PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **178**, 373–384 (1985)

Heparin Inhibition of Human Neutrophil and Eosinophil-Enriched Leukocyte Acid β-Glycerophosphatase (42020)

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Abstract. Heparin inhibited acid β-glycerophosphatase (EC 3.1.3.2) from human blood leukocytes, eosinophil-enriched leukocytes, and neutrophils. The inhibition interfered in the hydrolysis of phosphorus from glycerophosphate, not in the formation or detection of colored complexes of phosphomolybdate in the second or color development step in two conventional assays. Heparin inhibited human hypereosinophilic syndrome leukocyte homogenate enzyme activity according to the equation: activity equals 0.946 − 0.087 ln heparin (units/assay) when heparin was varied from 1 to 100 units per assay. At 100 units of heparin per assay, 51% of the original activity remained. Enzyme activity was less in neutrophils than in eosinophils; moreover, the inhibition of neutrophil homogenate by heparin was considerably less than that seen in the eosinophil-enriched leukocyte preprations. In neutrophil homogenates containing 100 units of heparin per assay, 77.1% of activity without heparin was retained. When neutrophil lysates were utilized, less inhibition was observed: e.g., at 1 unit of heparin per assay, 91.7% enzyme activity was retained and at 1000 units, 76.2%; here, activity equals 0.289 − 0.007 ln heparin. The data allowed more precise consideration of the inhibition of acid β-glycerophosphatase by heparin, and, while confirming quantitatively the greater content of acid β-glycerophosphatase in eosinophil-enriched leukocyte preparations than in neutrophil preparations, provide experimental support for an acid β-glycerophosphatase in human eosinophils, which is different from that in human neutrophils. It is more highly susceptible to heparin inhibition than acid β-glycerophosphatase in human neutrophils from which it is apparently distinct. © 1985 Society for Experimental Biology and Medicine.

Acid phosphatase (EC 3.1.3.2) is the original marker enzyme for lysosomes (1, 2). Acid phosphatase utilizing β-glycerophosphatase as substrate is known to be present in the primary granule of the rabbit heterophile leukocyte (3). A similar association of this enzyme to human neutrophil azurophil granules (4–9) and eosinophil granules (10) has been made. Of the two populations of azurophil granules (5, 8, 11, 12), one at density 1.22 g/ml (band A granules) and one at density 1.20 g/ml (band B granules), the principal association of acid β-glycerophosphatase is with the more dense band A granules (5). Unequivocal localization is impeded because of inhibition of acid β-glycerophosphatase by heparin (5, 13, 14), which is required in some granulocyte lysis procedures to prevent agglutination of cell-free granules (4–6).

In this report, the assay for acid β-glycerophosphatase is a modification (5) of the method of Applemans *et al.* (15), utilizing the formation of phosphomolybdate to detect the release of inorganic phosphorus according either to the method of Fiske and Subbarow (16) or Chen *et al.* (17). The effect of heparin upon both steps (cleavage of phosphorus and color development) in the assay of acid β-glycerophosphatase in human granulocytes was assessed. A preliminary experiment showed inhibition of human eosinophil-enriched leukocyte acid β-glycerophosphatase. In this experiment leukocytes (mostly eosinophils) from one patient with the hypereosinophilic syndrome (HES) were the source of acid β-glycerophosphatase. The investigation of this inhibition of this enzyme activity from leukocytes and purified neutrophils from healthy men and the preliminary study which prompted it are reported herein. Heparin inhibition of both human eosinophil-enriched leukocyte and neutrophil acid β-glycerophosphatase was observed. Less susceptibility of neutrophil acid β-glycerophosphatase to heparin inhibition was observed. Heparin inhib-

0037-9727/85 $1.50
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ited the cleavage of phosphorus, not the formation and detection of phosphomolybdate.

Materials and Methods. *General.* Blood collection and leukocyte preparation were in sterile plasticware. All reagents were made utilizing triply distilled water or deionized water made from distilled water and reagent-grade chemicals. Sodium heparin of beef lung origin (Upjohn Co., Kalamazoo, Mich.) was utilized. Permission for venipuncture was granted after informed consent was obtained in keeping with established institutional regulations.

1. Preparation of HES leukocyte homogenate. Sixty milliliters of peripheral venous blood were drawn from a man with the HES (case 14, Ref. 18) in this experiment performed in 1970. His white blood cell (WBC) count was 23,152 ± 593 (mean ± SD) with 77% eosinophils, 2% neutrophil bands, 13% neutrophils, 6% lymphocytes, and 2% monocytes. Of the 77% eosinophils, 2 were small bizarrely shaped eosinophils judged to be very immature, 3 were promyelocytic eosinophils, 1 was a myelocytic eosinophil, 1 was a metamyelocytic eosinophil, 5 were juvenile eosinophils, and 65 were mature eosinophils. The patient's blood, anticoagulated in 0.2% Na_2H_2EDTA, pH 7.3, was mixed promptly with an equal volume of 3% dextran (approx. mol wt 250,000, Pharmacia, Uppsala, Sweden) in 0.9% NaCl, and sedimented at room temperature for 25 min. The leukocyte-rich supernatant was centrifuged at 400*g* for 10 min at 4°C and the pellet was subjected to three cycles of hypotonic lysis as previously described (5). The resulting leukocyte pellet was homogenized in a Teflon pestle homogenizer (A. H. Thomas Co., Philadelphia, Pa.) in ice. Aliquots of this homogenate were utilized as the source of acid β-glycerophosphatase after storage at −20°C for 1 month.

