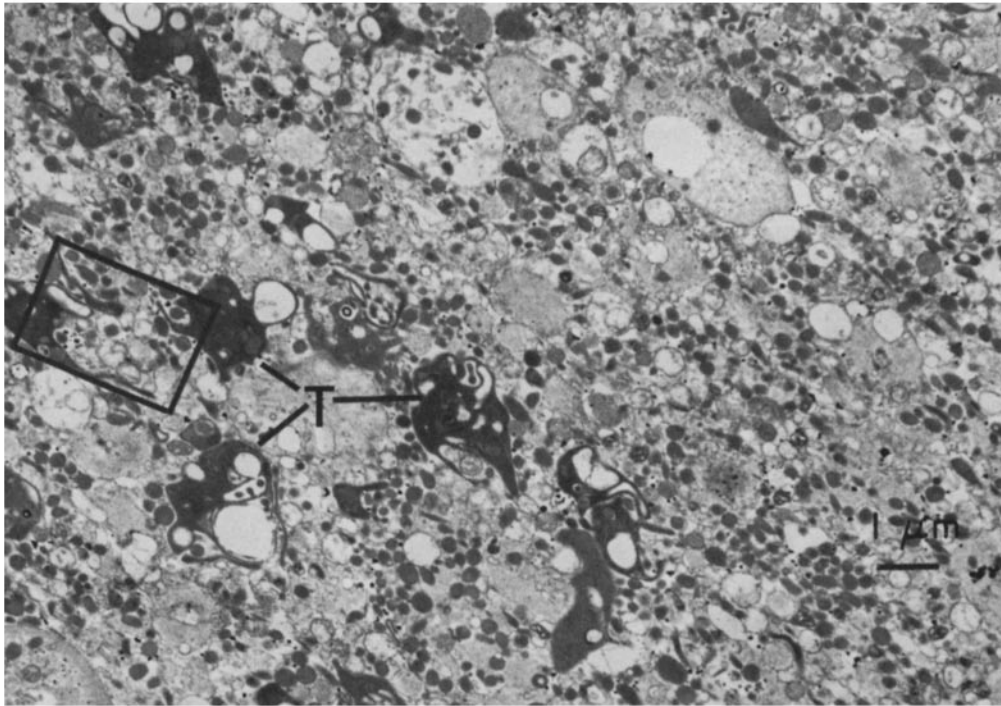
FIGURE 4 (Zone 6.) At low power, this zone shows great heterogeneity. Besides many α-granules and some mitochondria, there are two types of larger structures, one vesicular and the other thrombocytoid (*T*). The vesicles, some of which are possibly platelet ghosts, contain electron-lucent, amorphous material interspersed with clear vacuoles. The thrombocytoid elements are also vacuolated, but have strikingly electron-dense interiors and long pseudopods. There are also abundant α-granules.

