ISOLATION OF GRANULES FROM EOSINOPHIL LEUCOCYTES AND STUDY OF THEIR ENZYME CONTENT*

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Plates 17 to 19

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Perhaps the most distinguishing feature of eosinophil leucocytes is the presence in their cytoplasm of numerous acidophilic granules. This investigation was undertaken in the hope that knowledge about the nature of these granules might shed light on possible functions of eosinophils.

We report here techniques that allow separation of horse and rat eosinophils free of other cell types, and methods for isolating their cytoplasmic granules. Peroxidase and a variety of hydrolytic enzymes were found to be associated with eosinophil granules.

RESULTS

Isolation of Eosinophils from Horse Blood and from Rat Peritoneal Washings.—Eosinophils constitute only a minor proportion of the cells in normal horse blood, and are present together with mast cells and macrophages in rat peritoneal fluid. Our first efforts were accordingly directed towards separation of eosinophils from other cell types. Such a separation was accomplished by centrifugation in media of high specific gravity, thus taking advantage of the unusually high density of the eosinophil as compared to erythrocytes and other leucocytes. Detailed aspects of the procedures employed were as follows:—

Horse Blood Eosinophils.—A horse was bled from the jugular vein, 250 ml of blood being collected and mixed with one-tenth volume of 0.1 M sodium citrate to prevent clotting. The blood was then dispensed into test tubes and allowed to stand for 45 minutes, during which time erythrocytes sedimented spontaneously. The supernatant plasma, containing platelets, leucocytes, and a few red cells, was then aspirated and spun at 100 *g* for 10 minutes, depositing white cells but not platelets. After decanting the supernate, the leucocyte button was suspended in 5 ml 36 per cent bovine albumin.

Albumin powder (Bovine fraction V), obtained from Armour Laboratories, Chicago, was dissolved in isotonic saline at 60 per cent, and adjusted to pH 7 by addition of 1 N NaOH. The albumin solution was then dialyzed twice with mixing for 2 hours against 100 volumes of 0.9 per cent saline. Increase in volume of the albumin solution resulting from neutralization

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and dialysis brought the final concentration of albumin to approximately 36 per cent. Specific gravity of this solution was measured, and, when required, adjusted to 1.10 by addition of saline. Reasonably good separation could also be achieved using sterile 35 per cent bovine albumin solution (Pentex, Inc., Kankakee, Illinois).

The horse leucocyte suspension in 5 ml of albumin was then layered over a 5 ml column of 36 per cent albumin in a 17 × 125 mm test tube and spun at 200 *g* for 10 minutes. The deposit was usually composed of about 90 per cent eosinophils, the main contaminants being erythrocytes and neutrophils. Both layers of supernatant albumin solution, containing the other cell types, were removed by aspiration. The deposit was mixed with a few drops of saline and then resuspended in 5 ml of new 36 per cent albumin. This cell suspension was again layered over 5 ml 36 per cent albumin and spun as described above. The leucocytes recovered in this second pellet were over 99 per cent eosinophils. The small numbers of red cells occasionally present could be removed by hypotonic lysis without apparent damage to the eosinophils. This was accomplished by suspension of the deposit in 1 ml of 0.25 per cent saline, followed by addition of 10 ml isotonic saline after 15 seconds, and centrifugation. The eosinophils were then washed three times in saline on the centrifuge. Total recovery of eosinophils by this technique was approximately 30 per cent. All operations were performed at room temperature.

Rat eosinophils were harvested by peritoneal lavage. This involved intraperitoneal injection and prompt removal of 50 ml physiological saline every 3 days in Long-Evans strain rats. With repeated washing of the same animal the mast cell count fell progressively and the eosinophil count rose; usually 2×10^7 cells, 60 per cent of which were eosinophils, were obtained from each rat. For isolation of eosinophils, washings from 6 rats were pooled and centrifuged at 100 *g* for 5 minutes. The deposit was then suspended in 3.5 ml of 36 per cent bovine albumin and spun at 250 *g* for 15 minutes to bring down and remove any mast cells; rat eosinophils did not sediment under these conditions. Exactly 3.0 ml of the supernatant albumin solution, containing eosinophils and mononuclear cells, was diluted with 0.5 ml of isotonic saline and centrifuged at 500 *g* for 15 minutes, depositing the eosinophils but not the macrophages and lymphocytes. The eosinophils in the pellet were then washed three times in saline on the centrifuge. All operations were at room temperature.

