Isolation and Partial Characterization of Human Eosinophil Granules

Comparison to Neutrophils

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Human blood eosinophils obtained from untreated patients with large numbers of circulating eosinophils were purified and lysed. An eosinophil contains 2.65 times as much peroxidase, 2.44 times as much β-glucuronidase, approximately two times as much acid β-glycerophosphatase, and 1.2 times as much protein as a neutrophil. Lysate filtration allowed isolation of eosinophil granules by isopycnic ultracentrifugation in sucrose. The granules had a mean density of ρ 1.24 g/ml, and contained peroxidase, β-glucuronidase, and acid β-glycerophosphatase. They totally lacked muramidase and alkaline phosphatase. Electron micrography confirmed the isolation. (Am J Pathol 81:575–588, 1975)

GRANULES FROM HUMAN EOSINOPHILS have not heretofore been characterized in detail. The small number of eosinophils normally circulating in peripheral blood has limited their study. Horse eosinophil granule study has been possible because of the large volume of blood available.[1,2] The induction of rat [2,3] and guinea pig [4] eosinophils by peritoneal lavage has made study of their respective granules feasible. Similar guinea pig eosinophils are preferentially attracted by a recently described chemotactic stimulus,[5] while human blood eosinophils have been found at least as motile as neutrophils in stimulus-free experiments.[6,7] However, the human eosinophil is functionally inferior to the neutrophil in phagocytic and certain bactericidal activities.[8,9] Only recently has a good technique for purifying eosinophils from peripheral blood been available,[10] although it is only really helpful when an abnormally high number and percentage of circulating leukocytes are eosinophils.

Eosinophilia in human disease implies a special function for eosinophils, at least in the associated illnesses. Considerable clinical reporting and discussion of disease with eosinophilia has occurred in the 119 years since the eosinophil was described; nevertheless, the unique, non-

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neutrophil properties of blood eosinophils, particularly from man, have not been elucidated.[11,12] The relative rarity of massive eosinophilia in persons suitable for study has further limited study of this cell, although a very brief recent report includes certain enzyme data from a patient with an eosinophilic leukemoid reaction.[13]

Fourteen patients with the hypereosinophilic syndrome, clinically reported elsewhere,[14] form the base population from which 5 untreated men were selected for the current study. The authors previously observed a separate band of granules in isopycnic sucrose gradients (at density 1.24 g ml) of filtered granulocyte lysates; it was present only in a small group of normal human donors with mild (5 to 8%) eosinophilia.[15] The studies reported here confirm that "extra" band as the eosinophil granule-rich band. Leukocyte suspensions containing 90% eosinophils were lysed, compared to neutrophil lysates regarding protein and enzyme content, filtered, and centrifuged to equilibrium density, yielding a single optically dense band at 1.24 g/ml. That the isolated band consisted of eosinophil granules was confirmed by electron microscopy and defined by assay of protein, peroxidase, muramidase, β-glucuronidase, and acid and alkaline phosphatases.

Materials and Methods

Untreated patients with marked eosinophilia of unknown cause were the source of blood eosinophils (Table 1). Twenty- to 130-ml samples of venous blood were drawn into polypropylene syringes wet with 0.2% Na_2H_2 EDTA, pH 7.3. Total and differential WBCs were recorded, and the peripheral eosinophil counts varied from 3,600 to 32,000 cu mm. The blood was mixed with an equal volume of 3% dextran (Dextran T-250, average molecular weight approximately 250,000; Pharmacia, Uppsala, Sweden) in sterile 0.85% NaCl and allowed to stand for 25 minutes at room temperature. The resulting supernatant was utilized as it was if the initial eosinophil count exceeded 75%. If the differential blood eosinophil count was less than 75%, the method of Day [10] was adopted to further concentrate eosinophils. Ninety percent sodium diatrizoate (Hypaque, Winthrop Laboratories, Division of Sterling Drug, Inc., New York, N.Y.) was diluted to density 1.138 to 1.139 g ml with sterile distilled water according to Day's formula. Three milliliters of diatrizoate (1.139 g ml) was placed in cellulose nitrate or polypropylene tubes and carefully overlaid with 3 ml of leukocyte-rich dextran supernatant. Eosinophils were gently pelleted by centrifugation at 400*g* for 40 minutes at 4 C. Eosinophils were resuspended in modified Hanks' solution (MHS) that was lacking calcium, magnesium, phenol red, and antibiotics and was freed of contaminating red blood cells (RBCs) by two 20-second cycles of hypotonic lysis with MHS equivalent to 0.2% and 1.6% NaCl and washed in 0.34 M sucrose. The pellet was resuspended in 0.34 M sucrose [15] to yield 5×10^7 leukocytes 4 ml to which was added 5000 units (0.5 ml) sodium heparin (The Upjohn Co., Kalamazoo, Mich.). The cells were lysed by repeated passages through a No. 18, 9-cm metal needle attached to a 6 ml polypropylene syringe. The resulting lysate was passed through both 5-μ and 2-μ pore size polycarbonate filters (Nuclepore, General Electric Corp., Pleasanton, Calif.), aliquots being taken in some experiments before and after each filtration. Phase microscopy was utilized to monitor lysis of leukocytes and to review filtrates for purity. The filtrate, approximately 2.5 ml of the original 4.5 ml volume, was layered upon a waiting