At high power, the pseudopods show the electron density, shape, and size of the anisometric bodies previously noted in large numbers in zones 4 and 5. The α-granules (α) differ in size and shape, but have nearly the same electron density as the anisometric bodies and pseudopods.
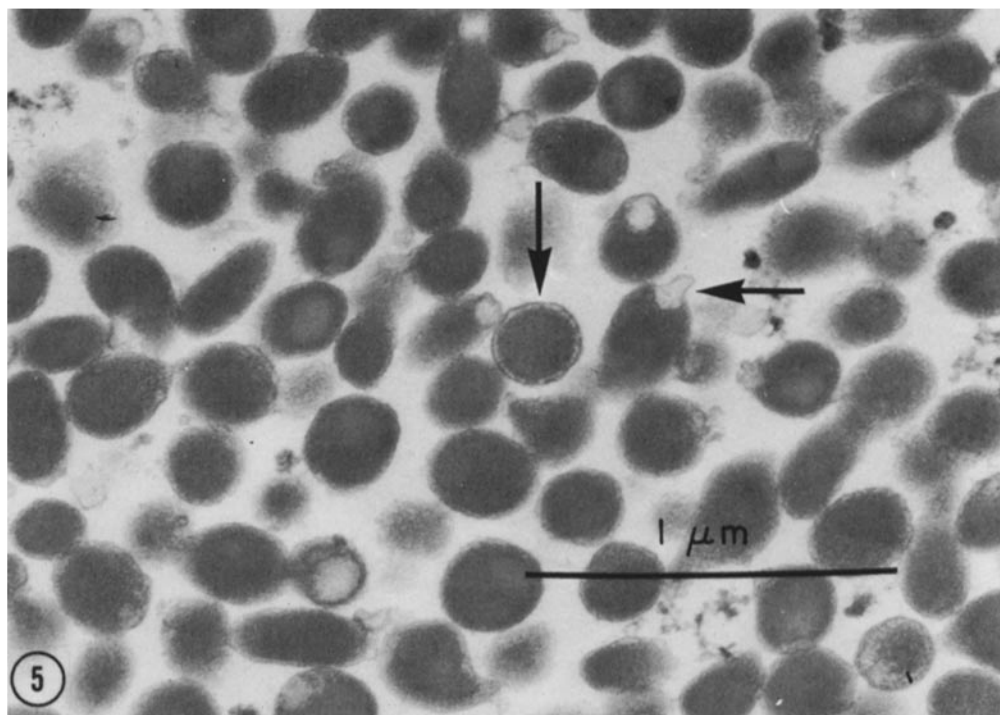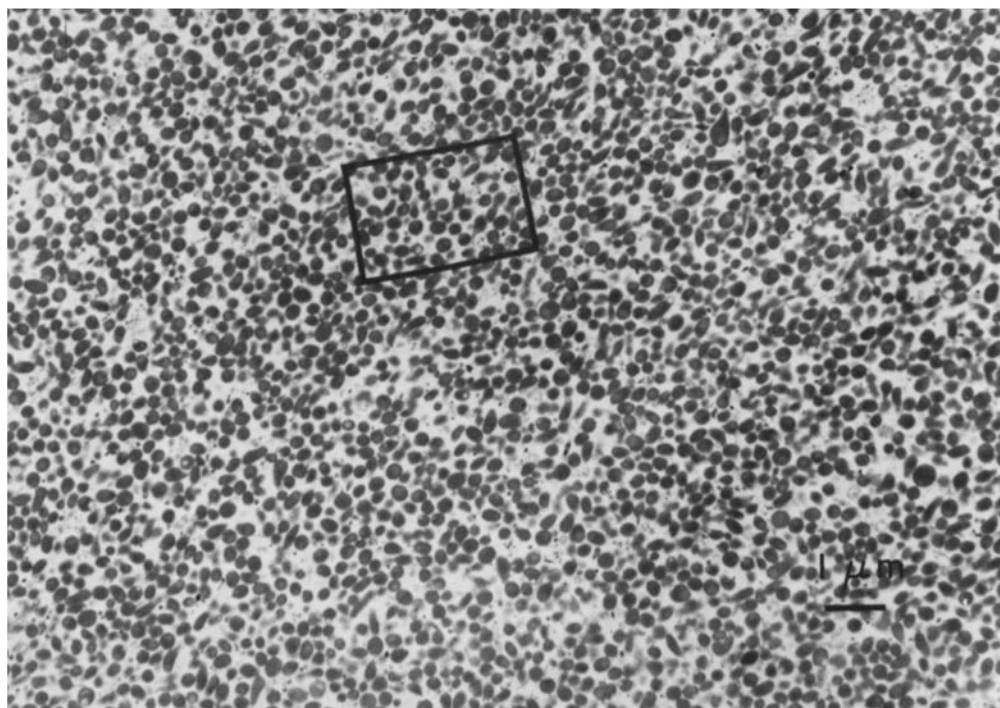
FIGURE 5 (Zone 7.) At low power, there is a remarkably pure array of electron-dense, round or oval α-granules. At high power, a number of the α-granules show round or oval areas which are less electron dense than their peripheral matrix. Many of the granules have halos (vertical arrow); a few have peripheral vacuoles, one of which appears to be disrupted (horizontal arrow).
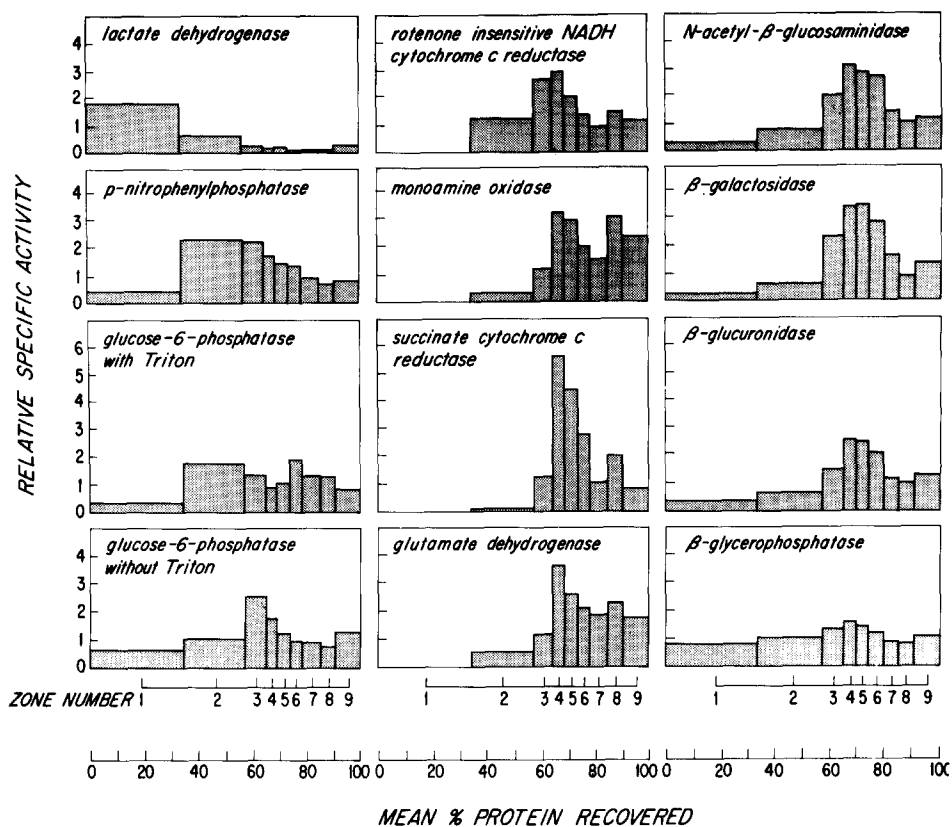
FIGURE 6 Marker enzyme distributions over the nine zones shown in Fig. 1. The data are plotted according to de Duve et al. (10). On the ordinate is the relative specific activity (percent activity recovered/ percent protein recovered); on the abscissa, the protein recovered in each zone. Shown are the means of two experiments for the mitochondrial markers (center group) and three experiments for the remainder.