These procedures permitted separation of horse and of rat eosinophils essentially free of contamination with other cell types. Eosinophils so processed appeared to be undamaged; their morphology on stained smears was identical to that seen in blood or peritoneal washings, and they were capable of locomotion and active phagocytosis as described in the following communication (1).

Fig. 1 shows a photomicrograph of the isolated horse eosinophils, stained by Wright's procedure. The homogeneity of the cell type and the morphologic features of normal horse eosinophils are demonstrated. Particularly notable was the large size of the horse eosinophil granule. In the original preparation these granules stained red to pink, and stood in sharp contrast to the blue cytoplasm and dark purple nucleus of the cell.

Fig. 2 illustrates the appearance of rat eosinophils after isolation as described above. Cytoplasmic granules in these cells were much smaller and more numerous than those in horse eosinophils. The characteristic ring or doughnut nucleus of the rat eosinophil was also evident. In the stained slide the granules were red to pink, the cytoplasm showed little color, and the nucleus was dark brown in shade.

Isolation of Cytoplasmic Granules from the Eosinophil Preparations.—Techniques previously employed by others for disrupting various types of cells in a manner permitting recovery of cytoplasmic elements were tried on the eosinophil suspensions. Mechanical grinding, freezing and thawing, and sonic disintegration all were found to damage or disrupt the eosinophil granules whenever applied sufficiently vigorously to break most of the cells, in line with the previous experience of Vercauteren (2). The sucrose lysis method used for disrupting rabbit polymorphonuclear leucocytes (3) did not rupture horse or rat eosinophils. It thus became necessary to search for a new method in order to harvest granules from the eosinophils.

The following procedure, based on rapid passage of the cells through a fine mesh screen under negative pressure, was finally adopted.

The device used was the Swinny hypodermic adapter (Millipore Filter Corp., Bedford, Mass.) in which the Millipore filter was replaced by a thin plastic washer (10 mm diameter with a 5 mm central hole). The washer was placed on top of the metal screen normally used to support the Millipore filter. The holes in the screen were approximately 150 μ in diameter. The adapter was connected to an 11 gauge needle which was in turn inserted through a rubber stopper into a flask maintained under 25 inches of Hg negative pressure. The vacuum flask was placed in an ice bath, and all subsequent operations on granules were at 0–4°C. The assembled device permitted rapid passage of the cells through a fine mesh metal screen under negative pressure.

Horse eosinophils were suspended in 0.05 M sodium citrate and added dropwise to the inlet of the Swinny adapter. Examination of the fluid collected in the vacuum flask revealed numerous intact granules and a small amount of stringy debris. Only rare intact cells or nuclei were found.

The citrate solution, containing horse eosinophil granules and cell debris, was collected from the vacuum flask and spun at 500 *g* to deposit the granules. They were then washed once in 0.1 M sodium citrate on the centrifuge, and finally suspended in a known volume of 0.1 M sodium citrate and counted by phase contrast microscopy, employing the high dry objective and AO Spencer thin hemacytometer for use with phase microscopes.

Rat eosinophils suspended in 0.1 M sodium citrate were disrupted in a similar manner, but the small size of their granules required a slightly different procedure for collection. The broken cell suspension was first spun at 200 *g* for 20 minutes to sediment debris. The supernate, containing rat eosinophil granules, was then spun at 5000 *g* for 20 minutes, and the deposit suspended in a known volume of 0.1 M sodium citrate for counting under phase contrast.

These procedures allowed collection of horse and rat eosinophil granules relatively free of contamination by whole cells or other structural elements. The size, shape, and staining properties of these granules were identical to those seen in intact cells.

Fig. 3 shows a photomicrograph of a Wright's stain of horse eosinophil granules obtained by this technique.

The Association of Various Enzymes with Eosinophil Granules.—The availability of eosinophils free of other leucocytes, and of cytoplasmic granules from these cells made possible investigations on localization of various enzymes in the granules.