2. Preparation of leukocyte homogenates. Sixty milliliters of blood, minimally anticoagulated in 0.2% of Na_2H_2EDTA, pH 7.3, were obtained by venipuncture from each of four healthy white men. The WBC counts ranged from 4750 to 10,850/mm^3 (7088 ± 2753). The differential WBC counts were normal (except one donor with 15% eosinophils) with 67 ± 8% neutrophils, 25 ± 11% lymphocytes, 1 ± 1% monocytes, 6 ± 6% eosinophils, and 1 ± 1% basophils. The blood was mixed with an equal volume of 3% dextran in 0.85% modified Hanks' solution (MHS: Hanks' solution lacking calcium, magnesium, phenol red, and antibiotics, adjusted to 8.5 g/liter NaCl concentration), and allowed to sediment at room temperature for 20 min. The supernatant was aspirated, diluted in an equal volume of 0.85% MHS, and centrifuged at 400*g* for 10 min at 4°C. The resulting pellets were subjected to two cycles of hypotonic lysis and a WBC count was done. The yield was 324 ± 115 × 10^6 (mean ± SD) leukocytes, which included 69 ± 8% neutrophils, 29 ± 9% mononuclear cells, and 2 ± 3% eosinophils, although each donor's cells were kept separate for assay. Cells from the donor with 15% eosinophilia contained 7% eosinophils after these steps, and results from his homogenates were analyzed separately, by combining them with the three others', and by excluding them from analysis of the three normal donors' results. The pellets were diluted in 0.34 *M* sucrose containing Triton X-100, homogenized, further diluted with 0.34 *M* sucrose to a concentration of 10^7 WBC/ml in 0.1% Triton X-100, and frozen at −20°C until assayed which was within 2 weeks.

3. Preparation of neutrophil homogenate. Another white male volunteer was similarly bled of 100 ml and the neutrophils were purified according to the method in section 4, except that once the neutrophil pellet was obtained, the neutrophils were subjected to homogenization as described above. The WBC count was 14,550/mm^3 with 68% neutrophils, 26% lymphocytes, 5% eosinophils, 2% monocytes, and 1% basophils. After cell separation, there were 561 × 10^6 WBC which were 98% neutrophils and 2% eosinophils. Of 594 × 10^6 neutrophils, 550 × 10^6 were obtained for a yield of 92.6%.

4. Preparation of neutrophil lysates. Blood (100 ml) minimally anticoagulated with 0.2% Na_2H_2EDTA, pH 7.3, was obtained by venipuncture from each of four healthy adult white men. The WBC counts ranged from 4716 to 8890/mm^3 (6178 ± 1858). The differential WBC counts were normal with 53 ± 6% neutrophils (range 47–60%); 41 ± 5% lymphocytes; 2.3 ± 1.0% monocytes; and 3.3

± 2.5% eosinophils. The neutrophils were prepared by Hypaque–Ficoll gradients, dextral sedimentation, and hypotonic lysis as previously described (5, 11). This yielded 189.5 ± 55.9 × 10^6 leukocytes, 98 ± 2.0% neutrophils, and 2.0 ± 2.0% eosinophils. The yield of neutrophils was thus 185 × 10^6 per donor. The mean number of neutrophils available per donor was calculated by multiplying the volume of blood (60 ml), times the mean WBC (6178), times the mean ratio of neutrophils to leukocytes (0.53), times 1000, and was 202.8 × 10^6. Approximately 94% of the available neutrophils were recovered. Neutrophils were suspended in 0.34 *M* sucrose at a concentration of 10^7/ml. Neutrophils were then subjected to five cycles of freezing at −20°C and thawing which was done in ice to prevent excess warming. Assays were performed within 2 weeks, lysates being stored at −20°C until assayed.

Effect of heparin upon the color development step of assays for acid β-glycerophosphatase. The assay for acid β-glycerophosphatase was a modification (5) of the method of Applemans *et al.* (15) utilizing in its second stage, i.e., color development, one of two methods for determination of released inorganic phosphorus. The standard aminonaphtholsulfonic acid method of reducing phosphomolybdate of Fiske and Subbarow (16) was compared to the eightfold more sensitive method of Chen *et al.* (17) which depends upon ascorbic acid for reduction of phosphomolybdate. Because a commercial aminonaphtholsulfonic acid solution yielded erratic results, this reagent was made as needed. Phosphate standard stock solution was made from potassium phosphate monobasic (Lot No. 786903, Fisher Scientific Co., Fairlawn, N.J.). About 1 g was dried overnight at 110°C, 0.4400 g was weighed while hot on a precision balance and dissolved in triply distilled water and 10 ml of 10 *N* H_2SO_4 to a final volume of 1000 ml. A 1/10 v/v dilution of the stock solution contained 10 μg *Pi*/ml and was used in the color development experiments and steps in the assay as phosphate standard.

Assay for acid β-glycerophosphatase in HES leukocyte homogenates. Utilizing the method of Fiske and Subbarow, 1 ml leukocyte homogenate equivalent to 10 million leukocytes; 0.5 ml 0.5 *M* sodium acetate buffer, pH 5.0; 0.5 ml 1% Triton X-100 (Packard Instrument Co., Downers Grove, Ill.); 0, 1, 10, or 100 units of heparin; and water to make a 2.5-ml volume were mixed and preincubated at 37°C. Sodium β-glycerophosphate, 0.05 *M* (Fisher Scientific Co.), was adjusted to pH 5.0 with a few drops of concentrated acetic acid and preincubated at 37°C. The reaction was started by addition of 2.5 ml substrate to the buffered leukocyte homogenate, giving a final concentration of 0.05 *M* acetate buffer, pH 5.0, 0.1% Triton X-100, and 0.025 *M* β-glycerophosphate. The reaction was carried out in a 37°C agitating water bath. One-milliliter aliquots were removed at 0, 15, 30, and 60 min and placed in cold 10% trichloroacetic acid. Precipitated protein was removed by filtration through Whatman No. 42 filter paper. Inorganic phosphorus release was measured by color development at room temperature for 10 min and read at 660 nm (16). A Gilford 240 spectrophotometer equipped with digital readout and rapid sampler accessories (Gilford Instrument Laboratories, Oberlin, Ohio) was used. A standard curve using 10, 20, and 40 μg of phosphorus was determined with each assay. The slope of this curve was used to determine enzyme activity according to the formula: (absorbance − absorbance at zero time/min of incubation) × (total volume of the reaction mixture in ml/volume of reaction mixture in ml assayed at an interval) × (volume of the 10% trichloroacetic acid plus the volume of the reaction mixture added to it/volume of this mixture assayed for *Pi*) × (slope of standard curve: μg *Pi*/absorbance)/(volume of enzyme solution used in reaction mixture), which provides enzyme units in micrograms *Pi* released per minute per milliliter.