Homogenization and Fractionation

Marcus et al. unsuccessfully tried a variety of homogenizing procedures (1), including nitrogen decompression (23), before devising their no-clearance pestle technique. With ultracentrifugation on a linear sucrose gradient they defined two particulate fractions, membranous and granular, by electron microscopy, marker enzyme, and coagulation studies. Day et al. followed with a study confirming the superiority of the no-clearance pestle as compared with a blender for the homogenization step (2). They extended the work of Marcus et al. (1) by achieving clearer separations, morphologically and biochemically, between mitochondria and α-granules. Later, Siegel et al. revived blender homogenization by showing morphology similar to that of Day et al., but did not further identify their fractions biochemically (3). Subsequently, Minter and Crawford (24),

in studies with pig platelets, suggested that the effectiveness of homogenization procedures depends upon the volume of the homogenate; they favor the blender for large volumes and the no-clearance pestle for small volumes. Earlier, focusing on the isolation of platelet membranes, Barber et al. (25) had found the glycerol lysis technique (26) superior to all other methods, nitrogen decompression included.

Our fractionation technique has three principal features: (a) use of a Tris-citrate suspension medium for the platelet homogenate; (b) use of cavitation, as opposed to shear forces, for cell disruption; (c) the elimination of a preliminary centrifugation to remove debris before ultracentrifugation. One or all of these steps could account for the uniqueness of our findings. However, our electron microscopic and biochemical data are not sufficiently exhaustive to precisely assess the impact of each of our experimental departures.

TABLE I

Enrichment of Enzymatic Activities in Human Platelet Fractions as Compared with Whole Homogenate

	Specific activity in homogenate	Highest specific activity		Purification factor	% Recovery
		Value	Zone		
Lactate dehydrogenase (13)	706.7	1178	1	1.7	74
p-Nitrophenylphosphatase (2)	119	323	2	2.7	103
Glucose 6-phosphatase in presence of Triton (10)	3.95	8.90	2	1.6	134
Glucose 6-phosphatase in absence of Triton (10)	5.79	11.30	3	2.0	79
Rotenone insensitive NADH cytochrome c reductase (12)	84.1	226	3	2.7	83
Monoamine oxidase (14)	0.0023	0.0053	4	2.3	75
Succinate cytochrome c reductase (10)	6.17	28.3	4	4.6	107
Glutamate dehydrogenase (9)	12.3	17.2	4	1.4	32
N-Acetyl-β-glucosaminidase (2)	10.7	28.4	4	2.7	69
β-Galactosidase (15)	3.10	8.23	4	2.6	85
β-Glucuronidase (16)	1.93	4.91	4	2.5	78
β-Glycerophosphatase (17)	8.63	12.69	4	1.5	87