Table 1—Clinical Data on Five Male Patients With Eosinophilia*

Patient	Age/race	WBC (% eosinophils)	Duration of known eosinophilia (yrs)	Major signs and symptoms	Diagnosis
DP	65/C	25,000(53%)	1	1. Angioneurotic edema-like disease with subacute edema of head, neck and airway (not hereditary) 2. Interstitial Pneumonitis 3. Perivascular eosinophilic infiltration	Hypereosinophilia syndrome of unknown etiology, symptomatic
GM	50/C	12,900(65%)	18	Pruritis with mild papulosquamous eruption	Idiopathic hypereosinophilia
LM	50/C	8,000(45%)	6	Asymptomatic	Idiopathic hypereosinophilia
EW	43/B	36,000(85%)	1	1. Congestive heart failure 2. Eosinophilic nephritis with necrotizing arteriolitis	Hypereosinophilia syndrome with Loffler's endocarditis and eosinophilic nephritis
DC	43/C	41,000(78%)	4	1. Peripheral sensory neuropathy 2. Splenomegaly (removed) 3. Hilar and abdominal adenopathy 4. Ventricular irritability	Hypereosinophilia syndrome of unknown etiology, symptomatic

* Four of the patients (except DP) had serum IgE determinations, which ranged from 40 to 104 ng/ml, the normal human value being 105 ng/ml, with 90% confidence limit being 15 to 748 ng/ml.

sucrose gradient continuous from density 1.287 to 1.10,[15] and centrifuged for 4 hours at 4 C in an SW 27 rotor with standard cups at 27,000 rev/min (Model L2-65B Centrifuge, Beckman Instruments Company, Palo Alto, Calif.). The gradient was photographed and collected from below by standard techniques.[15] Fractions were assayed (or frozen at −20 C and assayed later) for enzyme activity or fixed for electron microscopy by previously described methods.[15]

Enzyme activity was expressed as follows: peroxidase, micrograms of horseradish peroxidase (HRP) equivalents per milliliter; β-glucuronidase, micrograms of phenolphthalein released per hour per milliliter; muramidase, micrograms of egg white muramidase equivalents per milliliter; alkaline *p*-nitrophenylphosphatase and acid *p*-nitrophenylphosphatase, micromoles of *p*-nitrophenol (pnp) released per hour per milliliter; and acid β-glycerophosphatase, micrograms of phosphorus released per minute per milliliter.[15,16] All enzyme activities were linear under the assay conditions employed. Standard error was used as an estimate of variation, and comparisons of means were made using the Student *t* test.

Results

Leukocyte purification procedures resulted in preparations containing a mean of 90.9 ± 2.3% eosinophils (Table 2). The recovery of eosinophils

Table 2—Total and Differential Counts of Twelve Peripheral Blood and Purified Eosinophil Preparations From Five Patients

	Peripheral WBC/cu mm	Percentage of total WBC					Eosinophil yield†
		Eosinophils	PMNs	Lympho-cytes	Mono-cytes	Basophils	
Peripheral blood	28009* ±3844	69.1 ± 4.6	11.3 ±2.7	16.9 ±2.6	1.5 ±0.5	1.2 ±0.4	
Eosinophil-enriched prepara-tions		90.9 ±2.3	5.5 ±2.0	2.5 ±0.6	0.2 ±0.2	0 —	54.2 ±7.3

* Mean and standard error.
† Eosinophil yield is the mean percentage recovery of twelve separations.

from blood varied considerably (mean 54.2 ± 7.3%), generally being a function of the number and percentage of circulating eosinophils. Hypotonic lysis increased the percentage of eosinophils, reflecting the relatively superior resistance of eosinophils to hypotonicity compared to other leukocytes, and accounting in part for the increase in the eosinophil percentage in the purified preparations.