Assay for acid β-glycerophosphatase in leukocyte homogenates. The assay was the same as described above except that 1.4 ml of leukocyte homogenate, which was equal to 10^7 leukocytes/ml, was used in each assay, that 1.0-ml aliquots were removed at 0, 30, 60, and 90 min incubation, and that heparin in each assay amounted to 0, 1, 10, 100, or 200 units.

Assay for acid β-glycerophosphatase in neutrophil homogenate. The assay for acid β-

glycerophosphatase was the same as described above for leukocyte homogenates.

Assay for acid β-glycerophosphatase in neutrophil lysates. The total reaction mixture volume was proportionately reduced to 2.0 ml (from 5.0 ml), and color development was only performed according to Chen *et al.* (17). To the preincubation mixture was added 0.2 ml freeze–thawed neutrophils, containing approximately 10^7 neutrophils/ml. This mixture also contained 0.2 ml 0.5 *M* sodium acetate buffer, pH 5.0, 0.2 ml 1% Triton X-100, and water to make a 1.0-ml volume. The reaction was started by addition of 1.0 ml of 0.05 *M* sodium β-glycerophosphate, previously adjusted to pH 5.0 with a few drops of concentrated acetic acid and preincubated at 37°C. The final concentration of reagents was equal to that described in the previous section, but the volumes were reduced 40%. A standard curve using 1, 2, and 4 μg of phosphorus was determined with each assay (17).

Protein. Protein was determined in duplicate by the method of Lowry *et al.* (19), using bovine serum albumin as the protein standard.

Statistical analysis. Standard deviation was used as an expression of variation. Comparisons of means were made using the program for the paired *t* statistic, the linear regression curve fitting program calculated through the origin was used to describe the standard phosphorus concentration vs absorbance curves, and the logarithmic curve fitting program was used to relate heparin concentration, excluding the zero concentration of heparin, and enzyme activity (HP97 programs and programmable calculator, Hewlett–Packard, Corvalis, Ore.).

Results. *Effect of heparin upon color development.* Color development, which is proportionate to the concentration of inorganic phosphorus, is the final step in both methods. The aminonaphtholsulfonic acid method of reducing phosphomolybdate (16) and the ascorbic acid method to reduce phosphomolybdate to blue complexes (17) were tested in the presence of 0–20 μg of phosphorus and of 0–4 μg of phosphorus, respectively, and heparin ranging from 0 to 200 units per assay and were unaffected.

Effect of heparin upon HES leukocyte homogenate acid β-glycerophosphatase activity. Heparin added to the reaction mixture caused a dose-related inhibition of eosinophil-enriched leukocyte acid β-glycerophosphatase (Fig. 1). The relationship of heparin concentration to enzyme inhibition as derived from Fig. 1 is: μg *P*i released per minute per milliliter at concentrations of heparin 1 to 100 units is equal to 0.946 − 0.087 ln heparin (units/assay). The activity at each concentration of heparin is provided in Table I. When comparisons of mean enzyme activity at each concentration of heparin are made using the paired *t* statistic, significant differences ($P < 0.05$) are observed for all such comparisons (based upon values of *t* and upon the area in two tails). For example, comparing enzyme activity in assays having no heparin with assays having 1 unit of heparin, 10 units of heparin, or 100 units of heparin showed a significant difference for each comparison. Mean enzyme activity at each heparin concentration was different from all others. By defining the mean activity with no heparin 100%, the percentage activities remaining were: 89.6% at 1 unit of heparin; 73.7% at 10 units; and 51% at 100 units. This clear

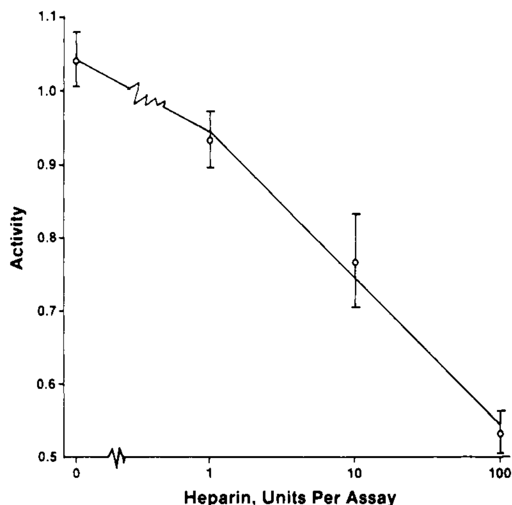

FIG. 1. Human hypereosinophilic syndrome leukocyte homogenate acid β-glycerophosphatase activity and its inhibition by heparin are shown. Activity expressed on the ordinate is μg *P*i released per minute per milliliter (mean ± SD). The abscissa is units of heparin per reaction mixture added to the leukocyte homogenate of 10^7 leukocytes just prior to the initiation of the timed assay.

result prompted a more thorough investigation of heparin inhibition of the enzyme activity.

