Specific and Azurophilic Granules from Rabbit Polymorphonuclear Leukocytes. I. Isolation and Characterization of Membrane and Content Subfractions

WILLIAM J. BROWN, W. ALLEN SHANNON, JR., and WILLIAM J. SNELL
Department of Cell Biology, The University of Texas Health Science Center at Dallas; and Veterans Administration Medical Center, Dallas, Texas 75235. Dr. Brown's present address is the Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510.

ABSTRACT The specific and azurophilic granules of rabbit polymorphonuclear heterophils (PMNs) have been isolated and fractionated into membrane and extractable subfractions. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) revealed several features of the protein composition of the two granules: (a) Whereas each type of granule had 40-60 proteins separable on one-dimensional gradient gels, few of the proteins were common to both granules. (b) The proteins of the extractable fractions (which comprised ~98% of the total granule protein) of each granule were distinct from the proteins of the membrane fractions (which comprised ~2% of the total granule protein). (c) The extractable proteins co-migrated with those collected from the medium of ionophore-treated, degranulating PMNs and therefore were defined as content proteins. These results were confirmed by radiolabeling studies. Lactoperoxidase-catalyzed iodination of intact granules did not label the content proteins but did label proteins that co-migrated with major granule membrane proteins. Moreover, disruption of the granules before iodination led to labeling of both content and membrane proteins. We conclude that the membranes of specific and azurophilic granules, which arise from different faces of the Golgi complex, are composed of unique sets of membrane proteins some of which are exposed on the cytoplasmic face of the granules.

Rabbit polymorphonuclear heterophils (PMNs) contain at least two well-defined classes of cytoplasmic granules, specific (SpG) and azurophilic (AzG), whose contents are delivered to phagocytic vacuoles (PV) by fusion of granules with nascent PV membranes (derived from the plasma membrane) during phagocytosis (1). In addition, granules fuse with the plasma membrane upon exposure to soluble stimuli or substrate-bound immune complexes (2), thereby releasing granule contents into the extracellular space; thus, under certain pathological or experimental conditions, PMNs function as secretory cells. Although the postsecretory disposition of secretory granule membranes of PMNs and other cell types has been extensively studied by ultrastructural methods, there is little if any biochemical information about the fate of the individual membrane proteins of secretory granules (2-4). Moreover, due to the paucity of cell types suitable for this line of investigation, the compositional relationship between secretory granule membranes and the plasma membranes with which they interact remains unknown.

We decided to take advantage of the secretory properties of the PMN to learn more about the fate of individual membrane proteins of SpG and AzG during secretion and to investigate the cell surface changes that result from the fusion of these granules with the plasma membrane. To do these studies required that the granules first be carefully purified and fractionated to identify membrane and content proteins. In the present report, the membrane and content proteins of SpG and AzG from rabbit PMNs have been characterized by ultrastructural, electrophoretic and radiolabeling methods. In the accompanying report we examine the radiolabeling pattern of the cell surface of rabbit PMNs before and after secretion (5). A preliminary account of this work appeared earlier (6).

MATERIALS AND METHODS

Materials

The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): shellfish glycogen Type II, cytochalasin B, diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), Micrococcus lysodeikticus, p-ni-
Relative concentration was calculated from the relation trophenylphosphate, fl-nicotinamide adenine dinucleotide (NAD), o-dianisidine, V from polycarbonate bottles in an IEC CRU-5000 centrifuge (Damon/IEC Division, consisted of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, 0.26 mM Na~HPO4·7H2O, 0.44 mM KH2PO4, 0.4 mM MgSO4·7H2O, 1 mM sucrose.

Collection of Cells
Rabbit peritoneal exudate PMNs were collected after injection of a sterile peritonitis with glycogen as described (7). Routinely, 300-500 ml of exudate fluid with a concentration of 1-3 x 10^6 cells/ml was collected from each rabbit. Exudate cells were harvested by a 500 g, 10-min centrifugation at 4°C in 250 ml polycarbonate bottles in an IEC CRU-5000 centrifuge (Damon/IEC Division, Needham, MA) fitted with a No. 269 rotor. Contaminating erythrocytes were lysed by two cycles of treatment with 10 volumes of ice-cold hypotonic saline as previously described (8). The sedimented white cells were washed twice in 0.15 M NaCl at 4°C and either resuspended in HBSS or prepared for homogenization. Cells obtained in this manner were always >98% viable as determined by trypan-blue dye exclusion and contained >98% heterophil polymorphonuclear leukocytes. Before homogenization, cells were treated, without loss of viability, with the protease inhibitor DFP by resuspending cells in 5 ml of 0.15 M NaCl and adding to that 0.02 volume of DFP according to the method of Armean and Stossel (9).

