

STUDIES ON ISOLATED MEMBRANES OF AZUROPHIL AND SPECIFIC GRANULES FROM RABBIT POLYMORPHONUCLEAR LEUKOCYTES

RALPH NACHMAN, JAMES G. HIRSCH, and MARCO BAGGIOLINI

From The New York Hospital-Cornell Medical Center, New York 10021, The Rockefeller University, New York 10021, and the Research Institute, Wander Limited, CH-3001 Berne, Switzerland

ABSTRACT

Membranes were prepared from rabbit polymorphonuclear leukocyte azurophil and specific granules separated by zonal differential centrifugation. The two types of granule membranes were quite similar in ultrastructural appearance, but they showed distinct differences in cholesterol-phospholipid ratios and in protein components demonstrable in polyacrylamide gels.

INTRODUCTION

In recent years, two distinct types of cytoplasmic granules, the azurophils and the specifics, have been characterized morphologically and biochemically in rabbit polymorphonuclear leukocytes (PMN) (1-7). The azurophil granules are formed first, during the promyelocyte stage of PMN maturation. They arise from the inner, concave face of the Golgi apparatus, and contain myeloperoxidase, lysozyme, cationic bactericidal proteins, and a wide variety of acid hydrolases commonly found in lysosomes. The specific granules form later in the maturation process, during the myelocyte stage, budding from the outer, convex Golgi face. They are smaller and less dense than azurophils and contain alkaline phosphatase, lysozyme, and lactoferrin, but lack lysosomal hydrolases.

Azurophil and specific granules are both bounded by a single unit membrane. This study reports the isolation of membrane fractions from pure preparations of azurophil and specific rabbit PMN granules (5), and presents results indicating that there are significant differences in the molecular composition of the two membranes.

MATERIALS AND METHODS

Materials

Shellfish glycogen was obtained from Amend Drug and Chemical Co., Inc., New York. The substrates used for the determination of marker enzymes were purchased from different sources: *o*-toluidine from MC&B Manufacturing Chemists, (Norwood, Ohio); *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide from Sigma Chemical Co. (St. Louis, Mo.); and *p*-nitrophenyl phosphate (disodium salt 5 H₂O, A grade from Calbiochem (La Jolla, Calif.).

Cell Fractionation

Azurophil and specific granules from rabbit PMN obtained from glycogen-induced peritoneal exudates were separated by zonal differential centrifugation at 6500 rpm for 15 min in a B-XIV rotor under the experimental conditions described in a previous paper (5). The composition of the fractions was established by measuring the distribution of protein, alkaline phosphatase, myeloperoxidase, and β -acetyl-glucosaminidase (5). Two gradient zones were further processed. A 20-25 ml fraction adjacent to the cushion, containing 70-80% of the myeloperox-

idase activity of the starting material, was used for the isolation of membranes from the azurophil granules, and 3-4 contiguous 20-ml fractions from the middle of the gradient having the highest relative concentrations in alkaline phosphatase were used for isolation of specific granule membranes. Studies done on granules separated from two different leukocyte homogenates gave essentially identical results on morphology, on the protein components, and on the cholesterol/phospholipid ratios of the granule membranes.

Preparations of the Granule Membranes

The granules of the two fractions were spun down in a Beckman LB 65 ultracentrifuge using a SW 25.2 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 25,000 rpm for 30 min. Before centrifugation, the fraction of the azurophils, partly contaminated by the 60% sucrose cushion, was diluted with water to approximately 20% sucrose. The pellets were suspended in 2 ml of isotonic saline, rapidly frozen and thawed three times at -70°C and 38°C , diluted to 4 ml with saline, and spun in the same centrifuge using a Beckman SW 65 rotor at 40,000 rpm for 30 min. The pellets were then extracted for 30 min with 1 ml of 1 mM citric acid (pH 3.2) in order to completely solubilize cationic proteins (8, 9), diluted to 4 ml with 1 mM citric acid, and spun under the above conditions. Saline and citric acid supernates were concentrated by ultrafiltration and kept at 4°C for analysis

Electron Microscopy

Granule membrane pellets which formed a film at the bottom of the centrifuge tube were fixed by exposure to 1% osmium tetroxide in phosphate buffer at 0°C for 30 min, followed by saline rinsing and postfixation in uranyl acetate. The films were then scraped from the tube and broken into small pieces for dehydration and embedding in Epon. Sections were stained with lead citrate and uranyl acetate. Osmium tetroxide was used rather than glutaraldehyde as the primary fixative in order to aggregate the packed membranes and facilitate recovery from the small quantity of starting material.