Effect of heparin upon leukocyte homogenate acid β-glycerophosphatase activity. Heparin caused a dose-related but less striking inhibition of leukocyte homogenate β-glycerophosphatase activity, as shown in plots of enzyme activity vs heparin concentration in Fig. 2. The results from one normal donor with eosinophilia of 15% in peripheral blood and 7% of his purified leukocytes were considered separately (Fig. 2A), combined with the others (Fig. 2B), and excluded from consideration of the three normal donors' leukocyte homogenates (Fig. 2C).

When the single donor with 7% eosinophils in the leukocyte homogenate was separately examined (Fig.2A), the enzyme activity at all heparin concentrations was significantly higher than leukocyte homogenate activities (Figs. 2B, C) ($P < 0.05$), but less than activity in HES leukocyte homogenates (Fig.1) ($P < 0.05$). The enzyme activity of this donor's leukocytes (Fig. 2A) at all concentrations of heparin, especially at 10 and 100 units of heparin, was closer to that seen in the HES leukocyte homogenates than to non-eosinophil-enriched leukocytes or neutrophil homogenates (Table I).

Comparisons of each point within Fig. 2B reveal significant differences between mean activities both at 100 and at 200 units of heparin per assay and every other mean, but no significant difference existed between activities at 100 and at 200 units or any other pair of means (Table I). Within the experiment (Fig. 2B), comparison of means revealed the same significant differences observed as for the experiment depicted in Fig. 2A (Table I). By defining activity without heparin as 100%, the greatest decrease in activity was with 100 units, which was 74.7%.

When the donor with eosinophilia was excluded (Fig. 2C), the final leukocyte suspensions from the three normal donors contained, respectively, 1, 0, and 1% eosinophils. Less acid β-glycerophosphatase activity was observed and the same pattern of significant differences held as for the experiments depicted in Figs. 2A, B. Within the experiments displayed in Fig. 2C, significant decreases in activity only occurred at 100 and at 200

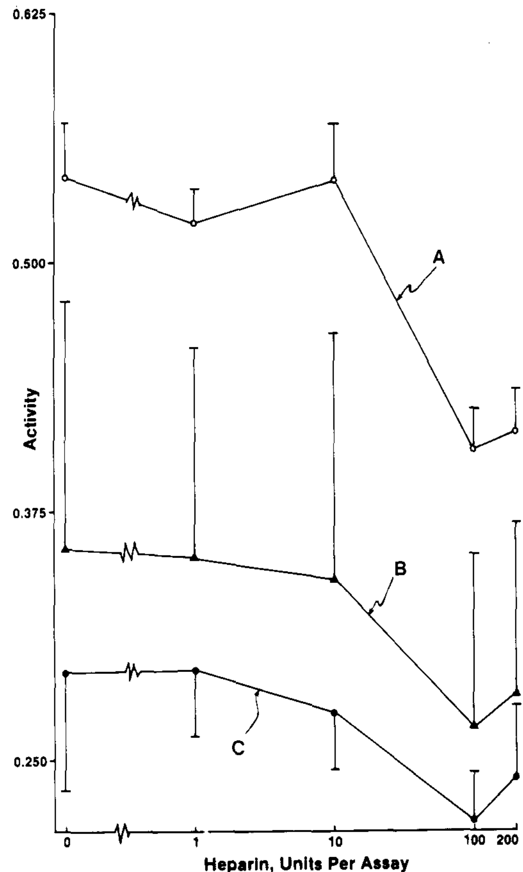

FIG. 2. Human leukocyte homogenate acid β-glycerophosphatase activity and its inhibition by heparin are shown. Activity expressed on the ordinate is μg Pi released per minute per milliliter (mean ± SD). The abscissa is units of heparin per reaction mixture added to the leukocyte homogenate of 10^7 leukocytes just prior to the initiation of the timed assay. (A) the donor had 15% peripheral blood eosinophilia and 7% of the homogenized leukocytes were eosinophils. (B) data from four donors including the one with eosinophilia shown in (A) are combined demonstrating the inhibition of enzyme activity by heparin. (C) excludes the data from the patient with eosinophilia and displays that from three normal donors demonstrating less overall activity but a similar dose-response of inhibition by heparin.

units of heparin per assay, compared to 0, 1, or 10 units of heparin, and the enzyme activities at these two concentrations of heparin were not different from each other (Table I).

The mean enzyme activity of these three leukocyte homogenates with zero heparin (depicted in Fig. 2C) contained 34% (0.356