Isolation of Granules
SpG and AzG were isolated from rabbit peritoneal PMNs by a modification of described procedures (10). All steps in the fractionation procedures were carried out at 4°C. Purified exudate PMNs (1-4 ml packed cells; 1-5 x 10^7 cells) were washed twice in 0.34 M sucrose containing 5 mg/ml heparin sodium, resuspended in 4 volumes of 0.34 M sucrose-heparin and homogenized with a motor-driven teflon pestle in a Potter-Elvehjem homogenizer (10 or 25 ml capacity) at 1,800 rpm. Two complete strokes lasting 1 min each resulted in ~75% cell breakage. The homogenates were centrifuged at 2,000 g for 10 min (IEC CRU-5000 centrifuge fitted with a No. 269 rotor) in 15-ml conical glass tubes to remove nuclei, unbroken cells and large debris. Based on the recovery of marker enzyme activities, these postnuclear supernatants contained ~65% of the total SpG and ~85% of the total AzG activities of whole cell homogenates. Portions (2.5 ml) of this postnuclear supernatant were layered over 20-55% (wt/wt) linear sucrose gradients prepared in round-bottomed cellulose nitrate tubes (57 ml total gradient volume). After centrifugation at 19,000 g (13,500 rpm) for 30 min at 4°C in a Beckman L3-50 ultracentrifuge (Beckman Instruments-Sorvall Biomedical Div., Newington, CT). The granule suspensions were gently swirled every 2 min and the reactions were stopped by the addition of 40 volumes of ice-cold 0.25 M sucrose containing 0.5 mM Na2S04. Granules were collected by centrifugation at 48,200 g (20,000 rpm) for 1 h at 4°C in an RC-5B centrifuge (Du Pont Instruments-Sorvall Biomedical Div.) fitted with an SS-34 rotor, and washed twice by centrifugation as above in fresh suspension. The final granule pellets were resuspended in distilled H2O (dH2O) by freezing and thawing (liquid nitrogen and a 23°C water bath) and a small sample was removed for determination of 125I incorporation. The bulk of the sample was solubilized in electrophoresis sample buffer.

In some experiments granule suspensions were lysed by freezing and thawing, as above, before radioiodination. The labeling conditions were the same as above except that reactions were carried out in 1.5-m1 Beckman microfuge tubes and were stopped by the addition of an equal volume of 20% trichloroacetic acid (TCA) containing 0.2 M KI. A small sample from each tube was removed to determine 125I incorporation and the remaining sample was left on ice for 1 h. TCA-insoluble material was collected by centrifugation for 10 min in a Beckman microfuge. The TCA pellets were washed four times with ice-cold 10% TCA containing 0.1 M KI, and prepared for electrophoresis (see below).

Subfractionation of Granules into Membrane and Extractable Components
To lyse the granules and separate membranes from extractable components, granule preparations were subjected to freeze-thawing in liquid nitrogen and 23°C water-baths (three cycles) in three successive extraction solutions (see Fig. 1). After each extraction, the membranes were harvested by centrifugation and resuspended by freeze-thawing in the next extraction solution. Portions of the extraction supernatants were saved for biochemical analysis while the bulk of the pooled supernatant material was dialyzed at 4°C against 1,000 volumes of 2 mM PMSF, 0.1 mM EDTA, lyophilized, and solubilized in electrophoresis sample buffer. Following the last extraction step, membranes were washed once in 1 mM EDTA containing 2 mM PMSF, pH 7.0, and resuspended by freeze-thawing at 0.08 M sucrose in 20 mM NaHCO3 and 0.5 mM EDTA, pH 7.2. The membrane suspensions were layered over a 7.0 M sucrose step gradient (see Fig. 1) and centrifuged at 81,000 g (25,000 rpm) for 1 h at 4°C in a Beckman L3-50 ultracentrifuge fitted with a SW 27.1 rotor. Membranes collected from the 0.6 M/1.5 M sucrose interface were diluted with 10 volumes of 1 mM EDTA in 2 mM PMSF, pH 7.0, and harvested by centrifugation in a Beckman type 42 rotor at 125,000 g (40,000 rpm) for 1 h at 4°C.

Collection of PMN Secretions
Purified exudate PMNs were washed twice in 0.15 M NaCl by centrifugation and washed twice again in HBSS at 4°C by centrifugation at 250 g for 10 min at 4°C. Cells were resuspended to a density of 10^9 cells/ml in HBSS containing 5 μg/ml cytochalasin B and incubated for 30 min at 37°C in siliconized 250-ml glass Erlenmeyer flasks. Secretion was initiated by adding the calcium ionophore A23187 to a final concentration of 10 M M. At various times thereafter, 1 ml samples from the incubation flask were removed and cells were collected by a 10-s centrifugation in a Beckman Microfuge B. To measure the release of granule contents, the sedimented cells and the supernatants were assayed for lysozyme, a SpG enzyme and myeloperoxidase, an AzG enzyme.