Phase microscopy of lysates revealed monodispersed phase-dense particles, spherical nuclei, a few unbroken granulocytes, and rare RBCs. The 5-μ filtrate was cleared of nuclei and cells, yet it was viscous. Sequential filtration through the 2-μ filter eliminated the viscous nature of the 5-μ filtrate, and revealed by phase microscopy innumerable monodispersed phase-dense particles, which were consistent with eosinophil granules inasmuch as they were larger and more uniform than neutrophil granules prepared similarly. By electron microscopy this preparation contained little morphologically recognizable contaminating material (Figure 1).

Analysis of the enzyme data was performed on cell lysates (Table 3) comparing the content of eosinophils to the content of normal neutrophils purified according to slightly different methods.[15] One milliliter of lysate contained 11.1 × 10^6 leukocytes; for neutrophil preparations, 96.1 ± 0.81% were neutrophils, and for eosinophil preparations, 90.9 ± 2.3% were eosinophils. Eosinophils contained approximately 20% more protein than neutrophils, presumably reflecting their larger size. Differences between the lysate content of neutrophils and eosinophils were significant for protein and each enzyme measured ($P < .01$, except acid β-glycerophosphatase, where $P < .05$). Alkaline phosphatase and muramidase were nearly absent from the eosinophil lysate, while protein and the acid hydrolases were significantly greater in eosinophil lysate.

Specific activities (Table 3) were determined for individual enzyme determinations by taking the ratio of enzyme activity to protein content

Table 3—Protein Content, Enzyme Activity, and Specific Enzyme Activity of Human Blood Neutrophils and Eosinophils Compared for Lysates, 5-μ Filtrates, and 5-μ-2-μ Filtrates*