TABLE I. ACTIVITY OF ACID β-GLYCEROPHOSPHATASE IN HUMAN LEUKOCYTE PREPARATIONS, AND ITS INHIBITION BY HEPARIN

<table>
<tr><td></td><td></td><td></td><td colspan="6">Enzyme activity in the presence of heparin (units/assay)</td></tr>
<tr><td>Expt</td><td>Donors</td><td>Assays</td><td>Heparin-free</td><td>1 unit</td><td>10 units</td><td>100 units</td><td>200 units</td><td>1000 units</td></tr>
<tr><td>1. Hypereosinophilic syndrome leukocyte homogenate</td><td>1</td><td>3</td><td>1.042 ± 0.038</td><td>0.934 ± 0.038</td><td>0.768 ± 0.064</td><td>0.533 ± 0.030</td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td colspan="2">$P < 0.001$</td><td></td><td></td><td></td><td></td></tr>
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<tr><td>2A. Leukocyte homogenate from donor with eosinophilia</td><td>1</td><td>3</td><td>0.542 ± 0.028</td><td>0.518 ± 0.018</td><td>0.541 ± 0.028</td><td>0.406 ± 0.020</td><td>0.414 ± 0.021</td><td></td></tr>
<tr><td></td><td></td><td></td><td colspan="2">$P =$ ns</td><td></td><td></td><td></td><td></td></tr>
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<tr><td></td><td></td><td></td><td colspan="4">$P < 0.02$</td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td colspan="5">$P < 0.05$</td><td></td></tr>
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<tr><td>2B. Leukocyte homogenates including donor with eosinophilia</td><td>4</td><td>12</td><td>0.356 ± 0.123</td><td>0.351 ± 0.105</td><td>0.341 ± 0.124</td><td>0.266 ± 0.087</td><td>0.284 ± 0.085</td><td></td></tr>
<tr><td></td><td></td><td></td><td colspan="2">$P =$ ns</td><td></td><td></td><td></td><td></td></tr>
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2C. Leukocyte homogenates excluding donor with eosinophilia	3	9	0.295 ± 0.060	0.295 ± 0.034	0.274 ± 0.028	0.220 ± 0.024	0.241 ± 0.036	
			P = ns	$P < 0.05$	$P < 0.001$	P = ns		
			P = ns					
			$P < 0.001$					
			$P < 0.05$					
				$P < 0.001$				
				$P < 0.01$				
					$P < 0.01$			
3. Neutrophil homogenate	1	3	0.349 ± 0.004	0.369 ± 0.014	0.355 ± 0.025	0.269 ± 0.014	0.252 ± 0.009	
			P = ns	P = ns	$P < 0.02$	$P < 0.05$		
			P = ns					
			$P < 0.02$					
			$P < 0.01$					
				$P < 0.05$				
				$P < 0.02$				
					$P < 0.02$			
4. Neutrophil lysates [method of Chen *et al.* (17)]	4	8	0.316 ± 0.038	0.290 ± 0.030	0.271 ± 0.034	0.261 ± 0.027	0.259 ± 0.029	0.241 ± 0.026
			$P < 0.01$	$P < 0.02$	P = ns	P = ns	P = ns	
			$P < 0.001$					
			$P < 0.001$					
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				$P < 0.01$				
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					$P < 0.01$			
						$P < 0.05$		

Leukocyte homogenate, neutrophil homogenate, or neutrophil lysate from 10^7 leukocytes or neutrophils was assayed under standard conditions for acid β-glycerophosphatase activity in the absence and presence of various concentrations of heparin. Assays refer to the number of data points or time intervals at which separate enzyme activity determinations are made. Enzyme activity (mean ± SD) is expressed as μg P_i released per minute per milliliter. The abbreviation for not significant ($P > 0.05$) is ns.

units) of the enzyme activity in the HES leukocyte homogenate without heparin (1.042 units). The 34% ratio is comparable to the 42% ratio of neutrophil lysate to eosinophil lysate acid β-glycerophosphatase activity previously reported (10).

Effect of heparin upon neutrophil homogenate acid β-glycerophosphatase activity with Fiske and Subbarow method. The mean activities of the three timed intervals of the assay are presented in Table I. Slight, nonsignificant increases in activity were noted at 1 and at 10 units of heparin compared to no heparin. Compared to each of these, the activity was significantly decreased at 100 units of heparin and at 200 units of heparin. In contrast to the leukocyte homogenate data above, the activity at 200 units of heparin was significantly less than at 100 units (Table I). Enzyme activity at 100 units of heparin represented 77.1% of the control without heparin and at 200 units of heparin, the activity was 72.3% of the control without heparin.

Effect of heparin upon neutrophil lysate acid β-glycerophosphatase activity. This set of experiments is different from the preceeding in that neutrophil lysates were prepared and then assayed according to the method of Chen *et al.* (17). In contrast to the immediately preceeding pilot experiment, four donors were used. The mean acid β-glycerophosphatase activity of the donors was 0.316 ± 0.038 units when no heparin was present, similar to the activity of Experiment 2C (Table I), which was 0.295 ± 0.060 units.

Mean activity consistently decreased despite larger variation at each incremental heparin concentration (Fig. 3). When activity data, paired regarding the time interval, were compared using the *t* statistic, the heparin-free neutrophil lysate acid β-glycerophosphatase activity was significantly higher than each of the enzyme activities with heparin added (Table I). Similarly, mean activity with 1 unit of heparin was significantly greater than each mean activity with a larger amount of heparin present. Significantly different also were 10 vs 200 units, 10 vs 1000 units, and 100 vs 1000 units (Table I). Other comparisons yielded no difference. By identifying heparin-free mean enzyme activity as 100%, heparin in the amount stated left the following

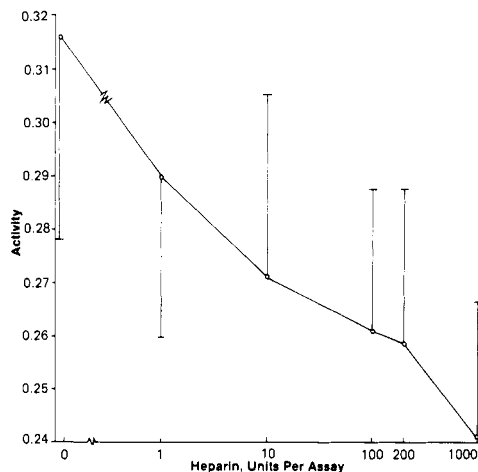

FIG. 3. Human neutrophil lysate acid β-glycerophosphatase activity and its inhibition by heparin are shown. Activity expressed on the ordinate is μg *Pi* released per minute per milliliter (mean ± SD). The abscissa is units of heparin per reaction mixture added to the leukocyte homogenate of 10^7 leukocytes just prior to the initiation of the timed assay.

residual activity: 1 unit, 91.7%; 10 units, 85.7%; 100 units, 82.5%; 200 units, 81.8%; and 1000 units, 76.2%. As illustrated in Fig. 3, a significant although modest enzyme inhibition was seen in these neutrophil lysates although it is substantially less than was observed in the eosinophil-enriched leukocyte homogenate. By applying a logarithmic curve-fitting program, enzyme activity expressed as micrograms *Pi* released per minute per milliliter of neutrophil lysate at 1–1000 units of heparin per assay is equal to 0.289 − 0.007 ln heparin (units/assay).