Downloaded from jcb.rupress.org on September 22, 2017
granule and membrane suspensions were obtained as described above and spotted on Whatman GF/C filters (Whatman Inc., Clifton, N J) filters were placed in 400 ml ice-cold 10% TCA, 0.1 M KI for 1 h, washed three times in 300 ml of cold 10% TCA, and extracted with 250 ml of 90% acetone at ~20°C. Filters were air dried, and counted directly in an Isodyne Model 1185 Automatic Gamma Counter. Control filters carried through the washing procedure with a batch of filters containing TCA-precipitable material always contained <3% of the CPM found in the least radioactive filter.

PAGE of Granules and Their Subfractions

SDS PAGE was performed on slab gels according to the Jarvik and Rosenbaum (14) modification of the Laemmli procedure (15). The resolving gel (13 cm x 13 cm x 0.1 cm) was a linear 6-16% acrylamide gradient gel containing a gradient of 3-8 M urea, and the stacking gel was 3% acrylamide. There was no SDS in either the stacking or resolving gels but SDS was included in the sample and running buffers. The sample buffer contained 2% SDS, 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. After electrophoresis at 12 mA for ~12 h, the gels were fixed in 50% TCA for 30 min and stained for protein with 0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid solutions. Alternatively, gels were fixed and stained by the silver acetate method of Merril et al. (17).

TCA-precipitable material from 125I-labeled granules was solubilized in SDS-sample buffer by vigorous mixing with magnetic flea stirring bars. When necessary, samples were brought to approximately the correct pH by addition of small amounts (1-2 µl) of 1.0 M Tris-base, and, after solubilization, samples were immersed in a boiling water bath for 2 min. Following electrophoresis, proteins were visualized on gels by silver staining. Autoradiographs of gels dried onto sheets of dialysis membrane (Bio-Rad Laboratories) were made by exposing Kodak X-OMAT R film with the aid of a Dupont Cronex Lightning Plus intensifier screen (Dupont at -80°C. Using this procedure, 10^3 cpm per lane gave a suitable exposure in 5 h.

Enzymatic and Chemical Assays

Myeloperoxidase (18), alkaline p-nitrophenolphosphatase (18), β-glucuronidase (19), lysozyme (20), and lactate dehydrogenase (21) activities were determined as described. All reaction mixtures contained 0.2% Triton X-100 (final concentration), and in all enzyme assays only the initial rates were used for calculating activities. Initial rates were linear throughout the incubation times and proportional to the enzyme concentrations.

Phospholipids were determined by Prussian-blue complex formation using phosphatidylcholine as a standard (22). Protein was measured according to the method of Lowry et al. (23) using crystalline bovine serum albumin (Sigma Chemical Co.) as a standard.

Processing of Samples for Electron Microscopy

To prepare samples for electron microscopy, granule fractions were collected as described above and granule membrane subfractions were collected from sucrose gradients, diluted with 0.25 M sucrose to ~0.4 M, and harvested by a 100,000 g, 1-h centrifugation at 4°C. The pellets were treated with a combination glutaraldehyde-osmium tetroxide fixative for 1 h at 4°C (24), re suspended in the fixative by pipetting, and transferred to Beckman 1.5-ml microfuge tubes (all subsequent steps were carried out in these tubes). The fixed material was sedimented by a 1-min centrifugation as above in 0.15 M NaCl. The samples were en bloc stained with 0.25% aqueous uranyl acetate, dehydrated in a graded series of ethanol and embedded as previously described (25). Embedded pellets were cut parallel to the direction of centrifugal force and these pieces were re-embedded in flat embedding molds. Silver sections were cut through the full depth of the pellets, stained with uranyl acetate and lead citrate, and examined with a Philips 301 electron microscope.

RESULTS

The approach for these studies was to obtain pure fractions of SpG and AzG, separate the granules into membrane and extractable subfractions, and examine the protein and glycoprotein composition of each fraction by SDS PAGE. As a further means of distinguishing between and identifying membrane and content proteins, surface iodination methods were used (which also gives topological information); and the extractable fractions were compared to PMN secretions collected from the medium of ionophore-treated cells.