Polyacrylamide Gel Electrophoresis

The final membrane pellet after granule freeze and thawing in saline and acid extraction was solubilized in 1% sodium dodecyl sulfate (SDS) in 0.1 M sodium phosphate buffer, pH 7.0, and analyzed by gel electrophoresis (10). The gels were 5 mm in diameter and 6 cm long, 5% cross-linked, and contained the same concentrations of SDS and sodium phosphate buffer as used in the medium. Gels were

pre-run for 1 hr before application. Sucrose was added to the sample to permit layering between the buffer and the top of the gel. The gels were run at 10 ma/gel for 2 hr 15 min. They were then fixed in 50% Trichloroacetic Acid overnight, stained for 2.5 hr in 20% Trichloroacetic Acid containing 0.25% Coomassie blue (Coomassie Acid Dyes, ICI America Inc., Stamford, Conn.), and finally rinsed with 7% acetic acid using a diffusion destainer (Bio-Rad Laboratories, Richmond, Calif.). Molecular weights were estimated using known markers (11). Densitometric scanning of the stained polyacrylamide gels was performed at 550 nm in a Gilford model 240 recording spectrophotometer, full scale being equivalent to 1.0 OD, equipped with a Gilford linear transport gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Chemical Assays

In the granule subfractions, protein was measured by the method of Lowry et al. (12). Chloroform-methanol extraction for lipid analysis was performed as described by Folch et al. (13). Phospholipids were ashed to inorganic phosphate by the method of Chen et al. (14) and measured as described by Ames and Dubin (15). Cholesterol determinations were performed by Dr. Zena Werb using gas-liquid chromatography (16).

RESULTS

Separation of Azurophil and Specific Granules

The results of the cell fractionation experiments were identical to those obtained previously (5), and need not be described in detail. The average reciprocal contamination of the two preparations, as calculated from the relative concentrations of alkaline phosphatase and myeloperoxidase, was 5.5% for the fraction of the azurophil granules and 4.5% for the specific granules.

Morphologic Characterization of the Membrane Preparations

Several clumps of membrane material from different parts of the pellets were examined. All samples had the same appearance, illustrated in Figs. 1 and 2. In the electron microscope, the pellets were observed to consist in the main of circular or elongated profiles of membranes, with varying amounts of amorphous electron-opaque material distributed in between or attached to these membranes. The circular empty membrane