Specific activity. Since all enzyme units are expressed per ml of leukocytes containing 10^7 leukocytes per ml, the expressions of activity in Figs. 1–3, in Table I, and in the text are already in the form: activity per 10^7 leukocytes. Mean protein concentration for neutrophil lysates (Experiment 4, Table I, and Fig. 3) was 711 ± 49 μg/ml. Thus, the heparin-free acid β-glycerophosphatase activity of 0.316 units represented a specific activity (based upon protein) of 0.44 μg *Pi* released/min/milligram protein.

Discussion. Heparin was first reported to inhibit acid β-glycerophosphatase from nonleukocyte, nonhuman sources in 1957 (20).

This observation was made *de novo* as a problem in the assay of leukocyte acid β-glycerophosphatase using leukocyte and granule fractions which depended upon the use of heparin for their preparation (4–6, 10, 13, 14, 21, 22). In addition, heparin inhibits certain other human neutrophil enzymes, including acid *p*-nitrophenylphosphatase (4–6, 10, 23), β-glucuronidase (EC 3.2.1.31) (4–6, 10, 13, 14, 21–24), *N*-acetyl-β-glucosaminidase (EC 3.2.1.30) (11, 13, 14, 21, 22, 25), glutamate dehydrogenase (EC 1.4.1.3) (West, B. C., unpublished observations), and others (13, 14, 21, 22).

Earlier reports placed leukocyte acid β-glycerophosphatase activity as a percentage of control at 70% in the presence of 100 units of heparin/ml (13), 55% in the presence of 6 m*M* heparin (at pH 4.0) (21), and 91% (at pH 4.0) and 108% (at pH 5.0) when heparin was present in a 2:1 ratio to lysosomal protein at a concentration of 45–90 μg/ml (14). Those studies, which also addressed the effect of heparin on other neutrophil enzymes, provided less information about acid β-glycerophosphatase than about other enzymes, implying difficulties with this phosphatase or its assay (13, 14, 21, 22). Our experience confirmed that implication that great care is required to control variables and to achieve consistent results.

The present report indicated substantial inhibition of human eosinophil acid β-glycerophosphatase by heparin. Considerably less inhibition of human neutrophil acid β-glycerophosphatase was observed. The differences in the susceptibility of acid β-glycerophosphatase from different granulocyte sources indicate a high probability that acid β-glycerophosphatase in neutrophils is different from that in eosinophils. Both neutrophil and eosinophil acid β-glycerophosphatase are associated with lysosomal granule fractions. Although the associations are imperfect, neutrophil acid β-glycerophosphatase is associated with band A granules at density 1.22 g/ml (5), while eosinophil acid β-glycerophosphatase is associated with the more dense eosinophil granule (band E) at density 1.24 g/ml (10). Therefore, in addition to the observation that human eosinophils contain approximately twice as much total and specific activity of acid β-glycerophosphatase as do human neutrophils (10), the present results support an identity of eosinophil acid β-glycerophosphatase separate from neutrophil acid β-glycerophosphatase, the latter being about one-half as susceptible to heparin inhibition. Precedents for separating similar enzymes partly on the basis of differential susceptibility to heparin inhibition, for example, type I casein kinase from type II casein kinase, are known (26). Similarly, neutrophil myeloperoxidase has been distinguished from eosinophil peroxidase by recording different peroxidase activities when the same conditions and substrate were used (10, 27). Hence, the data also support separate enzyme names: neutrophil acid β-glycerophosphatase and eosinophil acid β-glycerophosphatase. Complex relationships of acid β-glycerophosphatases, such as one enzyme common to the two granulocytes in addition to two or more separate granulocyte-specific enzymes, are possible. Seven acid phosphatase isoenzymes from human leukocytes have been separated sufficiently to suggest that at least three and possibly four of them are present in neutrophils (28). However, β-glycerophosphatate was not one of the substrates used, thus not elucidating our results. Acid *p*-nitrophenylphosphatase, which has been repeatedly distinguished from acid β-glycerophosphatase, is identified with membrane fractions, not with granules, in human neutrophils (5, 7, 8, 29) and in human eosinophils (10).

The mechanism of heparin inhibition of acid β-glycerophosphatase is not revealed by our data. The fact that heparin is a potent polyanionic organic acid containing at least 24 sulfate groups (30), suggests it might compete with substrate for enzyme active sites. Alternatively, it might interfere with the configuration of the acid β-glycerophosphatase structure, diminishing its affinity for the usual substrate.

Acid β-glycerophosphatase activity of neutrophil homogenate without heparin in Experiment 3 was 0.349 ± 0.004 units which was more than comparable activity in Experiments 2C and 4. Although each of these differences was nonsignificant, the trend toward higher activity in Experiment 3 might have related to the leukocytosis of the donor, thus giving him a higher proportion of young

neutrophils, as reflected in his differential WBC count which showed 68% neutrophils of 14,550 WBC/mm^3. Although no toxic granules were observed in his blood smear, neutrophils with toxic granules do contain more acid phosphatase when tested by a method which does not depend upon the detection of acid *p*-nitrophenylphosphatase (31). Acid *p*-nitrophenylphosphatase is known to be normal in neutrophils with toxic granulations (32). Thus, our data are consistent with increased activity and specific activity of acid β-glycerophosphatase which is present in young neutrophils and which accompanies leukocytosis.

