

IDENTIFICATION OF A MAJOR BASIC PROTEIN IN GUINEA PIG EOSINOPHIL GRANULES*

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Although the eosinophil has been recognized as a distinctive type of blood cell for over 100 yr, its specific functions not shared by neutrophils remain unknown (1). One approach to the elucidation of the particular functions of eosinophils is the isolation and analysis of the constituents of granules from this cell. However, studies of eosinophil granules have been hampered by the difficulties of obtaining adequate numbers of eosinophils for study and of securing intact granules. Vercauteren took advantage of the resistance of horse eosinophil granules to lysis and isolated granule preparations (2-3). His studies suggested the presence of arginine-rich proteins in granules. Archer and Hirsch purified rat and horse eosinophils and isolated granules from these cells (4). They showed that many of the same hydrolytic enzymes present in granules from rabbit polymorphonuclear leukocytes were also present in eosinophil granules. The eosinophil granules differed from neutrophil granules in their high content of peroxidase and the absence of lysozyme and phagocytin.

We have prepared granules from purified guinea pig eosinophils obtained by peritoneal lavage and have solubilized granule constituents by a variety of methods. Analysis of granule solutions has revealed the presence of a major protein component which accounts for over 50% of the protein in the granule. This material is very basic, has a molecular weight between 6,000 and 12,000, and does not possess peroxidase activity.

Materials and Methods

Materials.—Sodium diatrizoate (Hypaque) was obtained from Winthrop Laboratories, New York. Lipopolysaccharide B from *Salmonella enteritidis* was purchased from Difco Laboratories, Detroit, Mich. Hexadecylpyridinium chloride was from CalBiochem, San Diego, Calif. Hexadecyltrimethylammonium bromide was from Eastman Kodak Co., Rochester, N. Y. Brij 35 was from Schwartz/Mann Div., Becton, Dickinson, and Co., Orangeburg, N. Y. *O*-dianisidine was purchased from Sigma Chemical Co., St. Louis, Mo.

Eosinophilic Leukocytes.—Eosinophils were isolated from peritoneal fluids obtained from the guinea pig. These results will be described in detail elsewhere.¹ Briefly, the peritoneal cavities

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of guinea pigs were repeatedly lavaged with 50 ml of sterile, nonpyrogenic 0.9% NaCl essentially as described by Litt (5). After 4–6 wk of weekly or biweekly lavage the peritoneal lavage fluids contained 40–60% eosinophils. These fluids were essentially devoid of both neutrophils and basophils, and the only contaminating cells were monocytes and lymphocytes. Peritoneal cells were centrifuged at 400 g for 10 min and the cell button resuspended in saline. Eosinophils were purified by centrifugation through a layer of sodium diatrizoate, $d = 1.142$ g/ml, for 40 min at 400 g. The resulting purified cell suspensions contained greater than 93% eosinophils in all cases and usually greater than 95% as determined by total eosinophil counts.

Neutrophilic Leukocytes.—Neutrophils were purified from guinea pig peritoneal fluids induced by intraperitoneal injection of 100 μ g of lipopolysaccharide. 24 h after injection the peritoneal cavity was lavaged with 50 ml of normal saline. Cell suspensions were concentrated by centrifugation at 400 g and purified by sedimentation through a cushion of sodium diatrizoate, $d = 1.115$ g/ml essentially as described above for eosinophils. The resulting cell preparations were 90% neutrophils or greater as determined by staining with Wright's stain and differential counting.

Isolation of Eosinophil Granules.—Suspensions of eosinophils were treated to liberate granules and were centrifuged 10 min at 400 g at 4°C in a Sorvall RC-3 centrifuge (Ivan Sorvall, Inc., Newtown, Conn.) to sediment undisrupted cells. Attempts at sonic lysis of eosinophils were performed using a Bisonik Probe (Bronwell Scientific Co., Rochester, N. Y.) at settings of 4 and 90 for times from 0.5 to 5 min. Eosinophils were subjected to mechanical stress using an emulsifying device, the Mulsichurn (Mulsijet Inc., Elmhurst, Ill.), 60 strokes of the plunger, or by homogenization with a motor-driven Teflon pestle, clearance 0.1–0.15 mm, for 5 min. The supernatant, containing granules, and the sediment were examined by dark-field phase microscopy using an AO Spencer microscope (American Optical Co., Instrument Div., Buffalo, N. Y.). Samples of both supernatant and sediment were pelleted on microscope slides using a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.) at 2,500 rpm for 30 min and stained with Wright's stain using a Hema-Tek automatic slide stainer (Ames Co., Elkhart, Ind.). The quantity of granules liberated from cells was estimated by inspection of the suspensions and the stained slides as well as by absorbance of 400 g supernatants at 450 nm.