Isolation of Specific and Azurophilic Granules

Results of the fractionation procedure used to isolate SpG and AzG from rabbit PMNs are shown in Fig. 2. Measurement of turbidity (OD400) of gradient fractions showed two broad peaks; the distribution of alkaline phosphatase and myeloperoxidase indicated that the first peak corresponded to SpG and the second to AzG; the levels of cross contamination of the peak fractions were 2.5% for SpG and 3.5% for AzG, and there was a 4.9-fold purification of SpG granules and a 5.2-fold purification of AzG from the postnuclear supernatant. These results were typical of most experiments. Contamination of granule fractions by other organelles was found to be minimal; this was due in part to the paucity of mitochondria, endoplasmic reticulum, and other organelles in rabbit PMNs. In agreement with Baggioni et al. (26), glucose-6-phosphatase activity (KF-insensitive β-glucorophosphatase), a marker enzyme for rough endoplasmic reticulum, could not be detected. Small amounts (<0.001% of that found in mouse liver homogenates) of malate dehydrogenase (MDH) activity, a mitochondrial enzyme, were detected in postnuclear supernatants but the activity was not detected in gradient fractions. Electron mi-
specific and azurophilic granule fractions collected from sucrose gradients; the results of a typical experiment are shown. The amount of cross-contamination of specific and azurophilic granules, the postnuclear supernatant-fraction from homogenized rabbit PMNs was subjected to rate sedimentation on a linear 20-55% (wt/wt) sucrose gradient; the results of a typical experiment are shown. Fractions were assayed for turbidity, density, protein (102%), alkaline phosphatase (84%), ß-glucuronidase (91%), lysozyme (80%), and myeloperoxidase (75%) (percentage recovery given in parentheses). The amount of cross-contamination of specific and azurophilic granules in the experiment was 2.5% and 3.5%, respectively. The concentration of various components was plotted against the percentage of the total gradient volume collected (described in Material and Methods). The top of the gradient is to the left of the histogram. Specific granule fractions 11-13 and azurophilic granule fractions 17-18 were collected and separately pooled for electron microscopic examination and preparation of granule membranes.

Fig. 2 Turbidity, protein distribution, and enzyme analyses of specific and azurophilic granule fractions collected from sucrose gradients of rabbit PMN postnuclear supernatants. To isolate granules, the postnuclear supernatant-fraction from homogenized rabbit PMNs was subjected to rate sedimentation on a linear 20-55% (wt/wt) sucrose gradient; the results of a typical experiment are shown. Fractions were assayed for turbidity, density, protein (102%), alkaline phosphatase (84%), ß-glucuronidase (91%), lysozyme (80%), and myeloperoxidase (75%) (percentage recovery given in parentheses). The amount of cross-contamination of specific and azurophilic granules in the experiment was 2.5% and 3.5%, respectively. The concentration of various components was plotted against the percentage of the total gradient volume collected (described in Material and Methods). The top of the gradient is to the left of the histogram. Specific granule fractions 11-13 and azurophilic granule fractions 17-18 were collected and separately pooled for electron microscopic examination and preparation of granule membranes.

Granule Membrane Isolation

The effectiveness of the procedures for separating granules into membrane and extractable subfractions for a representative experiment is shown by the data in Table I. The first extraction removed the majority of the soluble marker enzymes, lysozyme and myeloperoxidase, and the final membrane pellets contained very small or undetectable amounts of these enzymes. In these experiments there were 107.0- and 75.2-fold decreases, respectively, in the specific activity (enzyme activity/mg phospholipid) of SpG lysozyme and AzG myeloperoxidase. In contrast, ~95% of the total phospholipids of the granules was recovered in the final membrane pellets, amounting to 38.8- and 81.8-fold increases in the phospholipid to protein ratio in SpG and AzG membrane preparations, respectively. The amounts of protein recovered with the final membrane pellets in this experiment were 2.5% of the total SpG, and 1.2% of the total AzG starting material. It was also determined that SpG membranes consistently had ~3.0 mg protein/mg phospholipid while AzG membranes had ~2.5 mg protein/mg phospholipid. Electron microscopic analysis of material collected from the 0.6 M/1.5 M interface of the sucrose gradients demonstrated that both SpG and AzG membranes (Fig. 3) were small (0.1-0.6 μm), closed membrane vesicles devoid of identifiable matrix material. The membrane vesicles of AzG lacked the fibrous material observed on some intact granules since higher magnification electron micrographs revealed a smooth surface on both SpG and AzG membrane vesicles.

Collection of PMN Secretory Products

Since separation of soluble granule components from granule membranes was based upon in vitro extraction procedures, it was essential to have a second, independent means to obtain soluble granule components. To do this we took advantage of the ability of PMNs to secrete SpG and AzG contents. By incubating the cells in cytochalasin B and the calcium ionophore A23187 (27) the cells were induced to release their granule contents, which could be harvested from the medium. After a 25-min incubation in 10^-6 M A23187, ~85% of the total cellular lysozyme and ~65% of the total myeloperoxidase were released from the cells. In contrast, <5% of the total cellular lactate dehydrogenase (LDH), a cytoplasmic marker enzyme, was released under these conditions. For SDS PAGE analysis, the medium, which contained secreted proteins, was cleared of debris (very little was observed) by high speed centrifugation (48,000 g, 1 h), dialyzed at 4°C against 1,000 volumes of 1 mM PMSF, 0.1 μM EDTA in 0.1 μM Tris, pH 8.0, lyophilized, and resuspended in electrophoresis sample buffer.