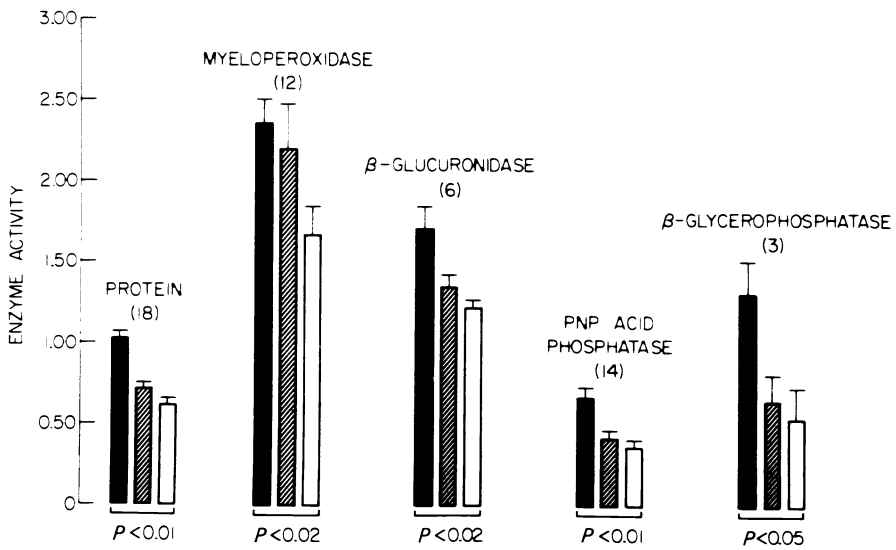
	Neutrophils		P	Eosinophils							Eosinophil S/A as percent of neutrophil S/A
Protein (mg/ml)											
Lysate	0.851 ± 0.029	(22)	< .01	1.012 ± 0.045	(18)						119%†
5-μ Filtrate	0.623 ± 0.035	(22)		0.705 ± 0.033	(18)						
5-μ-2-μ Filtrate	0.618 ± 0.024	(22)		0.608 ± 0.036	(18)						
	Enzyme activity‡					Specific enzyme activity§					
	Neutrophils		P	Eosinophils		Neutrophils		P	Eosinophils		
Peroxidase											
Lysate	0.94 ± 0.05	(23)	< .01	2.36 ± 0.16	(12)	1.09 ± 0.05	(20)	< .05	2.48 ± 0.24	(12)	228%
5-μ Filtrate	0.77 ± 0.04	(23)		2.19 ± 0.27	(13)	1.19 ± 0.06	(20)		3.30 ± 0.49	(13)	
5-μ-2-μ Filtrate	0.76 ± 0.04	(22)		1.67 ± 0.20	(12)	1.19 ± 0.06	(20)		2.75 ± 0.35	(12)	
β-Glucuronidase											
Lysate	74.6 ± 5.6	(11)	< .01	171.9 ± 14.8	(5)	92.9 ± 7.1	(11)	< .05	158.2 ± 12.2	(5)	170%
5-μ Filtrate	55.6 ± 6.5	(10)		134.3 ± 8.7	(6)	97.4 ± 8.2	(10)		178.8 ± 12.2	(6)	
5-μ-2-μ Filtrate	63.2 ± 5.4	(10)		122.3 ± 9.8	(6)	103.7 ± 7.1	(10)		175.0 ± 15.5	(6)	
Muramidase											
Lysate	72.1 ± 4.5	(18)	< .01	11.7 ± 1.4	(10)	82.6 ± 5.1	(17)	< .05	12.6 ± 1.7	(10)	15%
5-μ Filtrate	54.9 ± 4.2	(18)		10.9 ± 1.6	(10)	92.0 ± 7.1	(17)		17.7 ± 2.8	(10)	
5-μ-2-μ Filtrate	55.8 ± 3.6	(17)		9.4 ± 2.1	(10)	96.1 ± 6.3	(17)		19.0 ± 4.2	(10)	
Alkaline pnp phosphatase											
Lysate	1.76 ± 0.10	(22)	< .01	0.24 ± 0.03	(10)	2.14 ± 0.17	(22)	< .05	0.23 ± 0.04	(10)	11%
5-μ Filtrate	1.27 ± 0.09	(21)		0.16 ± 0.03	(11)	2.07 ± 0.15	(21)		0.22 ± 0.04	(11)	
5-μ-2-μ Filtrate	1.32 ± 0.10	(21)		0.16 ± 0.03	(12)	2.21 ± 0.17	(21)		0.25 ± 0.04	(12)	
Acid pnp phosphatase											
Lysate	4.49 ± 0.38	(23)	< .01	6.59 ± 0.63	(14)	5.26 ± 0.43	(23)	< .2	6.17 ± 0.50	(14)	117%
5-μ Filtrate	3.13 ± 0.28	(23)		4.16 ± 0.47	(15)	5.16 ± 0.46	(23)		5.85 ± 0.57	(15)	
5-μ-2-μ Filtrate	2.81 ± 0.24	(21)		3.64 ± 0.47	(14)	4.64 ± 0.36	(21)		5.57 ± 0.59	(14)	
Acid β-glycero-phosphatase											
Lysate	0.54 ± 0.08	(3)	< .05	1.30 ± 0.20	(2)	0.64 ± 0.10	(3)	< .05	1.30 ± 0.17	(2)	202%
5-μ Filtrate	0.48 ± 0.03	(3)		0.65 ± 0.14	(3)	0.77 ± 0.04	(3)		0.95 ± 0.17	(3)	
5-μ-2-μ Filtrate	0.46 ± 0.09	(3)		0.53 ± 0.14	(3)	0.74 ± 0.04	(3)		0.87 ± 0.28	(3)	

* Data are expressed as the mean ± standard error; numbers in parentheses indicate number of experiments.
† Eosinophil protein content expressed as a percentage of neutrophil protein content.
‡ Enzyme activity is expressed as enzyme units (see text) per milliliter of lysate or filtrate.
§ Specific enzyme activity (S/A) is expressed as enzyme units (see text) per milligram protein.

for that same sample. For each enzyme, the mean of individually determined specific activities was calculated and presented in Table 3. To compare the activity of eosinophils to neutrophils, the neutrophil-specific activity was made the denominator and the eosinophil-specific activity was made the numerator, with the ratio expressed as a percentage (Table 3). Differences in specific activities between neutrophils and eosinophils were significant ($P < .05$), except acid *p*-nitrophenylphosphatase ($P < .2$). The eosinophil lysates contained more than twice as much peroxidase and 1.7 times as much β-glucuronidase as neutrophil lysates when equalized for protein (Table 3). Roughly twice as much acid β-glycerophosphatase was present in eosinophil lysates. Although acid phosphatase measured with *p*-nitrophenylphosphate (pnp) as substrate was present in significantly greater amounts in the eosinophil lysates, specific activities were equal in lysates of both types of granulocytes. This represents an additional association between total cell protein and acid *p*-nitrophenylphosphatase. The small amounts of muramidase in the eosinophil lysates reflect the presence of small numbers of neutrophils as well as the inaccuracy of the assay at very low activities. The small amount of alkaline phosphatase can safely be attributed to leukocytes other than eosinophils, based on gradient data presented below.