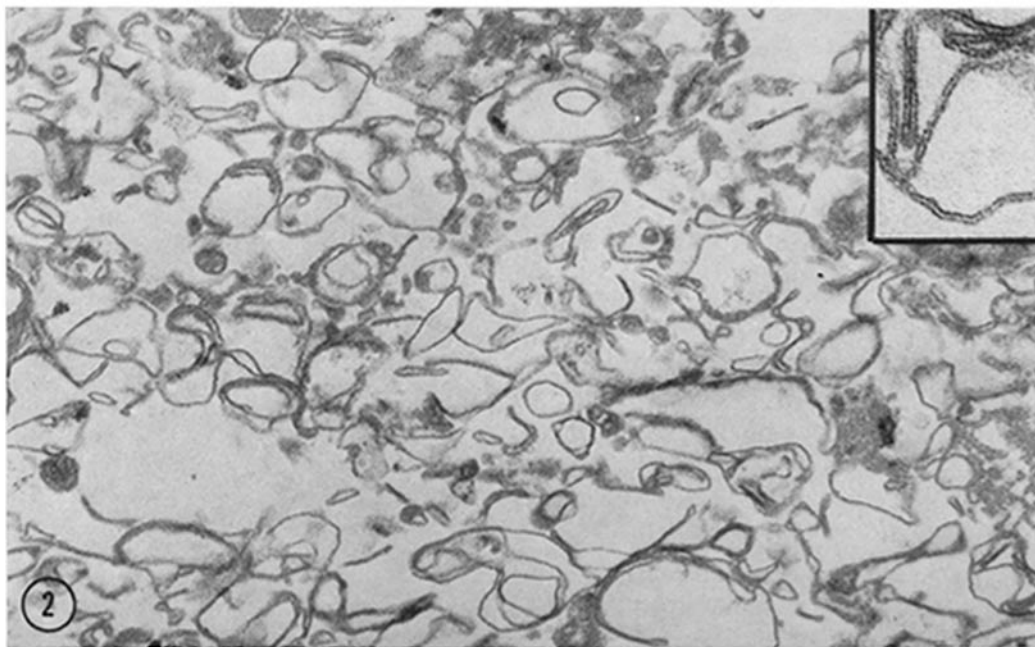
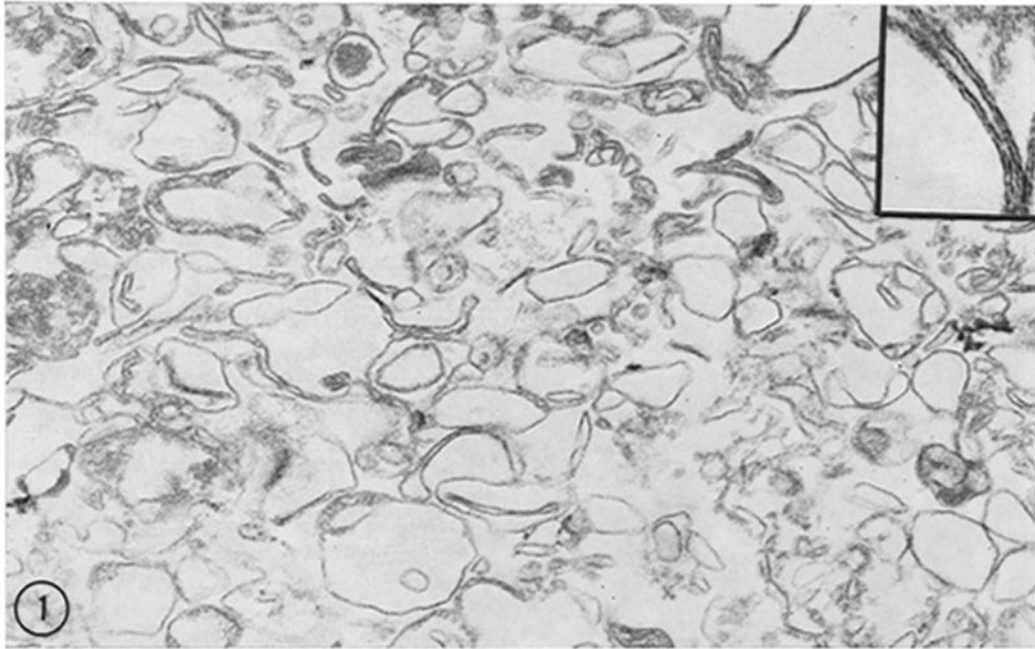


FIGURE 1 Membrane pellet obtained from the azurophil granules. Numerous membrane profiles are seen, mostly in form of round, irregular, and collapsed vesicles. Some amorphous, electron-opaque material is distributed randomly between membranes. $\times 46,000$. At higher magnification (inset, $\times 150,000$), suitable sectioned membranes have a typical trilaminar ultrastructure and are 6-7 nm thick.

FIGURE 2 Membrane pellet obtained from the specific granules. As in Fig. 1, circular and collapsed membrane vesicles are the dominant structures. Some amorphous material is also seen. The over-all appearance of the preparation as well as the membrane structure itself (inset, $\times 150,000$) appear to be the same as in Fig. 1. $\times 46,000$.

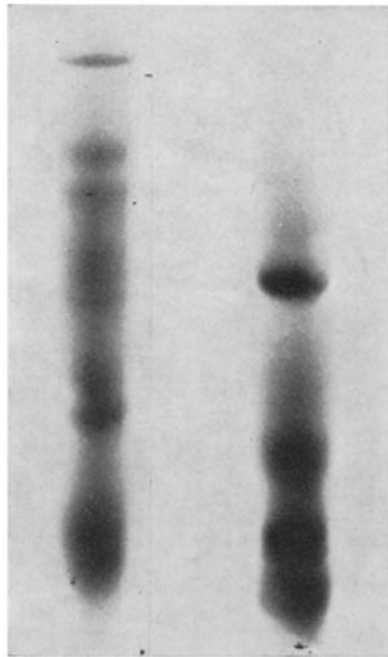


FIGURE 3 Polyacrylamide gel electrophoretic patterns of the proteins derived from the membranes of azurophil granules (right) and of specific granules (left). Each gel was loaded with 38 μg of protein. The positive pole is at the bottom.