Electrophoretic Analysis of Specific and Azurophilic Granules and Their Subfractions

To determine which of the proteins in the final membrane preparations were authentic membrane proteins, a comparative analysis using SDS PAGE was made of whole granules, pooled granule extracts, secretory products from ionophore-treated cells, and final membrane preparations from SpG and AzG. The results are shown in Fig. 4. Although up to 40-50 SpG proteins could be seen on overloaded gels, 8-10 polypeptides of mol wt 82, 80, 25, 24, 23, and a quintuplet at about 14-16 kdaltons were the major constituents of the extractable subfraction (Fig. 4, SPECIFIC, Ex). Fractionation of the granules revealed that 6-8 of these proteins were essentially missing from isolated granule membranes (Fig. 4, SPECIFIC, M) and were the major constituents of the extractable subfraction (Fig. 4, SPECIFIC, Ex). Moreover, most of these same proteins were the primary components of material secreted into the medium by ionophore-treated cells (Fig. 4, SPECIFIC, Sec). Analysis of the membrane subfraction revealed that there was a major group of unique polypeptides of mol wt 145, 96, 42, 32, and 27 kdaltons that we feel probably represent bona fide membrane proteins: SpG membranes were consistently (15 experiments) enriched with these five proteins and data from gel scans (not...
FIGURE 3  Electron micrographs of sections taken through nearly the full depth of pellets of isolated specific and azurophilic granules and their membrane subfractions. Specific granules (a) were 0.3-0.6 µm in diameter and round to oval in shape, whereas azurophilic granules (b) were 0.6-0.9 µm in diameter, generally round and more electron-dense than specific granules. Membrane subfractions purified from specific (c) and azurophilic (d) granules were composed of small (0.1-0.6 µm) closed vesicles and had no detectable matrix material attached to the membranes. a, x 20,000; b, x 20,000; c, x 29,500; d, x 27,700.
TABLE I

Fractionation of PMN Granules and Recovery of Components *

| % of Starting material | Protein   | Phospholipid | Lysozyme | Myeloperoxidase |
|------------------------|-----------|--------------|----------|----------------|
|                        | SP AZ     | SP AZ        | SP AZ    | AZ             |
| 1st Extraction supernatant | 78.4 75.5 | 1.5 4.5      | 88.1 81.9 | 88.3           |
| 2nd Extraction supernatant | 15.2 22.1 | -- --       | 0.6 5.0  | 5.1            |
| 3rd Extraction supernatant | 0.3 0.2   | -- --       | 3.3 6.7  | 7.1            |
| Final membrane pellet  | 2.5 1.2   | 96.2 97.7    | 0.9 0.5  | 1.3            |

* See text for details

SP, Specific granule components
AZ, Azurophilic granule components

Figure 4 SDS PAGE analysis of specific and azurophilic granule components. Polypeptides obtained from electrophoresis on 6–16% gradient gels of whole granules (Gr); content subfractions from granules extracted in vitro (Ex); proteins harvested from secreting cells (Sec); and purified granule membranes (Mb). The numbers with arrows indicate the mol wt (x 10^3) of granule proteins that were enriched in purified membrane preparations and not found in the content subfractions. The numbers with arrowheads indicate the mol wt of proteins found in the content subfractions, the secretory material, or both, but which nevertheless remained adherent in membrane preparations. The numbers with dots represent the mol wt of azurophilic granule proteins found both in secretions and extractable subfractions but not with membranes. The outer lanes (mw) are molecular weight markers: myosin, 200 kdaltons; β-galactosidase, 115 kdaltons; phosphorylase B, 96 kdaltons; BSA, 68 kdaltons; ovalbumin, 43 kdaltons; soybean trypsin inhibitor, 21 kdaltons; and lysozyme, 14 kdaltons.

shown) indicated that they represented at least 70% of the protein mass of the membranes. In addition these polypeptides were not found in the content subfraction nor in material secreted by cells, but they were present in small amounts in intact granules.

A second group of proteins (mol wt 80, 33, and a doublet at ~16 kdaltons) probably represent contaminating content proteins because polypeptides in this group co-migrated with proteins found in either the content or secretion fractions or both. In addition, in mixing experiments (not shown) in which content subfractions were mixed with isolated membranes, several of these proteins were able to re-associate with membranes. A third group of proteins representing upwards of 30–40 other minor proteins could also be visualized in gels of membranes but the amount of these varied among experiments.

SDS PAGE analysis of AzG (Fig. 4, AZUROPHILIC) showed that these granules were also dominated by a small group of major proteins (mol wt 80, 59, 48, 46, 33, 26, and 15 kdaltons) most of which did not co-migrate with SpG proteins. The majority of these proteins (mol wt 80, 59, 33, 26, 15 kdaltons) appeared in the extractable fraction and were also present in the cell secretions. The electrophoretic profile of the purified membrane preparation was dramatically different from those of both whole granules and the extractable fraction and was dominated by the 48- and 46-kdalton polypeptides. Based on gel scans these two proteins represented ~60% of the
total protein of these membranes. A second group of proteins consistently found in membranes but to a much lesser extent than the 48- and 46-kdalton proteins included polypeptides of mol wt 42 and 27 kdaltons. None of these proteins was found in the content subfraction. In contrast a group of proteins of 33, 26, and 15 kdaltons was also recovered in the extractable subfraction, and in mixing experiment could be reabsorbed to the membranes. As with the SpG, this second group of proteins in the membrane probably represents adsorbed content proteins.