TABLE I

Distribution of Protein in Eosinophil Fractions

Eosinophils from	Protein content (μg per million cells) of				
	Total cells	Granules	Frozen-thawed granules		
			Soluble fraction	Insoluble fraction	
				Soluble at pH 3.7	Insoluble at pH 3.7
Horse	176	78.5	22.2	28.8	27.5
Rat	65	31	7.1	11.5	12.4

TABLE II

Specific Activities of Various Enzymes in Horse Eosinophil Fractions

Enzyme	Specific activity in horse eosinophil fractions			
	Whole cell	Granules	Supernate (solubles) from disrupted granules	Deposit (insolubles) from disrupted granules
	units/ mg N	*units/mg N*	*units/mg N*	*units/mg N*
Cathepsin	1.1	1.93	5.3	0.5
Ribonuclease	1.6	2.7	7.2	0.94
Arylsulphatase	0.47	1.0	2.3	0.48
Beta glucuronidase	0.1	0.2	0.46	0.09
Acid phosphatase	0.11	0.2	0.19	0.21
Alkaline phosphatase	0.93	1.66	1.18	1.84
Peroxidase	23.0	43.0	0	60.0

TABLE III

Specific Activities of Various Enzymes in Rat Eosinophil Fractions

Enzyme	Specific activity in rat eosinophil fractions			
	Whole cell	Granules	Supernate (solubles) from disrupted granules	Deposit (insolubles) from disrupted granules
	units/mg N	*units/mg N*	*units/mg N*	*units/mg N*
Cathepsin	1.7	2.6	11.0	0.5
Ribonuclease	10.0	26.0	113.0	4.0
Arylsulphatase	1.7	3.9	13.0	1.2
Beta glucuronidase	1.1	1.9	6.0	0.7
Acid phosphatase	0.11	0.2	0.13	0.22
Alkaline phosphatase	0.65	1.0	0.74	1.05
Peroxidase	30.0	80.0	0	105.0

Thin smears of horse and rat eosinophils were made and granules were counted in 100 consecutive cells. Horse eosinophils contained 40 and rat eosinophils 250 granules per cell on the average. Using these figures and the direct granule counts in the suspensions of isolated granules, it was then possible to relate protein and enzyme content of granules to that in the intact cell.

Preparations of whole cells and of granules in saline were disrupted by exposure to 6 cycles of freezing and thawing in dry ice–alcohol and at room temperature before enzyme assay. The disrupted granules were in some instances centrifuged at 10,000 *g* to obtain a deposit (insoluble) and supernate (soluble). The insoluble residue from broken granules could be dissolved partially by extraction with acid (0.05 M acetate pH 3.7).

Protein was measured by the method of Lowry *et al.* (4), using crystalline egg white lysozyme as a standard. Nitrogen was estimated with Nessler's reagent (5).

TABLE IV

Distribution of Various Enzymes in Horse Eosinophil Fractions

Enzyme	Percentage of total horse eosinophil enzyme content recovered in		
	Granules	Supernate (soluble) from disrupted granules	Deposit (insoluble) from disrupted granules
Cathepsin	88	68	20
Ribonuclease	80	60	20
Arylsulphatase	103	67	36
Beta glucuronidase	100	65	35
Acid phosphatase	91	23	68
Alkaline phosphatase	89	18	71
Peroxidase	100	0	100

Alkaline phosphatase, acid phosphatase, cathepsin, ribonuclease, beta glucuronidase, lysozyme and phagocytin were estimated as described previously (3). Arylsulphatase was measured according to Tanaka *et al.* (6), using as substrate 2-hydroxy-5-nitrophenyl sulfate obtained from the California Corporation for Biochemical Research, Los Angeles. One unit was that amount of enzyme liberating 1 mg of 4 nitrocatechol per hour at 37°C. Peroxidase determinations were done following the method of Jermyn and Thomas (7), employing guaiacol as the chromogenic receptor. Crystalline horseradish peroxidase (Worthington Biochemical Corp., Freehold, New Jersey) served as standard. One unit of peroxidase was that amount causing decomposition of 1 μmole H_2O_2 per minute as determined by oxidation of guaiacol at 400 mμ at 25°C.

Distribution of protein in various eosinophil fractions is shown in Table I. As is seen, horse eosinophils contained about twice as much protein per cell as did rat. In both types of eosinophil, approximately half of the total cell protein was localized in the granules. Of this protein in the granules, roughly one-fourth was soluble after freezing and thawing, and approximately half of the remaining protein, insoluble after freezing and thawing, was dissolved on extraction with weak acid (acetate pH 3.7).