profiles measured approximately 0.3–0.6 μ in greatest diameter. At high magnification (insets) suitably sectioned membranes showed a typical 6–7 nm trilaminar structure. No morphological differences were detected between the membrane preparations from azurophil granules (Fig. 1) and those from specific granules (Fig. 2) with respect to over-all composition, size, and shape of profiles, or membrane structure.

Separation of Protein Components

The granule proteins extracted into saline, and the membrane-bound proteins solubilized in SDS were clearly different as shown in Figs. 3 and 4. The citric acid extract contained quantities of protein too small to allow electrophoretic analysis. Comparison of the scanning profiles of the SDS-solubilized azurophil and specific membrane proteins (Fig. 5) shows only one possible major overlap in the low molecular weight range where a shoulder of the profile of the azurophil membrane proteins could correspond to a major peak of the comparable specific granule membrane prepara-

tion. On the whole, however, the two profiles are strikingly different, especially in the middle molecular weight range, between approximately 30,000 and 100,000, suggesting that the membranes of the two granule types have few, if any, proteins in common.

Comparison of the membrane proteins and the saline-soluble proteins derived from the azurophil is shown in Fig. 6, and a similar study of the proteins derived from the specific granules is shown in Fig. 7. These studies demonstrate that the membranes of the azurophil and specific granules consist of proteins which differ from those present in the soluble fractions, thus eliminating the possibility that contaminating soluble proteins accounted for any of the major bands seen in the membrane preparations.

The azurophil and specific granule soluble protein fractions are compared in Fig. 8. Except for some overlap in the low molecular weight range, possibly due to lysozyme which is present in both types of granules (5, 6), the two profiles are highly different, as is to be expected from the previous data on the granule composition (3–7). It was of

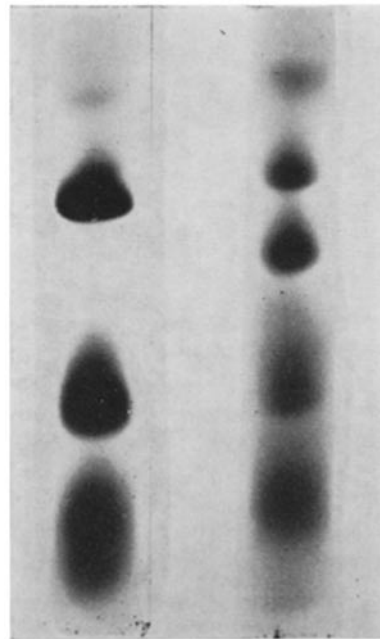


FIGURE 4 Polyacrylamide gel electrophoretic patterns of the saline-extractable proteins from the azurophil granules (right) and from the specific granules (left). Each gel was loaded with 30 μg of protein. The positive pole is at the bottom.

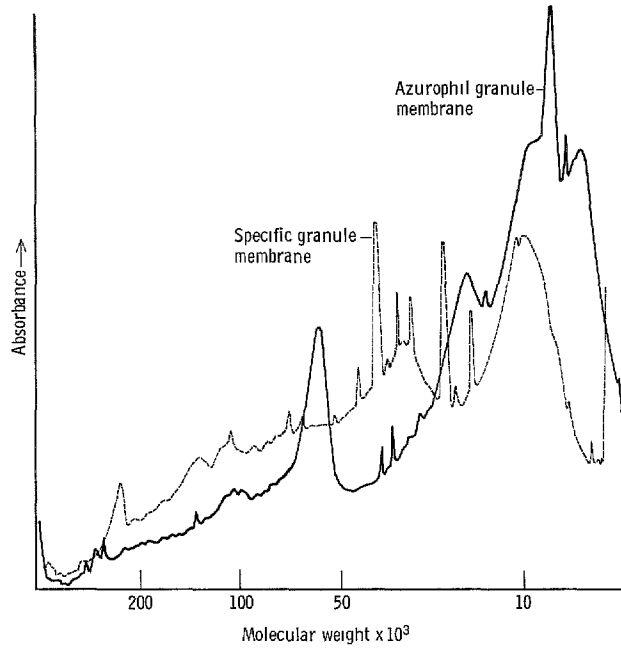


FIGURE 5 Densitometric scanning profiles of the polyacrylamide gel electrophoretic patterns (see Fig 3) of the membrane proteins from azurophil and specific granules. Each gel was loaded with 38 μg of protein. The positive pole is toward the right.