Electron Microscopy.—Eosinophil granules were sedimented at 5,000 g for 20 min and the pellet fixed in 3% glutaraldehyde in pH 7.3 0.1 M NaH_2PO_4 - Na_2HPO_4 at 23°C for 2 h. The fixed granules were washed in phosphate buffer containing 5% sucrose and postfixed in 2% osmic acid in phosphate buffer at 4°C. After dehydration in graded alcohols the preparations were embedded in Epon 812. Ultrathin sections were cut with a diamond knife using a LKB Ultratome III microtome (LKB Instruments, Rockville, Md.) and stained with uranyl acetate and lead citrate. The sections were examined with a Hitachi HU-12 electron microscope (Hitachi American Ltd., Indianapolis, Ind.) at 50 kv using 30 and 50 μ m apertures. After glutaraldehyde fixation some granule preparations were reacted with diaminobenzidine to localize peroxidase activity after the procedure described by Graham and Karnovsky (6). These preparations were subsequently processed as described above.

Electrophoresis in Polyacrylamide Gels.—Solubilized eosinophil granules were analyzed by electrophoresis in 15% polyacrylamide gels at pH 4.3 (7) or at pH 3 in the presence of 6.25 M urea (8). In the latter case the gels were always preelectrophoresed until the methyl green marker had migrated off the gel. Failure to preelectrophorese gels resulted in an absence of sharp band formation presumably due to interaction between residual products of the polymerization reaction and protein. Gels were fixed by exposure to 12% trichloroacetic acid for 0.5 h, rinsed several times with distilled water, stained with 0.25% Coomassie brilliant blue for 1 h, and destained with 7.5% acetic acid. Alternatively gels were fixed and stained with Coomassie brilliant blue as described by Fairbanks et al. (9). Gels were also stained for protein with aniline blue black (Allied Chemical Corp., Morristown, N. J.), for carbohydrate with the

periodic acid-Schiff procedure (PAS) (9),² for lipids with lipid crimson (Scarlet R; Pfaltz and Bauer Inc., Flushing, N. Y.) and with "Stains-All" (10). The last reagent stains RNA bluish-purple, DNA blue, and protein red.³ Gels were scanned using a Gilford 2400 S spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

The molecular weight of eosinophil granule proteins was estimated by electrophoresis of granules solubilized in distilled water on sodium dodecylsulfate (SDS) acrylamide gels (11) using the procedure described by Fairbank et al. (9). The isoelectric point of the eosinophil granule proteins was determined by electrofocusing in polyacrylamide gels using a pH gradient of 3–10 (12).

Peroxidase Assay.—Peroxidase activity was measured by the rate of decomposition of hydrogen peroxidase with *O*-dianisidine as a hydrogen donor (13). Horseradish peroxidase, type II, obtained from Sigma Chemical Co. served as a standard.

Gel Filtration.—Fractionation of solubilized eosinophil granule constituents was performed on columns of Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.).

RESULTS

Preparation of Eosinophil Granules.—We investigated a variety of methods to disrupt eosinophils and liberate granules. Treated cells were centrifuged at 400 *g* and the supernatant and sediment were examined microscopically. In three experiments samples of cells were freeze-thawed in pH 7.4 0.01 M K₂HPO₄-KH₂PO₄, 0.13 M NaCl phosphate-buffered saline (PBS), in 0.1 M sodium citrate, or in 0.34 M sucrose. In all cases one freeze-thaw cycle damaged the cells as judged by their microscopic appearance. However, the 400 *g* supernatants were water clear and examination of the sediment revealed clumps of cells with adherent granules. Sonic lysis of eosinophils almost completely disrupted the cells. Virtually no sediment was present after centrifugation and the supernatant was opalescent. However, we could not identify granules in the supernatant. We also tested two forms of mechanical stress for their ability to liberate granules. Cells suspended in 0.1 M sodium citrate were homogenized in a motor-driven tissue grinder or agitated in the Mulsichurn device and in both cases only slight opalescence of 400 *g* supernatants was found. In contrast to treatment by the above methods, suspension of cells in 0.34 M sucrose followed by repeated pipetting, approximately 10–15 times over a 2 min period, resulted in markedly opalescent supernatants. Absorbance at 450 nm after this treatment was eight times that after homogenization and almost five times that after treatment with the Mulsichurn device. Centrifugation of granule suspensions, 5,000 *g* for 20 min, pelleted the granules and, as shown in Fig. 1, examination of the 5,000 *g* sediment in the electron microscope revealed numerous granules with an electron-dense core and a less dense matrix characteristic of those in eosinophils (14, 15). Peroxidase staining was seen in the matrix but not in the core of the granules (16). Purified granule preparations were examined in the electron microscope on three occasions and the only contaminating cell

² Abbreviations used in this paper: PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate.