The results of enzyme assays on eosinophils and eosinophil granules are recorded in Tables II and III. The data are presented as specific activities (units per mg nitrogen). As is seen, the findings in horse and rat cells were essentially the same.

For each of the enzymes tested, specific activity in the granules was significantly higher than that in the whole cell, indicating that the enzymes were contained in or associated firmly with the granules.

The data from Tables II and III are presented as percentage of total eosinophil enzymes recovered in the granules and granule fractions in Tables IV and V. All these enzymes were localized to a high degree in the granules.

TABLE V

Distribution of Various Enzymes in Rat Eosinophil Fractions

Enzyme	Percentage of total rat eosinophil enzyme content recovered in		
	Granules	Supernate (soluble) from disrupted granules	Deposit (insoluble) from disrupted granules
Cathepsin	77	66	11
Ribonuclease	108	95	13
Arylsulphatase	122	92	30
Beta glucuronidase	87	63	24
Acid phosphatase	91	14	77
Alkaline phosphatase	77	15	62
Peroxidase	100	0	100

Cathepsin, ribonuclease, arylsulfatase and beta glucuronidase were for the most part released in soluble form on disruption of the granules by freezing and thawing in saline. Acid and alkaline phosphatase were partially soluble after granule rupture, but the major portion of activity remained associated with the insoluble granule debris. Peroxidase was not detected in solution following granule disruption. Ninety per cent of the peroxidase activity associated with the insoluble granule residue could, however, be extracted with weak acid (acetate pH 3.7).

No lysozyme or phagocytin were detected in any of the eosinophil granule extracts.

DISCUSSION

The eosinophil leucocyte was first recognized by Ehrlich and others more than 80 years ago on the basis of the striking affinity of granules in these cells for acid dyes. Much has been written about the eosinophil since then, but the specific function of this cell, if it has one, remains a mystery.

Alterations in distribution of eosinophils in blood or tissues occur in association with a wide variety of disorders such as allergic reactions, skin diseases, certain parasitic infestations, and some types of lymph gland tumors (8). Eosinophil physiology furthermore is strikingly influenced by adrenal cortical hormones (9). Recent investigations suggest that antigen-antibody complexes are chemotactic for eosinophils (10). The possible role of eosinophils in modifying or transporting antigen has also been stressed (11, 12).

Prior knowledge about the chemical composition of eosinophil granules may be summarized as follows. Electron microscopy reveals in human eosinophil granules a crystalline structure surrounded by a homogeneous matrix (13). The nature of the crystal is unknown. Behrens recently isolated eosinophil granules in non-aqueous solvents, and separated from them a zinc-containing protein or peptide which has affinity for eosin dye (14). Peroxidase has been localized in eosinophil granules by histochemical observations (15), and studies on enzymes in mixtures of leucocytes, with correlation of the results and eosinophil content, indicate the presence of arylsulfatase and beta glucuronidase in eosinophils (6, 16). Very recently plasminogen has been reported present in granules of bone marrow eosinophils as detected by immunofluorescent techniques (17).

Eosinophils normally constitute a minor fraction of the blood leucocytes, and chemical studies on these cells in the past have been hampered by the necessity of dealing with mixed cell populations. The technique reported here for separating eosinophils by centrifugation in isotonic, neutral albumin solutions of high specific gravity allows investigations on essentially pure populations of cells. Eosinophils so separated are, moreover, apparently undamaged as judged by their ability to move and their phagocytic capacity (1).

The range and specific activities of hydrolytic enzymes associated with horse or rat eosinophil granules are similar in many regards to those reported previously in rabbit polymorphonuclear leucocyte granules (3) and rat liver lyosomes (18). Eosinophil granules differ from those of rabbit polymorphonuclear leucocytes in their high content of peroxidase and in the absence of lysozyme and phagocytin. The absence of these two antibacterial agents might be taken to indicate that eosinophils do not have as one of their major functions the ingestion and destruction of microbes. The significance of the high peroxidase content is unknown.

Adequate disruption of liver lysosomes or of rabbit polymorphonuclear leucocyte granules by repeated freezing and thawing releases nearly all of the enzymes in saline-soluble form. After similar treatment of eosinophil granules many of the hydrolases are released, but most of the phosphatase activity and all the peroxidase remains insoluble.