The 48- and 46-kdalton polypeptides of AzG membranes did not co-migrate with any significant SpG membrane proteins and thus the major constituents of both membranes were unique to each organelle. However, proteins of mol wt 42 and 27 kdaltons were found in both membranes and may represent common constituents, although there is no other evidence for this.

Identification of Glycopeptides of Specific and Azurophilic Granule Membranes

Since analysis of SpG and AzG membranes by SDS PAGE revealed major differences in the polypeptide compositions of these membranes, this analysis was extended to include membrane glycopeptides by treating gels with PAS stain. The results shown in Fig. 5 indicated that the 145- and 96-kdalton polypeptides, which were unique to SpG membranes, were intensely stained by the PAS procedure. On the other hand, very few of the AzG membrane proteins were stained by PAS, although a faint PAS band was seen in the region of the 46 and 48 kdalton doublet. No PAS staining was detected on gels not treated with periodate.

Identification of Externally Disposed Granule Membrane Proteins Specific Granules

To identify externally (cytoplasmically) disposed granule membrane proteins, isolated SpG and AzG were surface-labeled by use of LPO-catalyzed iodination procedures. SDS PAGE analysis of labeled SpG components (Fig. 6) indicated that the 96-kdalton mol wt glycoprotein previously identified as a major SpG membrane protein (Fig. 6, lane A) was highly labeled on intact granules. Other granule-associated proteins of 145, 55, 32, 21, and 16 kdaltons were consistently labeled to a much lesser extent by surface iodination. Of these others, only the 145-kdalton glycoprotein and the 32-kdalton protein were previously identified as major membrane proteins. The 55- and 21-kdalton bands had corresponding protein bands in purified membrane preparations but were present in very small amounts. Their origin could not be determined by these data. The 16-kdalton band corresponded to the 15.5-16.5-kdalton triplet thought to be highly adherent content proteins. This
labeled protein(s) might have been released by leaky granules and adsorbed onto the outside of others. To demonstrate that intact granules were being labeled only on the outer surface, granules were disrupted by freezing and thawing and then iodinated. Under these conditions several proteins previously inaccessible to labeling on intact granule preparations and identified as the major granule content proteins became highly radiolabeled (Fig. 6, lane B) including the 82-, 80-, 23-, and 22-kdalton proteins. Other content proteins labeled to a lesser extent included those of 15 and 12 kdaltons.

In these lysis control experiments, nearly all of the granule proteins were radiolabeled, whereas only a certain class of these proteins was iodinated on intact granules. Notably absent, however, were the 42- and 27-kdalton proteins previously identified as major membrane proteins.

To identify granule membrane proteins that were not accessible to iodination on intact or disrupted granules, purified membrane preparations were iodinated. The results of this experiment (Fig. 6, lane C) demonstrated that iodination of purified SpG membranes resulted in the labeling of all of the previously identified membrane proteins. These results suggested that the membrane preparations were composed of right-side- and inside-out membrane vesicles. By comparing the results of radioiodination of intact SpG with those of lysed granule and membrane preparations it could be determined that the SpG membrane glycoproteins of 145 and 96 kdaltons and the 32-kdalton protein were accessible to surface iodination on intact granules, whereas the 42- and 27-kdalton proteins were not.

Azurophilic Granules

SDS PAGE analysis of iodinated intact AzO (Fig. 7) revealed that proteins of 48, 46, 40, 27, 20, and 15 kdaltons were highly labeled (Fig. 7, lane A). Of these polypeptides, the 48-, 46-, and 27-kdalton proteins were previously identified as major components of purified membrane preparations (Fig. 7, lane C), whereas the 40-, 20-, and 15-kdalton polypeptides were not identified in membrane preparations. Their origin could not be determined. As with SpG, radioiodination of lysed AzG (Fig. 7, lane B) resulted in a different pattern of labeled proteins when compared with labeled intact granules; most of the newly iodinated proteins, not previously accessible to labeling on intact granules, were identified as soluble content proteins including proteins of mol wt 80, 59, 30, 24, 16, and 14 kdaltons. Unexpectedly, however, the 27-kdalton membrane protein, iodinated on intact granules, became inaccessible in lysed granule preparations. Also, the 42-kdalton membrane protein was not accessible to iodination in either intact or disrupted granule preparations. Radioiodination of purified AzG membranes (Fig. 7, lane C) resulted in the labeling of nearly all the major membrane proteins including the 42-kdalton protein. The conclusions from these experiments were that the bona fide AzG membrane proteins of 48, 46, and 27 kdaltons were exposed on the external granule surface, whereas the 42-kdalton protein was not.