³ Peacock, A. C. Personal communication.

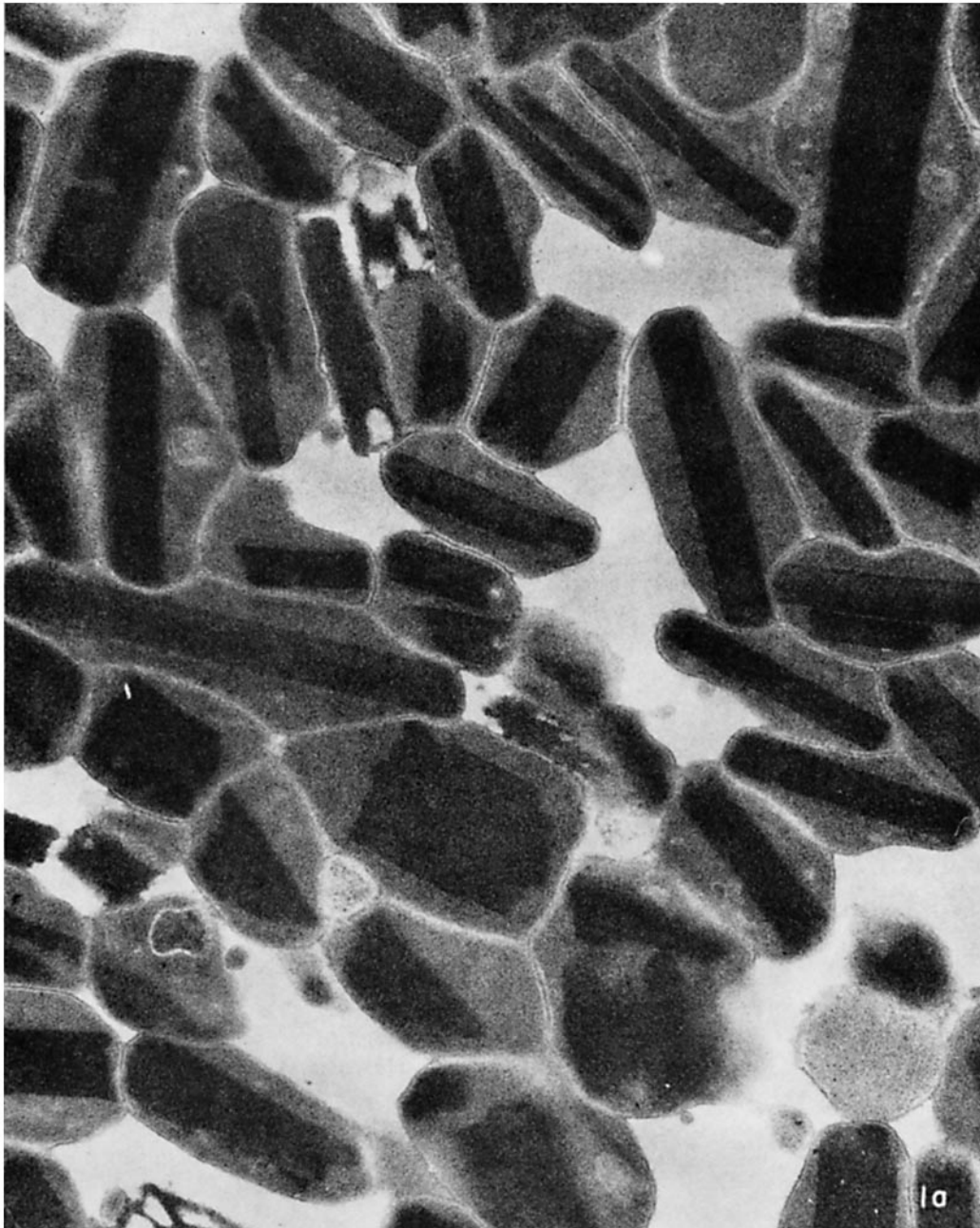


FIG. 1 *a*. Electron photomicrographs of eosinophil granules. Granules fixed with glutaraldehyde and osmic acid and stained with lead citrate and uranyl acetate. The central crystalloid is considerably more electron dense than the outer matrix for the granule. $\times 49,000$.