Except for peroxidase, the enzymes looked for in the eosinophil granules were those found previously in lysosomes or in rabbit polymorphonuclear leucocyte granules. It is of course possible that other enzymes remain to be detected. The

presence of other enzymes capable of attacking unusual substrates might provide a useful hint as to eosinophil function.

SUMMARY

Eosinophils were separated from other types of cells in horse blood or rat peritoneal fluid by centrifugation in concentrated albumin solutions. Eosinophils did not appear to be damaged by this separation procedure.

A technique was also devised for isolation of cytoplasmic granules from eosinophils, thus allowing studies on enzyme content of the granules.

Granules from both horse and rat eosinophils contained a number of hydrolytic enzymes, similar in variety and in concentration to those previously found in granules of rabbit polymorphonuclear leucocytes. Eosinophil granules differed from those of the rabbit granulocyte in their high content of peroxidase and the absence of lysozyme and phagocytin. On disruption of eosinophil granules by repeated freezing and thawing in saline, cathepsin, ribonuclease, arylsulfatase and beta glucuronidase were released into solution, but phosphatases were partially and peroxidase completely bound to the insoluble granule residue. Peroxidase could be extracted from the granule residue with weak acid.

Eosinophil granules thus are lysosome-like structures.

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EXPLANATION OF PLATES

PLATE 17

FIG. 1. Eosinophils separated from horse blood by the method described in the text. The uniformity of the cell type is evident. Horse eosinophils have, as is seen, huge cytoplasmic granules. In the original smear the granules were red to pink, the cytoplasm was light blue, and nuclear lobes purple. Wright's stain. × 2000.

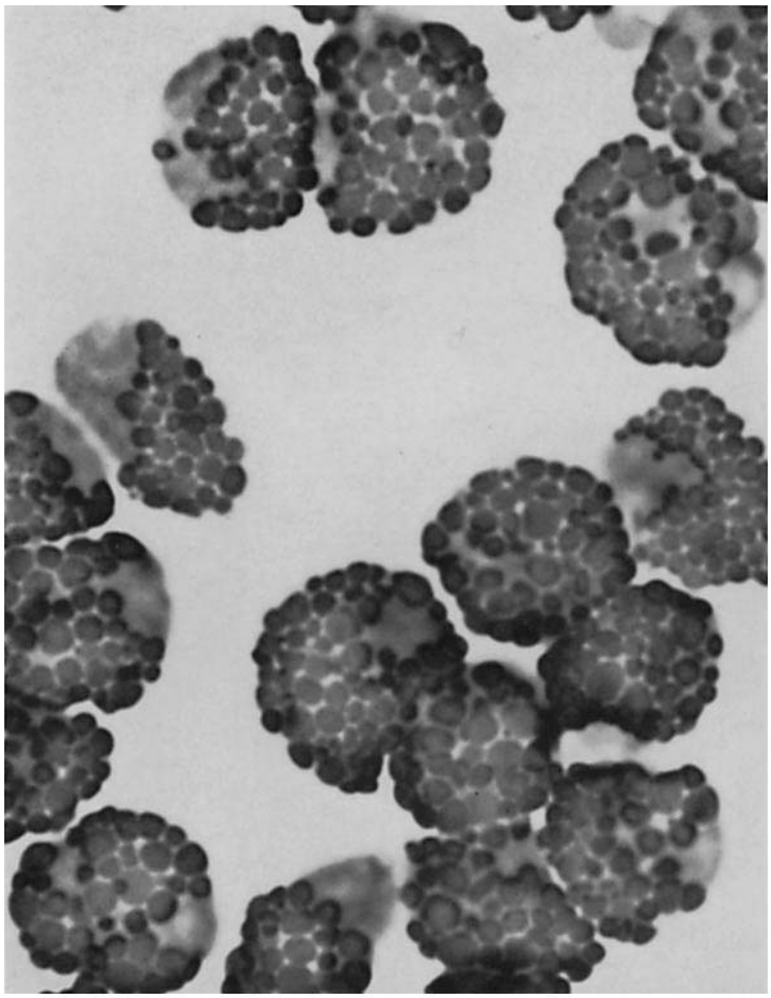

FIG. 1

(Archer and Hirsch: Enzymes in eosinophil granules)

PLATE 18

FIG. 2. A photomicrograph of rat eosinophils isolated from peritoneal washings. In the original preparation the numerous tiny cytoplasmic granules were red, the cytoplasm was faintly blue or colorless, and the nuclei had a brownish hue. The ring-shaped nucleus characteristic of the rat eosinophil is evident. Wright's stain. × 2000.