Controls

LPO-catalyzed iodination of AzG posed a special problem because these granules contain endogenous myeloperoxidase (MPO), which is capable of catalyzing the covalent bonding of halogens to proteins (28). In fact, as the results in Table II indicate, if AzG were lysed before iodination, exogenous LPO

| Granule | LPO | GO | Total CPM incorporated into granule suspensions |
|---------|-----|----|---------------------------------------------|
| Specific | +   | +  | 2.7 x 10^6                                  |
| Intact   |     |    | 1.5 x 10^6                                 |
| Disrupted| +   | +  | 2.2 x 10^6                                 |
| Azurophilic | + | +  | 8.8 x 10^6                                 |

* For determination of 125I-incorporation into protein, samples of the reaction mixtures from intact and lysed granules incubated in the presence or absence of exogenously added enzymes were applied to Whatman filters and processed as described in Materials and Methods.
was not necessary for incorporation of significant amounts of $^{125}$I. However, in the experiments with intact granules, the endogenous MPO was not very active since exclusion of LPO from intact granule preparations resulted in a >500-fold decrease in $^{125}$I incorporation. Based on these results two conclusions could be made: (a) preparations of intact AzG contained an insignificant amount of disrupted granules, and (b) under the conditions used, MPO was not labeling intact granules from the inside to any significant degree. Table III indicates that SpG preparations also required exogenous LPO for incorporation of significant amounts of $^{125}$I.

As another measure of the integrity of SpG and AzG, the levels of latent marker enzyme activities in intact granule preparations were determined. The results in Table III demonstrate that both SpG and AzG maintained high levels, >83% and >87% respectively, of latent enzyme activities throughout the course of the labeling procedures.

The results in Table IV demonstrate that extraction of $^{125}$I-incorporated material from intact SpG and AzG preparations with organic solvents released <5% of the total CPM incorporated in any sample and indicated that very little $^{125}$I was incorporated into lipid.

### Table III

Latency of Enzymes in Intact Granule Preparations

|                     | Lysozyme | Alkaline phosphatase |
|---------------------|----------|----------------------|
| Specific granules   |          |                      |
| Before incubation    | 89.6     | 90.5                 |
| After incubation     | 82.9     | 88.7                 |
| Azurophilic granules|          |                      |
| Before incubation    | 94.3     | —                    |
| After incubation     | 87.3     | —                    |

* The percentage of latent enzyme activity was calculated from the equation

\[
\text{Percentage Latent Enzyme Activity} = \left( \frac{\text{Total Activity} - \text{Free Activity}}{\text{Total Activity}} \right) \times 100.
\]

† Total activity

The free activity was determined by adding aliquots of granule suspension to substrates dissolved in 0.34 M sucrose while the total activity was assayed in granules suspended in 0.34 M sucrose containing 0.2% Triton X-100.

§ Latent enzyme activity in granule preparations before addition of iodination reagents.

### Table IV

Extraction of Lipids from Iodinated Specific and Azurophilic Granules

| Treatment        | CPM in TCA precipitate | % Control |
|------------------|-------------------------|-----------|
| Specific granules|                         |           |
| No extraction    | 217,696                 | 100.0     |
| CHCl₃: MEOH, acetone | 261,371             | 96.2      |
| Azurophilic granules|                        |           |
| No extraction    | 164,950                 | 100.00    |
| CHCl₃: MEOH, acetone | 157,873             | 95.7      |

Intact granules were labeled as described in Materials and Methods. The reactions were stopped by the addition of an equal volume of ice-cold 20% TCA, 0.2 M KI. After a 1-h incubation on ice, TCA-insoluble material was collected by a 10-min centrifugation in a Beckman Microfuge and the TCA pellets were washed three times with ice-cold 10% TCA. The TCA precipitable radioactivity was determined by direct counting of the pellets in the microfuge tubes; pellets were then extracted twice with chloroform: methanol (2:1) at 23°C followed by one extraction with acetone at −20°C and counted again.

### DISCUSSION

**Electrophoretic Analysis of Specific and Azurophilic Granules and their Subfractions**

In this report we have analyzed the membrane and content proteins of SpG and AzG obtained from rabbit PMN heterophils. The results indicated that each type of granule was comprised of unique sets of membrane and extractable or content proteins. An independent assessment of proteins constituting the soluble granule contents was obtained by inducing the release of SpG and AzG contents from PMNs by the use of the calcium ionophore A23187 as a secretagogue. Analysis by SDS PAGE demonstrated that all of the major secretory proteins had counterparts in the isolated granule extracts. And, just as significantly, none of the proteins identified as major membrane proteins were recovered in the secretory material.