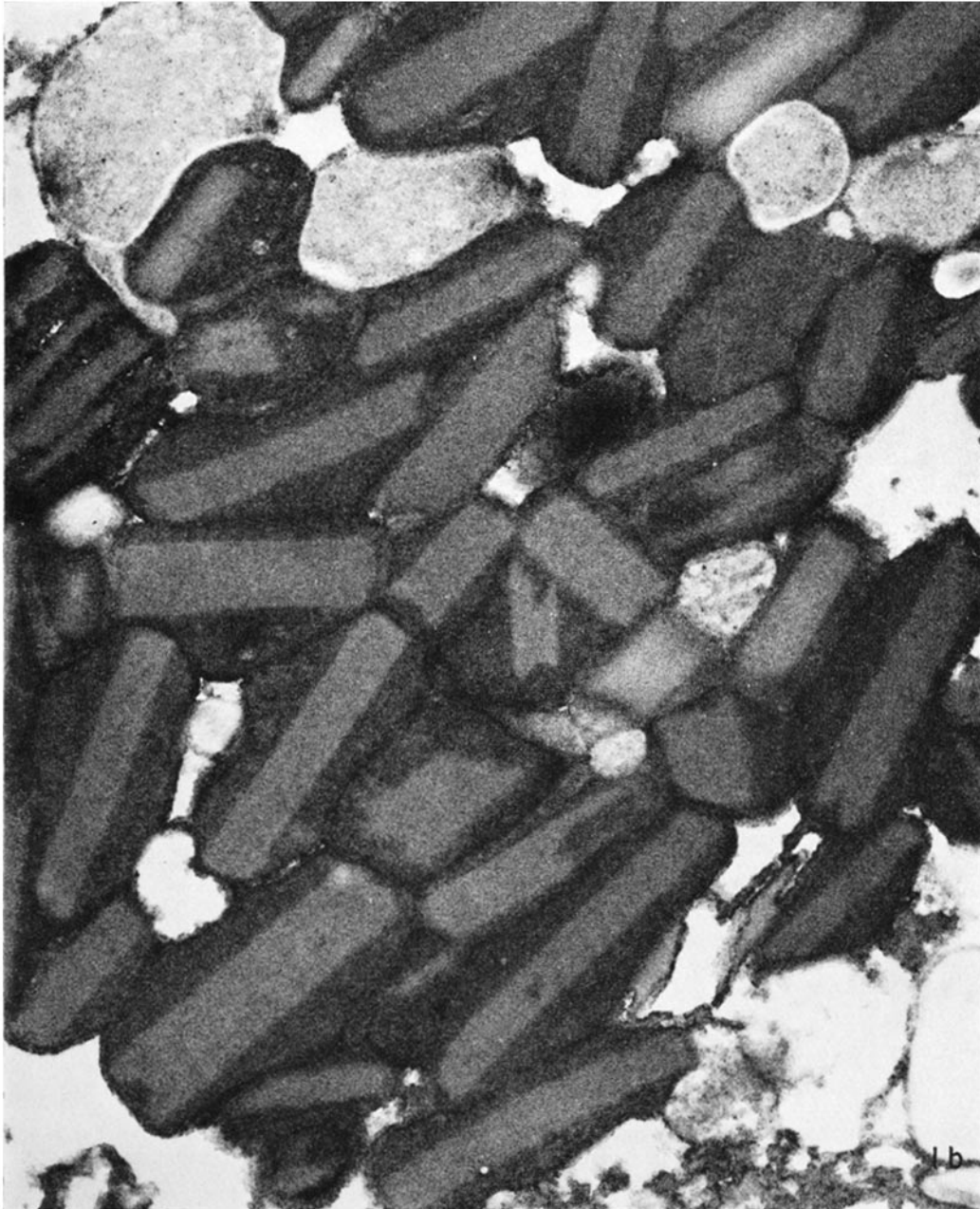


FIG. 1 *b*. Granules reacted for peroxidase activity. Although double stained (lead citrate-uranyl acetate) for contrast, the central crystalloid appears distinctly less electron dense than the peripheral matrix of the granule which is peroxidase positive. $\times 49,000$.

organelle identified was an occasional mitochondrion. In some areas of the sections amorphous debris probably derived from membranes was seen, but in none of the experiments were nuclei found.