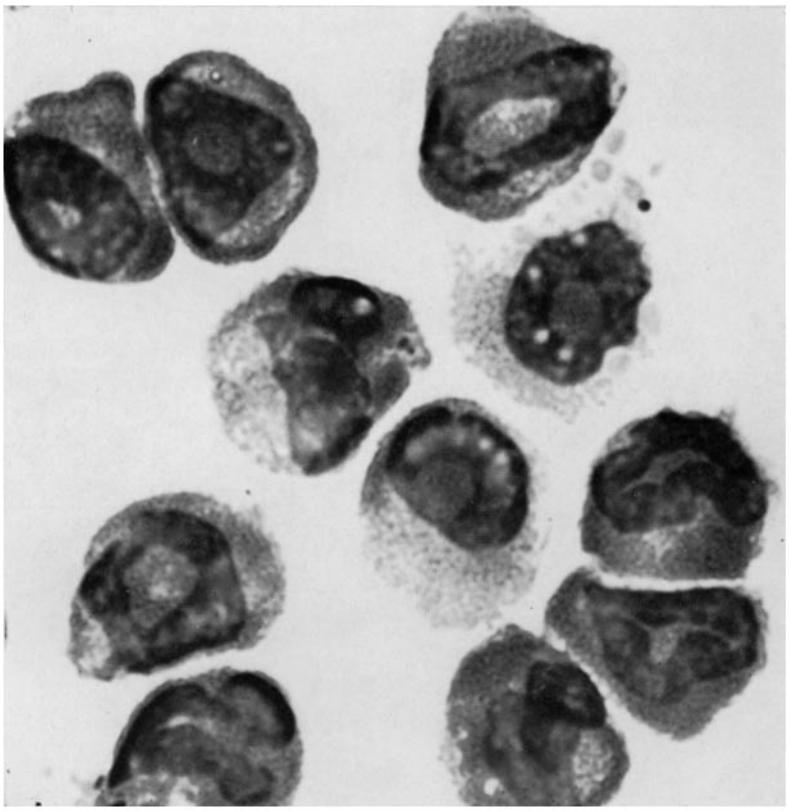

FIG. 2

(Archer and Hirsch: Enzymes in eosinophil granules)

PLATE 19

FIG. 3 illustrates the appearance of granules isolated from disrupted horse eosinophils. The size, shape, and staining properties of the granules were similar to those seen in intact cells (see Fig. 1). Wright's stain. × 2000.

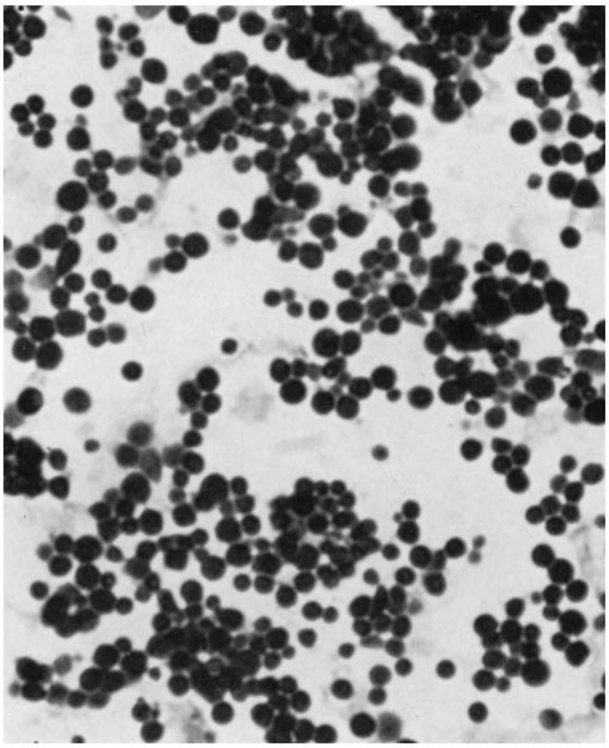

FIG. 3

(Archer and Hirsch: Enzymes in eosinophil granules)