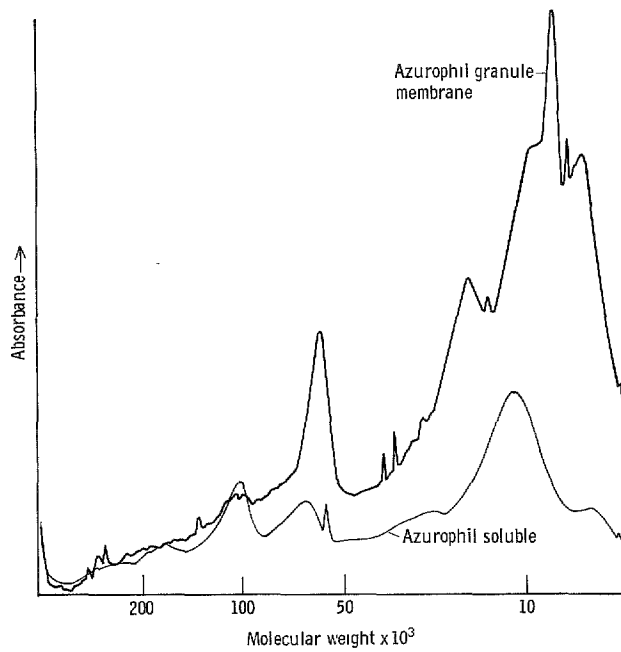


FIGURE 6 Densitometric scanning profiles of the polyacrylamide gel electrophoretic patterns of the membrane proteins and the saline-extractable (soluble) proteins from azurophil granules. 38 μg of membrane proteins and 30 μg of saline-extractable proteins were sampled on the gels. The positive pole is toward the right.

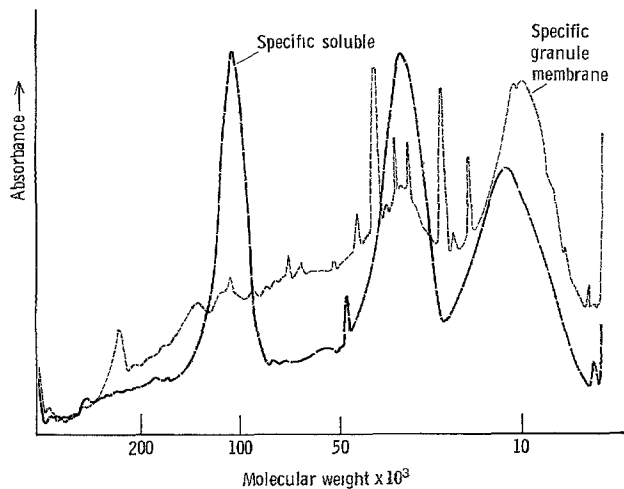


FIGURE 7 Densitometric scanning profiles of the polyacrylamide gel electrophoretic patterns of the membrane proteins and the saline-extractable (soluble) proteins from specific granules. 38 μg of membrane proteins and 30 μg of saline-extractable proteins were sampled on the gels. The positive pole is toward the right.