Disruption of Granules.—In preliminary experiments we found that the concentration of eosinophil granule suspensions could be estimated by absorbance at 450 nm; absorbance at this wave length was linearly related to granule content. We also found that opalescent suspensions of eosinophil granules rapidly clarified on acidification with HCl as previously reported for rabbit neutrophil granules (17). The most marked decrease in absorbance at 450 nm occurred at pH 3 or lower. Additional experiments revealed that HCl concentrations of 0.1 N, 0.01 N, and 0.001 N all produced equivalent decreases in 450 nm absorbance. When these solutions were neutralized to pH 6–8, a flocculant precipitate formed which only partially dissolved on addition of HCl.

To determine whether granule constituents could be solubilized at neutral or alkaline pH we exposed granules to various detergents and also subjected them to freezing and thawing. The results of these experiments are shown in Table I. Marked solubilization of granules was achieved by treatment with distilled water, as well as with three detergents, hexadecyltrimethylammonium bromide, hexadecylpyridinium chloride, and Brij. Other materials such as dioctyl

TABLE I
Lysis of Eosinophil Granules with Detergents (Exp. 1) and by Freezing and Thawing (Exp. 2)

	Granule suspension		Supernatant
	450 nm	Absorbance pH	277 nm
Experiment 1			
HDTA 0.1%	0.057	8.5	0.287
HDPC 0.1%	0.040	7.9	0.257
Brij 0.1%	0.046	4.7	0.230
H ₂ O	0.058	7.0	0.200
HCl, 0.01 M	0.009	2.0	0.255
Sucrose, 0.34 M	0.183	6.3	0.035
Experiment 2			
H ₂ O	0.310	7.0	1.495
0.15 M NaCl + freeze-thaw	0.308	6.5	1.800
0.001 M HCl	0.082	2.0	1.550
0.15 M NaCl	0.800	6.7	0.260
0.34 M Sucrose	0.990	7.6	0.150

In exp. 1 samples of eosinophil granules were suspended in the appropriate solution and after incubation at room temperature for 10 min the absorbance at 450 nm was determined. The tubes were placed at 4°C overnight and then centrifuged at 5,000 *g* for 10 min. The absorbance of the water clear supernatant at 277 nm was measured, using the individual solvents as blanks. HDTA refers to hexadecyltrimethylammonium bromide. HDPC refers to hexadecylpyridinium chloride. Brij refers to Brij 35. In exp. 2 granules were frozen and thawed six times in a Dry Ice acetone bath and a 37°C water bath.

sodium sulfosuccinate (Aerosol-OT), Triton X-100, sodium dodecyl sulfate, and sodium cholate at 0.1% and 1% concentrations failed to solubilize granules. Freezing and thawing of granules liberated as much absorbance at 277 nm as did exposure to 0.001 N HCl. The quantities of protein liberated from granules solubilized in 0.01 N HCl were measured by Biuret analysis (18) using human serum albumin as a standard. In the performance of this test granule constituents did not precipitate from solution. In three experiments an average of 2.7 mg of protein was derived from granules of 4×10^8 purified eosinophils.

Peroxidase Activity.—No peroxidase activity was detected in eosinophil granules solubilized in 0.01 N HCl even though the pH was adjusted to 6 in the assay itself. In contrast, peroxidase activity was readily detectable in neutrophil granules prepared by sucrose lysis and solubilized in 0.01 N HCl. When eosinophil granules were disrupted by sonic lysis in PBS, peroxidase activity was detectable and even greater activity was present in granule extracts suspended in PBS and subjected to six cycles of freezing and thawing. The bulk of this activity, 66 and 90% in two experiments, did not sediment at 5,000 g.

Analysis of Solubilized Granules.—Water clear solutions of eosinophil granules lysed in 0.001 N HCl were initially analyzed by polyacrylamide electrophoresis at pH 4.3 (7). There was intense staining of the sample gel, and only faint staining of broad bands in the separating gel was observed. Under these same conditions neutrophil granules prepared by sucrose lysis and solubilized in 0.1 N HCl yielded a series of discrete bands. Because the pH of the sample gel in this experiment was about 6 and because we had found in earlier experiments that eosinophil granule proteins precipitate at neutral pH, we suspected that the eosinophil granule constituents might have precipitated in the sample gel. Therefore we analyzed the eosinophil and neutrophil granule constituents by electrophoresis in polyacrylamide at pH 3 in 6.25 M urea (8) and directly applied the solutions in sucrose to the separating gel. As shown in Fig. 2 granule solutions from both cell types produced a series of discrete bands, but the pattern given by the eosinophil granule differed from that of the neutrophil granule in that there was one major band which migrated rapidly into the gel. Densitometric analysis of gels stained with Coomassie brilliant blue in two experiments and aniline blue black in two experiments revealed that 53.2 ± 3.9 ($\bar{x} \pm 1$ SE) of the absorbance was associated with the major band. We also found that the major band stained red with Stains-All, indicating protein, but was not stained with PAS or lipid crimson. In additional experiments eosinophil granules were solubilized by exposure to 0.01 N HCl or distilled water and samples were analyzed by polyacrylamide electrophoresis in SDS gels and by isoelectric focusing. As shown in Fig. 3 analysis on SDS polyacrylamide gels revealed that the major band protein migrated further than the marker with the lowest molecular weight, cytochrome *c* (12,400 daltons). Analysis by isoelectric focusing with ampholines spanning the pH range from 3–10 revealed a