The enzyme activities of eosinophil lysates were compared to the enzyme activities of the same samples following filtration in order to assess the effect of filtration. Text-figure 1 displays the activity of each filtrate (see Table 3) compared to the parent lysate for the enzymes present in significant quantity in eosinophils. Muramidase and alkaline phosphatase were omitted because of their low activities. In general there was a sequential reduction in both protein and enzyme concentration following each filtration. Proportionately more protein was eliminated than peroxidase or β-glucuronidase, although the rise in the specific activity of the 5-μ and 2-μ filtrate over that of the lysate was not significant.

The effect of filtration on granules was further assessed by partitioning enzyme activity between supernatant and pelleted granules. Aliquots of individual lysate and the lysate's 5-μ and 2-μ filtrate were centrifuged at 27,000*g* for 30 minutes at 4 C. The results are expressed as the mean (± standard error) total protein and enzyme specific activities comparing the pelleted parent lysates to the pelleted 5-μ and 2-μ filtrates of an aliquot of the same parent lysate. Three such experiments were conducted with all assays in duplicate. The mean total protein in the lysate pellets was 654 ± 15 μg compared to 228 ± 30 μg ($P < .001$) in the filtrate pellets. The mean peroxidase specific activity in the lysate pellets was 2.21 ± 0.17 μg HRP equivalents/mg protein as compared with 3.86 ± 0.37 ($P < .02$) in

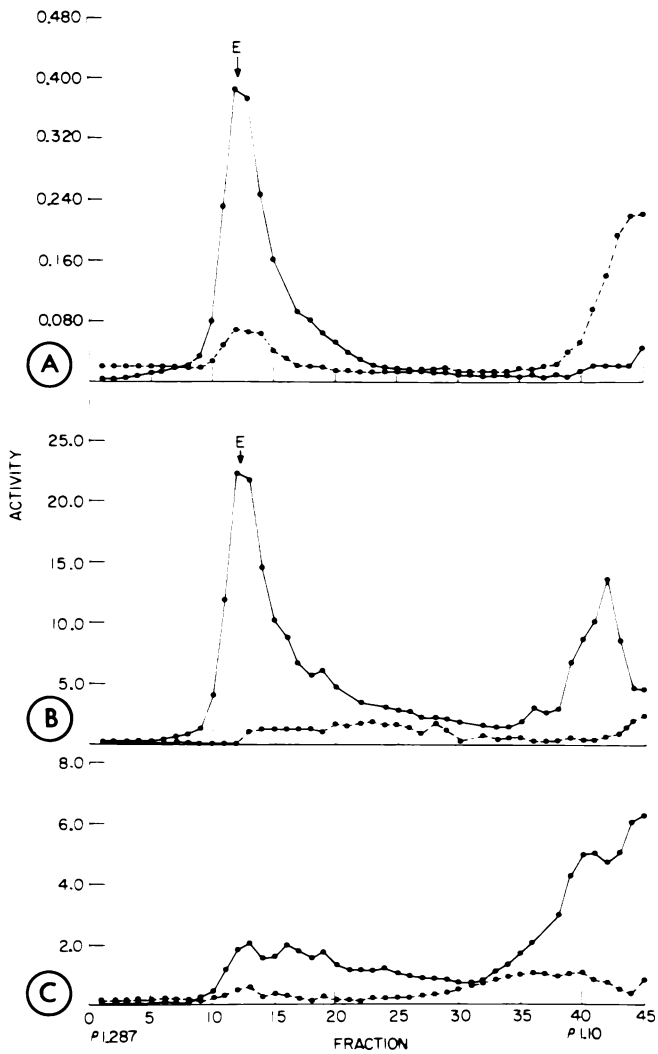


TEXT-FIGURE 1—Protein and enzyme activities of eosinophil lysates (*solid columns*), 5-μ filtrates (*hatched columns*), and 5-μ and 2-μ filtrates (*open columns*); the columns represent the mean of the number of experiments (in parentheses). Comparing the lysates with the 5-μ filtrates yielded $P < .05$ (similar to those shown comparing lysates with the 5-μ and 2-μ filtrates) except for the peroxidase, where there was no significant difference. $P < 0.05$ was significant. Protein is expressed as milligrams per milliliter, β-glucuronidase as 10^{-2} × units, and acid *p*-nitrophenylphosphatase as 10^{-1} × units; see text for units.

the filtrate pellets. This is consistent with the preservation of peroxidase as a sedimentable, tightly granule-bound enzyme, which is somewhat enriched *vis à vis* protein by filtration. β-Glucuronidase seemed less tightly bound to the granules since there was no difference between the mean specific activity of the lysate pellets, 199 ± 12 units/mg protein, and the filtrate pellets, 232 ± 31 ($P < .4$). The specific activity of acid *p*-nitrophenylphosphatase on the other hand, declined from 78.9 ± 10.2 units/mg protein in the pelleted lysate to 45.5 ± 2.9 in the pelleted filtrate ($P < .05$). Thus, of the enzymes tested, this enzyme appears to be the least granule associated, and it is probably not granule bound at all.