The present results suggest that inhibition of neutrophil acid β-glycerophosphatase by heparin might occur during clinical treatment with heparin. Heparin therapy transiently results in approximately 2 units of heparin per milliliter of blood when 10,000 units of heparin are administered rapidly by the intravenous route, provided the volume of distribution approximates a blood volume of 5 liters. Other enzymes, such as *N*-acetyl-β-D-glucosaminidase, might be more significantly inhibited (25). *In vitro,* heparin has been shown to inhibit properties of normal neutrophil function, including phagocytosis (33, 34), neutrophil adherence to glass (34), and the antibacterial effects of cationic, lysosomal proteins (35–37). Heparin promotes neutrophil aggregation and degranulation (38). Heparin therapy inhibits bactericidal activity apparently by diminishing complement activation, and results in an adverse *in vitro* effect (33). In contrast, heparin treatment *in vivo* enhanced survival of guinea pigs subjected to experimental gram-negative sepsis when heparin was combined with passive antibody treatment in comparison to treatment with passive antibody alone (39). Here, heparin treatment appeared to diminish complement activation and was beneficial (39). In another clinically pertinent interaction possibly with neutrophils, heparin does not inhibit the growth of bacteria when used to anticoagulate blood for blood cultures (40). In fact, heparin enhances the recovery of certain aerobic bacteria from blood culture media when present in the large concentration of 600 units/ml (41). Although the mechanism of this enhancement of recovery has not been examined, our data and that which we review suggest that the reason lies in heparin's adverse effect upon neutrophil lysosomal enzyme function and neutrophil cell function. Heparin is known to antagonize the adherence of bacteria to urothelium (42, 43), but this is not a true bactericidal effect. Despite its multiple activities (44, 45), heparin is not known to have useful direct antimicrobial activities either naturally (46) or therapeutically (47). Leukocyte extracts can inhibit the ability of heparin to induce lipoprotein lipase (48) and the anticoagulant activity of heparin (48–50). Little evidence exists in humans *in vivo* that heparin therapy impairs antiinflammatory or neutrophil (or eosinophil) functions. Nonetheless, our observations and others imply that certain nonanticoagulant aspects of heparin therapy in clinical medicine deserve study. Possibly the reason that no increased susceptibility to infection has been observed with short- or long-term heparin therapy is that heparin's "negative" effects *vis a vis* the human host, namely, inhibition of neutrophil enzyme activity, bactericidal activities, and phagocytosis and its "positive" effects, such as direct antimicrobial, anticomplementary, and anticoagulant activities, counteract one another.

The experiment using cells from a man with the hypereosinophilic syndrome was performed at the Laboratory for Clinical Investigation, NIAID, Bethesda, Md. The author thanks Carolyn A. Moore for technical assistance and Jan Webb and Tommie Lue Maddox for clerical assistance.

1. Berthet J, de Duve C. Tissue fractionation studies. 1. The existence of a mitochondria-linked enzymically inactive form of acid phosphatase in rat-liver tissue. Biochem J **50**:174–181, 1951.
2. de Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem J **60**:604–617, 1955.
3. Baggiolini M, Hirsch JG, de Duve C. Resolution of granules from rabbit heterophil leukocytes into distinct populations of zonal sedimentation. J Cell Biol **40**:529–541, 1969.
4. West BC, Gelb NA, Kimball HR. Human blood granulocyte granules. Fed Proc **31**:253 (abstract), 1972.
5. West BC, Rosenthal AS, Gelb NA, Kimball HR. Separation and characterization of human neutrophil granules. Amer J Pathol **77**:41–66, 1974.