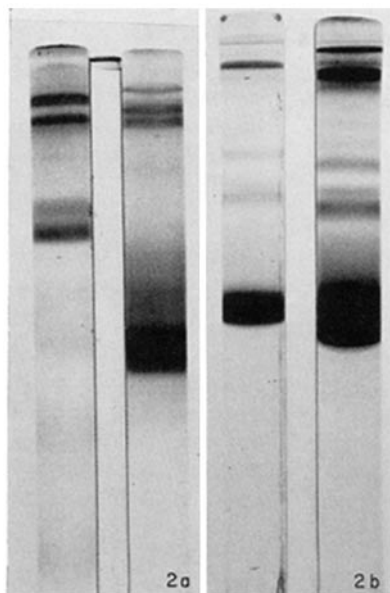


FIG. 2. Analysis of solubilized granules in polyacrylamide gels at pH 3.0 in the presence of 6.25 M urea. Granules from 3×10^8 eosinophil or neutrophils were prepared by sucrose lysis and solubilized in 0.001 M HCl. (a) Neutrophil and eosinophil granule solutions were tested, neutrophils (left) and eosinophils (right). Gels were fixed with 12% trichloroacetic acid and stained with Coomassie brilliant blue as described in Materials and Methods. (b) Eosinophil granule proteins were analyzed at two concentrations, 9 μg (left) and 26 μg (right) and stained with Coomassie brilliant blue as described by Fairbanks et al. (9).

broad band which stained intensely at the lower (pH 10) end of the gel.⁴ Finally, in several experiments solubilized granules were fractionated on columns of Sephadex G-50 and the results of a typical experiment are shown in Fig. 4. The bulk of the absorbance emerged in the second peak and a third peak was barely detectable. Fractions from the first peak and the second peak were analyzed by polyacrylamide electrophoresis and the major band protein was found, largely free of other materials, in the second peak (Fig. 4 *b*). The first peak contains a series of proteins which do not migrate into the gel as far as the major band. In other experiments solubilized granules were fractionated on columns of Sephadex G-50 calibrated with marker proteins. The first peak always emerged in the void volume fractions, the second peak containing the major band protein between the cytochrome *c* and protamine sulfate (6,500 daltons) markers, and the third peak after the bacitracin (1,411 daltons) marker. Finally as shown in Fig. 5 peroxidase activity was detected only in the first peak; no activity was associated with the second peak.

⁴ This experiment was performed by Dr. David Fass.

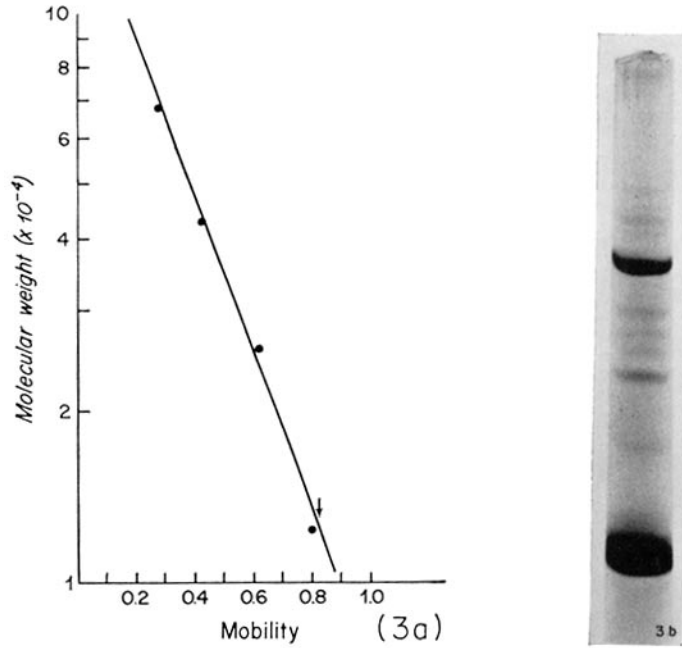


FIG. 3. Analysis of eosinophil granule proteins by electrophoresis on SDS polyacrylamide gels. (a) The mobility of the major band protein (arrow) in relationship to the mobility of the marker proteins, bovine serum albumin, 68,000 daltons; ovalbumin, 43,000 daltons; chymotrypsinogen, 25,700 daltons; and cytochrome *c*, 12,400 daltons. (b) The gel stained with Coomassie brilliant blue (11).