For details of procedures leading to the harvesting of nine subcellular fractions of human platelets, see Materials and Methods. The first column lists the enzymes with references to the techniques for their measurement; the second, the specific activity (nanomoles per minute per milligram of protein) of each enzyme in the whole homogenate; the third, the specific activity of each enzyme in the zone in which it peaked in activity; the fourth, the purification factor derived by dividing the value in the third column by that in the second column; the fifth, the combined recoveries of the total activities for each enzyme in all nine zones, expressed as a percentage of the activity in that amount of homogenate which was applied to the gradient. Shown are the means of two experiments for rotenone-insensitive NADH cytochrome c reductase, monoamine oxidase, succinate cytochrome c reductase, and glutamate dehydrogenase; and three experiments, for the remainder.

Membranes

Our membrane fractions were less pure than those of Barber and Jamieson who used several centrifugation steps before the final isolation of the membranes (26). We would expect preliminary centrifugations to result in considerable "cleaning up" of our membrane layers, possibly at the expense of our excellent yields of mitochondria and α-granules.

Microsomes

We have focused on two microsomal markers: rotenone-insensitive NADH cytochrome c reduc-

tase (12), and glucose 6-phosphatase (10). The former peaked maximally (Fig. 6) in zone 4 where mitochondria predominated (Fig. 2), but, in addition, showed considerable activity in zones 2 and 3, which were largely membranous. It is useful to point out that this enzyme has been found in outer mitochondrial membranes as well as microsomes (see reference 12), structures which may, in fact, share a common origin (27).

With regard to our second microsomal marker, four observations favor the presence of a "true" microsomal glucose 6-phosphatase: the high rate of enzymatic hydrolysis (Table I); the peaking of

TABLE II

Inhibition Studies of Three Human Platelet Phosphatases Using Sodium Fluoride and p-Hydroxymercuribenzoate

	p-Nitro-phenylphos-phatase (zones 2, 3)	Glucose 6-phos-phatase (zones 3, 4)	β-Gly-cerophos-phatase (zones 3–6)
Sodium fluoride (5 mM)	0	55	86
p-Hydroxy-mercuribenzoate (10 μM)	92	32	34

For details of procedures leading to the harvesting of nine subcellular fractions of human platelets see Materials and Methods. On each fraction, measurements of the activities of *p*-nitrophenylphosphatase (2), glucose 6-phosphatase (10), and β-glycerophosphatase (17) were done in the presence and absence of 5 mM NaF or 10 μM *p*-hydroxymercuribenzoate (23). Shown for each enzyme are the values of three separate studies in which the percent inhibition is expressed as the combined means from the zones where the enzyme had its highest relative specific activity: *p*-nitrophenylphosphatase (zones 2, 3), glucose 6-phosphatase (zones 3, 4), and β-glycerophosphatase (zones 3–6).

activity in zones 2 and 3, where microsomal membranes might be expected to collect; the inhibition of activity by Triton (Table III); the comparative inhibition studies in which, by contrast with glucose 6-phosphate, hydrolysis of β-glycerophosphate was inhibited more by NaF than by *p*-hydroxymercuribenzoate (Table II).

Thus, as opposed to rabbit heterophil leukocytes (28), platelets appear to have two biochemically distinct membrane fractions: one characterized by acid *p*-nitrophenylphosphatase; the other, characterized by Triton-inhibited glucose 6-phosphatase.

Mitochondria

The morphological integrity of the mitochondria in zones 4 and 5 (Figs. 2 and 3) was underscored by the superimposed activities of marker enzymes for matrix (glutamate dehydrogenase), inner membrane (succinate cytochrome *c* reductase), and outer membrane (monoamine oxidase) (Fig. 6) (12, 29).