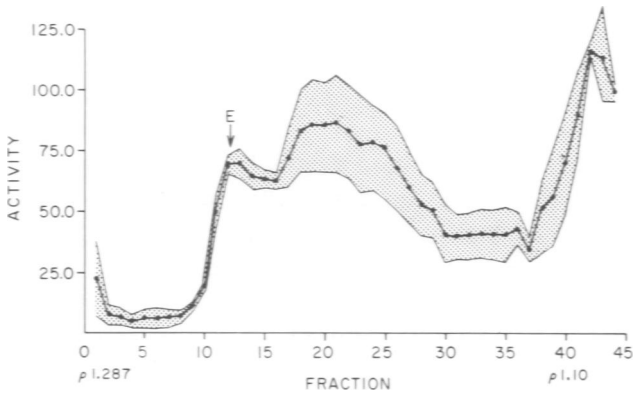
Gradients contained a single band (Text-figure 2), which was green, with mean density of 1.24. Electron microscopy of this band demonstrated a population of variably ellipsoidal granules most of which contained electron-dense cores, i.e. typical eosinophil granules.

Enzymatic assay of gradient fractions localized peroxidase, β-glucuronidase (Text-figure 2), and acid β-glycerophosphatase (Text-Figure 3) to Band E. Acid β-glycerophosphatase presented a particular problem because of its wide distribution in the gradient. The most precise peak (small standard error) did occur at Fractions 12 and 13, corresponding to Band E



TEXT-FIGURE 2—Protein and enzyme activity of human eosinophil granules in sucrose gradients from density 1.287 to 1.10, with Band E indicated by an *arrow*. **A**—Peroxidase, mean of eleven gradients (*solid line*) and protein, mean of ten gradients (*dotted line*) levels are shown. **B**—β-glucuronidase, mean of nine gradients (*solid line*) and muramidase, mean of three gradients, (*dotted line*) levels are shown. **C**—Acid *p*-nitrophenylphosphatase, mean of eight gradients, (*solid line*) and alkaline *p*-nitrophenylphosphatase, mean of six gradients (*dotted line*). Protein is expressed as milligrams per milliliter; see text for other units.

and the peak activities of peroxidase and β-glucuronidase. Since heparin inhibition could not easily be overcome,[16,17] and since the precise distribution of heparin could not be determined, the acid β-glycerophosphatase result is considered an estimate. Noteworthy was the parallel distribution of protein and acid *p*-nitrophenylphosphatase, reflecting the soluble na-



TEXT-FIGURE 3—Gradient distribution of acid β-glycerophosphatase of human eosinophil granules in sucrose gradients from density 1.287 to 1.10 with Band E indicated by an *arrow*, expressed as mean ± standard error (*shaded area*) of four gradients, in nanograms phosphorus released per minute per milliliter.

ture of the enzyme with amounts of both near the origin. In addition to its prominent localization in Band E, β-glucuronidase was also distributed near the origin, presumably reflecting either disruption of some granules or solubilization (separation from a sedimentable granule) of some β-glucuronidase from all granules. Thus, in contrast to peroxidase, which is concentrated by filtration and pelleting at 27,000*g* and which has virtually no residue at the origin of the gradient (Text-figure 2), β-glucuronidase is not concentrated by pelleting at 27,000*g* and has a significant soluble residue at the gradient origin. It probably is less tightly bound to granules and may exist in the outer shell of the granule as opposed to its inner peroxidase-containing core.

No appreciable muramidase or alkaline phosphatase was found in these gradients. Protein assay by the Lowry technique was considered an estimate because of the known interference with the Folin reagent by dense sucrose.[18,19] Distinguishing features of individual patient's eosinophil granule density or content were not identified.

Discussion

Separation of human blood neutrophils into three major bands occasionally resulted in a fourth, more dense band (Band E) at 1.24 g/ml.[15] Band E only occurred in linear sucrose gradients of neutrophil granules from normals with mild peripheral blood eosinophilia (5 to 8%), and corresponded to a small peak of peroxidase activity slightly out of proportion to the percentage of eosinophils involved, *vis à vis* the neutrophils. The studies reported here show conclusively that that band at density 1.24 does represent the eosinophil granule.