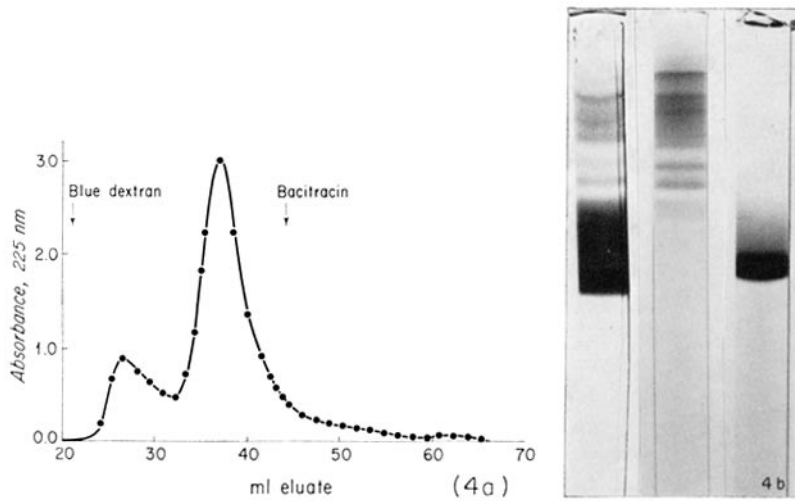


FIG. 4. Analysis of solubilized eosinophil granules on Sephadex G-50. (a) Granules prepared from guinea pig peritoneal cells were disrupted by freezing and thawing in pH 4.3 0.05 M sodium acetate-acetic acid and fractionated on a 1.2×50 cm column at 4°C . (b) Fractions with the greatest absorbance from the first and second peaks were electrophoresed in pH 3, 6.25 M urea, polyacrylamide gels, and stained with Coomassie brilliant blue (9). The crude granule extract is on the left, peak 1 fraction in the middle, and peak 2 fraction on the right.

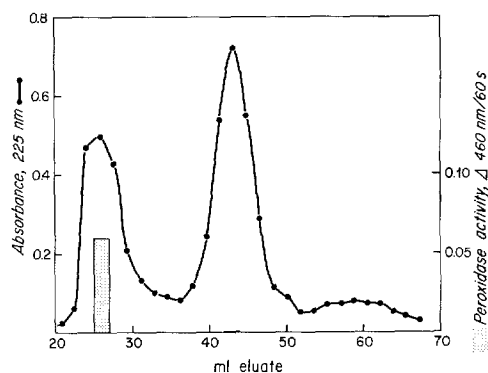


FIG. 5. Analysis of eosinophil granule proteins for peroxidase activity. Partially purified eosinophils (approximately 80%) prepared by sedimentation through a layer of sodium diatrizoate, $d = 1.115$ g per milliliter, were lysed in 0.34 M sucrose and the granules dissolved in pH 5.0 0.05 M sodium acetate-acetic acid buffer. The granule solution was fractionated on a 1.2×50 cm column of Sephadex G-50 equilibrated with pH 5 acetate buffer containing 0.4 M NaCl. The fractions with the greatest absorbance from the first and second peaks were tested for peroxidase activity.

DISCUSSION

In these studies we explored a variety of procedures to purify granules from guinea pig eosinophils and found one method, namely agitation of cells suspended in 0.34 M sucrose, which reproducibly yielded quantities of granules adequate for further study. In earlier studies of eosinophils, Petry described a method for purification of eosinophil granules from horse blood in which cells were incubated with trypsin at an alkaline pH and after approximately 24 h eosinophil granules were harvested (19). Vercauteren applied this procedure for granule isolation and found that trypsin was unnecessary (2). Subsequently Archer and Hirsch (4) studied a variety of methods for the isolation of horse and rat eosinophil granules and found that mechanical grinding, freezing and thawing, and sonic disintegration all damaged or disrupted granules whenever applied sufficiently vigorously to break most of the cells. They developed a method for liberation of granules in which eosinophils were rapidly passed through a fine mesh screen. We attempted to apply their procedure to guinea pig eosinophils, but even though granules were liberated we found that the method was more cumbersome than sucrose lysis. In our studies small quantities of granules were released after homogenization or treatment with the Mulsichurn device, but the yields were considerably less than after lysis in 0.34 M sucrose. In the case of rat and horse eosinophils the sucrose lysis method did not rupture eosinophils (4). Presumably the susceptibility of guinea pig eosinophils to this treatment reflects a species difference.