Heterogeneity of Acid Hydrolase-Associated Structures

Heterogeneity of platelet lysosomes on biochemical and morphological grounds was suggested by Siegel and Lüscher (30), and on biochemical grounds alone by Holmsen and Day (31) and Kaulen and Gross (32). We associate three different structures with acid hydrolase activity: the membranous vesicles in zones 2 and 3, the anisometric bodies in zones 4 and 5 (Figs. 2 and 3), and the α-granules in zones 6–8 (Figs. 4 and 5). This morphological heterogeneity, complemented by the biochemical heterogeneity depicted in Fig. 6, brings to mind recent studies with rabbit heterophil leukocytes (28).

First, the vesicles of zones 2 and 3 not only resembled those described by Baggiolini et al. (see Fig. 4 in reference 28), but were also rich in acid *p*-nitrophenylphosphatase which showed 30–35% latency (Table III).

Second, the anisometric bodies of zones 4 and 5 (Figs. 2 and 3) draw suspicion as repositories of the four acid hydrolases which peaked in those zones. In support of this we cite three observations: the enzymatic activities showed considerable latency (Table III), favoring a particulate origin; the two most abundant particles in zones 4 and 5 were mitochondria and anisometric bodies; the mitochondria of other tissues have not been regarded as loci of acid hydrolase activity.

Structures similar to our anisometric bodies (Figs. 2 and 3) were tentatively identified as pseudopods by Day et al. (2) and Siegel et al. (3). Our morphological observations lend some support to their stance, since the pseudopodal extensions of the thrombocytoids of zone 6 (Fig. 4) have the same electron density as the anisometric bodies. Further, it is worth noting that Webber and Firkin (33) found structures similar to our thrombocytoids by exposing platelets to distilled water or hypertonic saline. Our homogenization medium is isotonic; however, it is possible that the shock of entry into the hypertonic gradient could have produced similar osmotic stress.

Third, the platelet α-granules of zones 6–8 (Figs. 4 and 5) are morphologically quite similar to, although smaller than, purified rat liver lysosomes (34) or the alkaline phosphatase-containing structures described by Baggiolini et al. (see Fig.

	% Latency*									
	1	2	3	4	5	6	7	8	9	Homogenate
p-Nitrophenyl-phosphatase	35.5	**35.5**	31.9	33.1	27.3	24.6	20.1	26.7	14.5	39.8
Glucose 6-phosphatase	−5.3	39.8	**−118.1**	−119.6	37.6	52.2	52.3	51.7	−36.0	−37.5
N-Acetyl-β-glucosaminidase	−7.8	30.5	59.8	**63.4**	63.2	65.5	60.8	62.0	55.8	32.1
β-Galactosidase	48.4	25.2	34.9	**34.7**	30.8	31.5	37.2	40.3	35.1	19.2
β-Glucuronidase	6.5	33.3	60.3	**86.4**	75.4	79.5	74.5	59.4	65.7	28.1
β-Glycerophosphatase	18.2	7.3	54.0	**63.9**	75.6	61.9	68.9	38.3	56.6	26.7

For details of procedures leading to the harvesting of nine subcellular fractions of human platelets, see Materials and Methods. On each zone, measurements of the activities of p-nitrophenylphosphatase (2), glucose 6-phosphatase (10), N-acetyl-β-glucosaminidase (2), β-galactosidase (15), β-glucuronidase (16), and β-glycerophosphatase (17) were done in the presence and absence of Triton X-100 as described in Materials and Methods.

* Latency was expressed as a percentage according to the formula: (activity with Triton − activity without Triton)/(activity with Triton) × 100. Shown are the means of two experiments for glucose 6-phosphatase and β-glucuronidase; and three, for the remainder. Negative numbers indicate inhibition by Triton. The latencies in the zones of peak relative specific activities of the six enzymes are indicated by bold-faced numbers.

6 in reference 28). However, despite the rather pure harvest of apparently intact structures in zone 7, the relative specific activities and total activities of measured acid hydrolases were comparatively low (Fig. 6). Thus, we believe that the marker activities of zone 7 are incompletely defined, and that other markers, for example platelet factor 4 (35), may be found to peak sharply in this zone. However, we allow for the possibility that, despite the apparent intactness of most of our α-granules (Figs. 4 and 5), leakage of acid hydrolases (36) may have occurred during homogenization.

Through the cooperation of Dr. Charles E. Huggins the platelet concentrates were derived from blood drawn in the Blood Bank of the Massachusetts General Hospital. Dr. Manfred L. Karnovsky offered valuable advice in the early phases of the work. Also, Mrs. Barbara Burgess and Mrs. Claudia Starr gave excellent technical assistance with the preparations for electron microscopy.

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