6. Kimball HR, Ford GH, Wolff SM. Lysosomal enzymes in normal and Chediak-Higashi blood leukocytes. J Lab Clin Med **86**:616–630, 1975.
7. Bretz U, Baggiolini M. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. J Cell Biol **63**:251–269, 1974.
8. Spitznagel JK, Dalldorf FG, Leffell MS, Folds JD, Welsh IRH, Cooney MH, Martin LE. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab Invest **30**:774–785, 1974.
9. Lundgren E, Roos G, Tarnvik A. Enzymatic heterogeneity of granules in human leukocytes. Acta Pathol Microbiol Scand Sect A **83**:167–175, 1975.
10. West BC, Gelb NA, Rosenthal AS. Isolation and partial characterization of human eosinophil granules. Comparison to neutrophils. Amer J Pathol **81**:575–588, 1975.
11. West BC, Dunphy CH, Moore CA. Human neutrophil *N*-acetyl-β-D-glucosaminidase: Granule localization. J Lab Clin Med **104**:60–69, 1984.
12. Kinkade JM, Jr, Pember SO, Barnes KC, Shapira R, Spitznagel JK, Martin LE. Differential distribution of distinct forms of myeloperoxidase in different azurophilic granule subpopulations from human neutrophils. Biochem Biophys Res Commun **114**: 296–303, 1983.
13. Avila JL, Convit J. Studies on human polymorphonuclear leukocyte enzymes. I. Assay of acid hydrolases and other enzymes. Biochim Biophys Acta **293**:397–408, 1973.
14. Avila JL. The influence of the type of sulphate bond and degree of sulphation of glycosaminoglycans on their interaction with lysosomal enzymes. Biochem J **171**:489–491, 1978.
15. Appelmans F, Wattiaux R, de Duve C. Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. Biochem J **59**:438–445, 1955.
16. Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. J Biol Chem **66**:375–400, 1925.
17. Chen PS, Toribara TY, Warner H. Microdetermination of phosphorus. Anal Chem **28**:1756–1758, 1956.
18. Chusid MJ, Dale DC, West BC, Wolff SM. The hypereosinophilic syndrome: Analysis of fourteen cases with review of the literature. Medicine **54**:1–27, 1975.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
20. Buruiana LM. L'action de l'heparine sur les phosphatases. Naturwissenschaften **44**:306–307, 1957.
21. Avila JL, Convit J. Inhibition of leucocytic lysosomal enzymes by glycosaminoglycans *in vitro*. Biochem J **152**:57–64, 1975.
22. Avila JL, Convit J. Physicochemical characteristics of the glycosaminoglycan-lysosomal enzyme interaction *in vitro*. Biochem J **160**:129–136, 1976.
23. Wolff SM, Dale DC, Clark RA, Root RK, Kimball HR. The Chediak-Higashi Syndrome: Studies of host defenses. Ann Intern Med **76**:293–306, 1972.
24. Isenberg JN, Stjernholm RJ. Solubilization of β-glucuronidase from leukocyte lysosomes by heparin. RES-J Reticuloendothel Soc **9**:515–527, 1971.
25. West BC, Dunphy CH, Moore CA. Human neutrophil *N*-acetyl-β-D-glucosaminidase: Heparin inhibition. Biochem Med **29**:1–13, 1983.
26. Meggio F, Deana AD, Brunati AM, Pinna LA. Inhibition of rat liver cytosol casein kinases by heparin. FEBS Lett **141**:257–262, 1982.
27. Migler R, DeChatelet LR. Human eosinophilic peroxidase: Biochemical characterization. Biochem Med **19**:16–26, 1978.
28. Li CY, Yam LT, Lam KW. Studies of acid phosphatase isoenzymes in human leukocytes: Demonstration of isoenzyme cell specificity. J Histochem Cytochem **18**:901–910, 1970.
29. Avila JL, Convit J. Heterogeneity of acid phosphatase activity in human polymorphonuclear leukocytes. Clin Chim Acta **44**:21–31, 1973.
30. Goldstein J, Waldman AA, Marx G. Heparin as an inhibitor of mammalian protein synthesis. In: Bradshaw RA, Wessler S, eds. Advances in Experimental Medicine and Biology: Heparin. New York, Plenum, Vol 52:p289, 1975.
31. Schofield KP, Stone PCW, Beddall AC, Stuart J. Quantitative cytochemistry of the toxic granulation blood neutrophil. Brit J Haematol **53**:15–22, 1983.
32. McCall CE, Katayama I, Cotran RS, Finland M. Lysosomal and ultrastructural changes in human 'toxic' neutrophils during bacterial infection. J Exp Med **129**:267–293, 1969.
33. Edwards MS, Buffone GJ, Rench MA, Speer ME, Fuselier PA, Baker CJ. Effect of continuous heparin infusion on bactericidal activity for group B streptococci in neonatal sera. J Pediatr **103**:787–790, 1983.
34. Victor M, Weiss J, Elsback P. Heparin inhibits phagocytosis by polymorphonuclear leukocytes. Infect Immun **32**:295–299, 1981.
35. Zeya HI, Spitznagel JK. Cationic proteins of polymorphonuclear leukocyte lysosomes. II. Composition, properties, and mechanism of antibacterial action. J Bacteriol **91**:755–762, 1966.
36. Saba HI, Roberts HR, Herion JC. Anti-heparin activity of lysosomal cationic proteins from polymorphonuclear leukocytes. Blood **31**:369–380, 1968.
37. Clark JM, Higginbotham RD. Reversibility of heparin inhibition of the antibacterial activity of polymorphonuclear leukocyte lysosomal proteins. Infect Immun **1**:607–608, 1970.
38. Cairo MS, Allen J, Higgins C, Baehner RL, Boxer LA. Synergistic effect of heparin and chemotactic

factor on polymorphonuclear leukocyte aggregation and degranulation. Amer J Pathol **113**:67–74, 1983.
39. Dunn DL, Mach PA, Cerra FB, Ferguson RM. The role of heparin in guinea pig Gram negative bacterial sepsis. J Surg Res **34**:479–485, 1983.
40. Wright HD. The bacteriology of subacute infective endocarditis. J Pathol Bacteriol **28**:541–578, 1925.
41. Evans GL, Cekoric T, Searcy RL. Comparative effects of anticoagulants on bacterial growth in experimental blood cultures. Amer J Med Technol **34**: 103–112, 1968.
42. Hanno PM, Fritz RW, Mulholland SG, Wein AJ. Heparin—examination of its antibacterial adsorption properties. Urology **18**:273–276, 1981.
43. Chin JL, Sharpe JR. The anti-adherence effect of heparin: A visual analysis. Urol Res **11**:173–179, 1983.
44. Jaques LB. What is "heparin?" In: Bradshaw RA, Wessler S, eds. Advances in Experimental Medicine and Biology, Heparin. New York, Plenum, Vol 52: p139, 1975.
45. Busch C, Owen WG. Identification in vitro of an endothelial cell surface cofactor for antithrombin I. J Clin Invest **69**:726–729, 1982.
46. Skarnes RC, Watson DW. Antimicrobial factors of normal tissues and fluids. Bacteriol Rev **21**:273–294, 1957.
47. Corrigan JJ, Jr, Kiernat JF. Effect of heparin in experimental Gram-negative septicemia. J Infect Dis **131**:138–143, 1975.
48. Fekete LL, Lever WF, Klein E. Inhibition of lipemia clearing activity by human white blood cell and platelet components. J Lab Clin Med **52**:680–686, 1958.
49. Cacciola E, Ferrauto A. Presence of anti-heparin activity in leukemic cells. Boll Soc Ital Biol Sper **36**: 1317–1319, 1960.
50. Lisiewicz J. Antiheparin activity of normal leukocytes. Pol Tyg Lek **20**:1150–1152, 1965.

Received July 2, 1984. P.S.E.B.M. 1985. Vol. 178.
Accepted November 7, 1984.