We found that granules were solubilized by exposure to dilute HCl solutions, to distilled water, or to the detergents, hexadecyltrimethylammonium bromide, hexadecylpyridinium chloride, or Brij 35. In subsequent experiments we found

that freezing and thawing of granules also disrupted granules. Analysis of the granule constituents by electrophoresis on polyacrylamide gels at acid pH in the presence of urea demonstrated a marked difference between the patterns from eosinophil and neutrophil granules. Eosinophil granules contained a major constituent which accounted for over half of the absorbancy when the gels were stained with either Coomassie brilliant blue or aniline blue black. Because this material stained readily with protein stains, including Stains-All, and because it failed to stain with PAS and a lipid stain, we believe that it is proteinaceous in nature. Tentatively we have referred to the substance as the major band protein until further analyses reveal a definite function for the substance. The major band protein has a molecular weight between 6,000 and 12,000 as judged from its behavior on SDS polyacrylamide gels and on gel filtration. The isoelectric points of most of the proteins from the eosinophil granule are greater than pH 10. Finally the major band protein does not possess peroxidase activity. Disruption of eosinophil granules by freezing and thawing at pH 4.3 liberated peroxidase activity, and this was present in the first peak from the G-50 Sephadex column. This observation is in agreement with the work of Dresser and his associates (20) who found that the molecular weight of guinea pig eosinophil peroxidase was 80,000. A protein of this size would not be retarded by G-50. In contrast the low molecular weight major band protein was retarded on G-50 and eluted in the second peak.

The localization of the major band protein within the eosinophil granules is not evident from our studies. Nonetheless we suspect that the major band protein may be derived from the granule core. This tentative conclusion is based on three considerations. One, inspection of electron photomicrographs of eosinophil granules indicates that a sizeable portion of the structure is contained within the core of the granule. Two, the granule core or crystalloid has a regular structure and is likely composed of a single substance (15). And three, the granule core does not possess peroxidase activity (16). Clearly further studies are needed to determine the localization of the major band protein within the eosinophil granule. If, as we suspect, the major band protein is present in the granule core, then isolation of cores should yield solutions enriched in this component. In preliminary experiments we have attempted to isolate granule cores by the method described by Gessner and his associates (21), but we have not noted any marked difference between the electrophoretic patterns produced by these solutions and those from whole granules.

Studies of neutrophil granules have revealed the presence of a variety of enzymes associated with lysosomes (17) as well as basic proteins (22-26). The basic proteins have been investigated by several groups and at least four different proteins varying in molecular weight from 4,000 to 12,300 have been isolated (26). These basic proteins all possess the ability to increase vascular permeability and one of them liberated histamine from isolated peritoneal rat mast cells. Because the major cationic protein from the eosinophil granule accounts for over 50% of the protein derived from granules, studies of its bio-

logical activity are likely to provide important information about the function of the eosinophil. Presently we are engaged in studies of the biochemical properties and biological activities associated with the major band protein.

SUMMARY

Elucidation of the functions of the eosinophil might be accomplished by analysis of the granule constituents. We have purified eosinophils (93% or greater) from the peritoneal cavity of the guinea pig and have investigated a variety of methods to disrupt cells and liberate intact granules. Lysis in 0.34 M sucrose gave the best yield of granules and these had the characteristic morphology of eosinophil granules when examined by electron microscopy. Granules were solubilized by a variety of treatments and the solutions analyzed by polyacrylamide electrophoresis at pH 3 in 6 M urea. Comparison of the electrophoretic patterns of solubilized eosinophil and neutrophil granules revealed a difference: a major portion ($53 \pm 3\%$; $\bar{x} \pm 1$ SE) of the protein from the eosinophil granule migrated as a single component. This major band protein has a molecular weight between 6,000 and 12,000 daltons and a pI of 10 or greater. Analysis of eosinophil granule constituents on Sephadex G-50 revealed two main peaks; peak 1 possessed peroxidase activity and peak 2 contained the major band protein. These studies indicate that eosinophil granules contain a cationic protein of low molecular weight which lacks peroxidase activity and which accounts for greater than 50% of granule protein.

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