The peroxidase of eosinophils has been shown to be genetically distinct from neutrophil myeloperoxidase in humans [20] and biochemically distinct

in rats.[3] Hence, our observation that eosinophils contain 2.65 times as much peroxidase per cell as neutrophils has several possible explanations and implications. The slightly larger size of eosinophils, which contain approximately 20% more protein than neutrophils, does not alone explain the higher peroxidase content. The presence of a single granule type filling the cytoplasm, taken with the presence of peroxidase in all of these granules which is implied by the current data, is the best explanation for the higher content. However, the biochemical and genetic differences in peroxidases suggest that the higher content in eosinophils might be an artifact of different structures and disparate kinetics rather than strictly the amount of enzyme per cell. Furthermore, the absence of bactericidal activity of peroxidase in intact eosinophils [9] provides a functional difference between the two granulocytes' peroxidases. Packaging and availability of peroxidase to the eosinophil phagolysosome are probably quite different from neutrophils where myeloperoxidase is important to killing. For clarity, our data favor using separate distinguishing names for neutrophil myeloperoxidase (NMPO) and eosinophil peroxidase (EPO or EMPO).

Similarly the activity of the acid hydrolases (β-glucuronidase and acid β-glycerophosphatase) in the eosinophil indicates greater activity there than in the neutrophil. Eosinophils have been observed to contain only one granule type, as this study corroborates. In contrast, neutrophils have five granule types and subtypes,[15] two of which contain peroxidase.[15,21] Hence, it is not surprising that eosinophils have greater peroxidase and certain acid hydrolase activities than do neutrophils.

Muramidase was conspicuously absent from the eosinophil granule and cytoplasm, tending to indirectly confirm its location within the neutrophil granules lacking myeloperoxidase.[15]

Alkaline phosphatase is also noticeably absent from the eosinophil granule and other gradient fractions. Its absence is an additional distinguishing difference between eosinophils and neutrophils. The conventional histochemical test, *leukocyte alkaline phosphatase* (LAP), should be more precisely stated as *neutrophil alkaline phosphatase* (NAP) in light of these observations. This term—NAP—might aid in avoiding the clinical laboratory error of counting blood eosinophils negative for this enzyme when indeed, they do not contain this enzyme. Its absence from the eosinophil is possibly an additional reason for the quantitatively less effective phagocytic and bactericidal function and qualitatively distinct chemotactic response when compared to neutrophils.

By defining the density and some important enzyme markers of eosinophilic lysosomal granules, these studies emphasize distinguishing and nonneutrophil properties of the human blood eosinophil.

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[*Illustrations follow*]