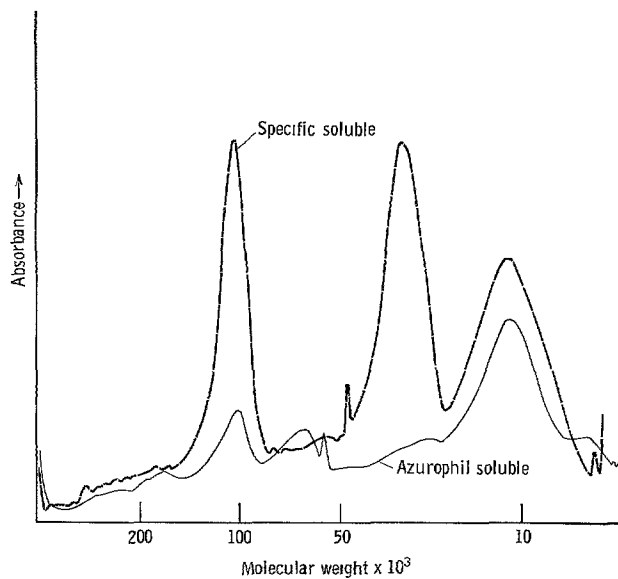


FIGURE 8 Densitometric scanning profiles of the polyacrylamide gel electrophoretic patterns of the saline-extractable proteins from azurophil and specific granules. Each gel was loaded with 30 μg protein. The positive pole is toward the right.

interest that despite equal protein loading, there was a marked difference in the total absorbance of the Coomassie blue staining of the gel patterns of the soluble proteins from azurophil and specific

granules (Fig. 8). The disparity is likely due to different dye binding of the various granule proteins. Also unexpected was the relatively small number of bands found in the soluble proteins

TABLE I
*Cholesterol and Phospholipid Content
of Granule Membranes*

Membrane preparation	Molar ratio cholesterol to phospholipid
Azurophil granule	0.49
Specific granule	0.36

derived from the azurophil granules. A similar pattern has been obtained by cellulose acetate electrophoresis of extract of azurophil granules (7). Many of the enzymes known to be present in this compartment (3-6) may have been present in too low a concentration to be detected in the gels by the method employed.

Lipid Studies

Studies on lipid composition of the membranes of azurophil and specific granules were limited to determination of cholesterol and phospholipid content because of the small amounts of material available. As is shown in Table I, the cholesterol to phospholipid molar ratios of these two membrane fractions were different, the membrane of the azurophil granules being richer in cholesterol than that of the specific granules.

DISCUSSION

Meaningful studies on PMN granule membrane composition require reasonable purity of the isolated granule populations, and of the membrane fractions recovered from these granules. The evidence for separation of the azurophil and specific granules by the methods used here has been presented above and elsewhere (4, 5). Our main consideration here, then, is directed towards the purity of the membranes recovered from these granules. The granules were disrupted by freezing and thawing in saline. The insoluble material was then extracted with dilute citric acid, since some granule components (e.g., cationic proteins) are extracted completely only at acid pH (8, 9). The citric acid extraction may well have damaged the membranes or extracted some of their proteins; however, the final membrane pellet examined by electron microscopy was composed for the most part of membrane sheets of size and thickness to be expected from observations made in fixed cells (1). More pertinent to the significance of the present observations is the question of contamination

of membranes by proteins, native or denatured, from the granule contents or elsewhere. Electron micrographs did show some amorphous material in addition to the membrane sheets. Significant contamination of the membranes by soluble granule proteins was ruled out by the fact that the gel patterns of proteins from the soluble and the membrane fractions showed no overlap. The limited number of major protein bands in the membranes, and the clear differences between the azurophil and specific membrane preparations make unlikely any major contamination with proteins not of granule origin. In general, the evidence indicates reasonable purity of the granule membrane fractions.

In PMN, it is reasonable to speculate about the nature of the Golgi membranes on the basis of results obtained from the analysis of the membrane of the granules in view of the studies of Bainton and Farquhar (1) establishing the origin of these granules from different faces of the Golgi complex. In our preparations, both azurophil and specific granules probably retained their original composition since there is no indication that they acquire or exchange materials with other structures when the cells have not been involved in phagocytosis. Therefore, the observed properties of azurophil and specific granule membranes may indicate that the Golgi apparatus of rabbit PMN consists of heterogeneous membranes, and that, in particular, considerable differences in membrane composition may exist between the inner cisternae where the azurophil granules are formed, and the outer ones from which the specific granules originate (1). Alternatively, since azurophil and specific granules are formed sequentially during PMN maturation (1, 2), the results could also indicate that the molecular structure of the entire Golgi apparatus undergoes considerable changes when the promyelocytes mature into myelocytes.

In view of the differences in membrane composition, one might expect that azurophil and specific granules would interact differently with membranes of endocytic vacuoles. Recent investigations have shown that this may indeed be the case, for, in rabbit (17) and guinea pig (18), specific granules seem to fuse with phagocytic vacuoles sooner after phagocytosis than do azurophils.

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