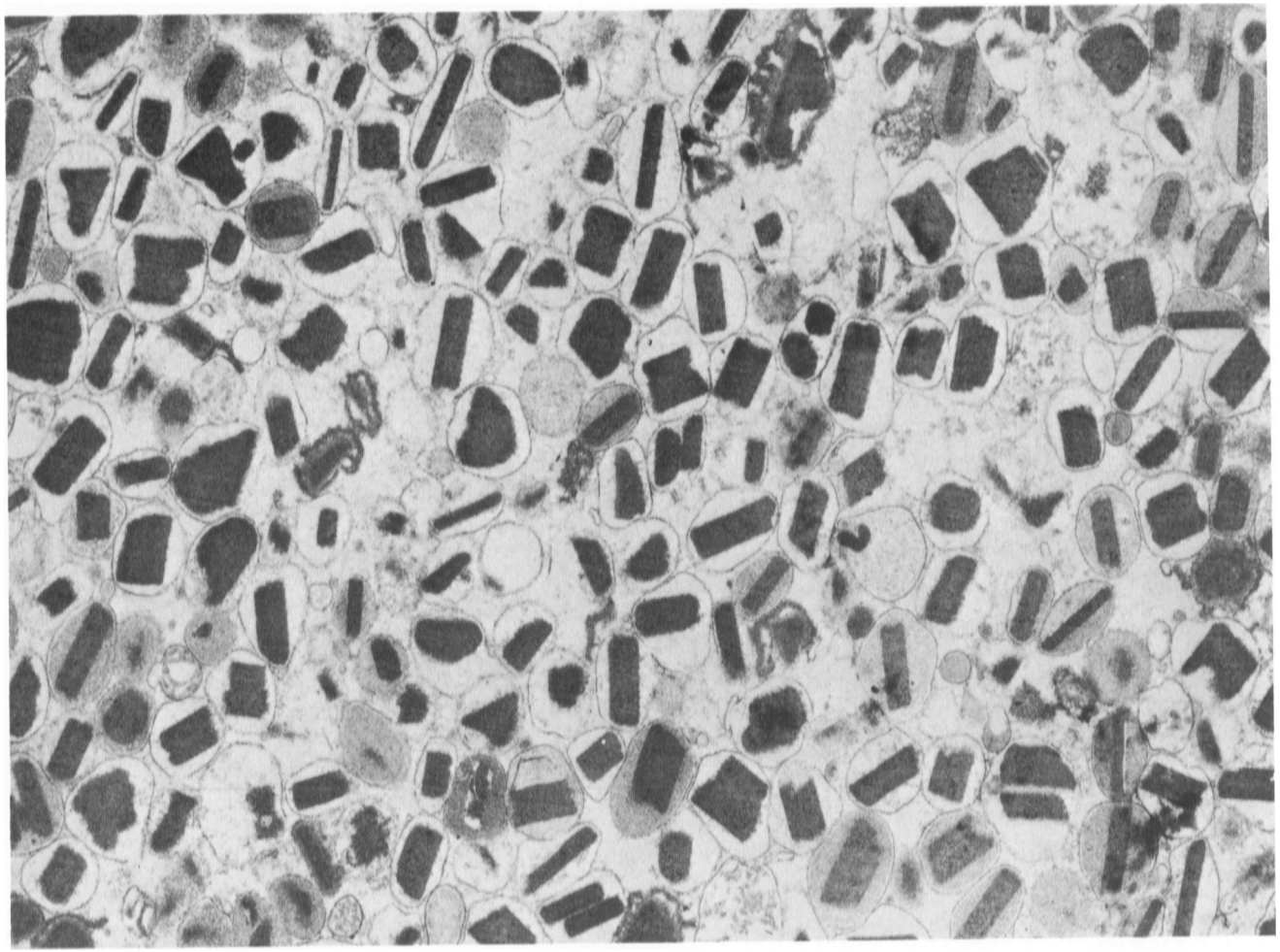

Figure 1—Electron micrograph of standard 5-μ and 2-μ filtrate of lysed human eosinophils illustrating their granule content (× 19,000).

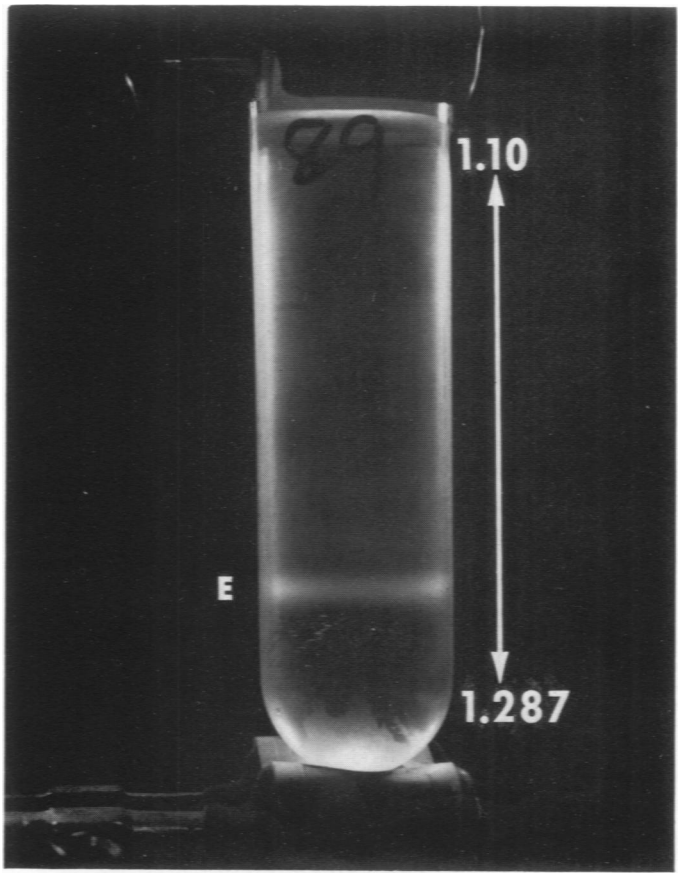

Figure 2—Linear sucrose gradient of density range 1.287 to 1.10, following a 4-hour sedimentation of a 5-μ and 2-μ filtrate of human eosinophil granules, showing Band E at density 1.24 g/ml.