The results described here support, in part, those reported earlier on the initial characterization of rabbit PMN cytoplasmic granules membranes (29, 30). Nachman et al. (29) and Baggilini et al. (30) concluded, as we have, that SpG and AzG were quite different in polypeptide compositions. However, these studies differ significantly in several respects. For example, Nachman et al. (29) reported the presence of a major AzG membrane protein of ~50-kdalton mol wt that we think has been resolved into two polypeptides of 46 and 48 kdaltons. Also, because the soluble content proteins were identified and several control experiments were conducted it was possible to state with reasonable certainty which proteins in membrane preparations were authentic membrane proteins and which were adsorbed soluble components. In this regard, a recent study of human PMN granules concluded that SpG and AzG membranes contained ~20% and ~40%, respectively, of the total granule protein (31). These high percentages may have been caused by nonspecific adsorption of soluble contents since extractions were done in low-salt, Tris buffers. In our hands, membranes derived from Tris-extracted rabbit PMN granules were found to contain the same polypeptides as whole granule preparations (data not shown). Moreover, the estimate reported here of the amount of total granule protein associated with the membranes falls within the range reported for other types of organelle membranes such as those of guinea pig pancreatic smooth and rough endoplasmic reticulum (32), guinea pig pancreatic zymogen granules (32, 33), and bovine adrenal chromaffin granules (34). Even though great lengths have been taken to remove soluble proteins in this and other studies, it should be recognized that because the protein concentration in these organelles may reach 200 mg/ml (35) these intracellular membrane preparations may still contain significant amounts of contamination from the soluble contents (e.g., specific granule 15-17-kdalton proteins); therefore, the warnings of Castle et al. (36) and Castle and Palade (37) should be heeded.

**Identification of Cytoplasmically Disposed Granule Membrane Proteins**

By the use of LPO-catalyzed iodination procedures, it was determined that of the five major proteins copurifying with isolated SpG membranes, the 145- and 96-kdalton glycoproteins and the 32-kdalton protein were accessible to iodination on intact SpG. Several other proteins were also labeled but these did not correspond to any major granule protein (with the possible exception of the 16-kdalton protein). These proteins could be externally disposed minor membrane proteins,
cytoplasmic proteins stuck to the outer granule membrane, or content proteins released from leaky granules that stuck to the outside of other granules. On intact AzG, the 48-, 46-, and 27-
dalton major membrane proteins were accessible to radioio-
dination. As with SpG, several other granule-associated pro-
teins were radiolabeled but were not identified as AzG mem-
brane proteins.

Both SpG and AzG membranes contained a protein of 42
daltons whose properties are noteworthy. SpG and AzG 42-
dalton proteins were enriched in membrane subfractions and
co-migrated on one-dimensional gradient gels, suggesting that
they may be the same protein. Although we have no direct
evidence to confirm or deny this suggestion, we suspect that
they may be the same polypeptide because the 42-kdalton
proteins gave the same unexpected results in the iodination
experiments. The 42-kdalton proteins were the only SpG and
AzG membrane proteins inaccessible to iodination in both
intact and lysed granule preparations. This result was not
attributable either to the absence of the proteins from granule
preparations or to the absence of tyrosine residues in the proteins because they were seen on gels stained for protein and
the proteins were iodinated in purified membrane preparations.

The 42-kdalton protein(s) could be buried in the lipid bilayer
in granule preparations, thus preventing accessibility to lacto-
operoxidase, but the orientation might have been altered during
the extensive freezing and thawing used to make purified membranes.

From these membrane characterization and iodination ex-
periments it can be concluded that SpG and AzG membranes
are very different in polypeptide composition and that some of
these membrane proteins are exposed on the external or cyto-
plasmic surface of the granules. In the accompanying paper we
compare PMN cell surface proteins and granule membrane
proteins and report on the fate of several of these membrane
proteins during degranulation.

We thank Drs. Richard G. W. Anderson, Anthony Bretscher, and Fred
Grinnell for discussions and criticisms and Ms. Helen Patterson and
Ms. Alicia B. Benitez for their excellent help in preparation of the
manuscript.

The research was supported in part by grants from the U.S. Public
Health Service (GM 25661 to W. J. Snell) and the Veterans Adminis-
trative Medical Research Service (to W. A. Shannon, Jr.).

Received for publication 19 August 1982, and in revised form 29 Novem-
ber 1982.

REFERENCES

1. Beintema, D. F., B. A. Nichole, and M. G. Farquhar. 1976. Primary lysosomes of blood
leukocytes. In Lysosomes in Pathology and Biology. J. T. Dingle and R. T. Deas, editors.
North-Holland Publishing Co., Amsterdam. 3-32.

2. Benson, P. M. 1976. Secretion of lysosomal enzymes induced by immune complexes and
complement. In Lysosomes in Biology and Pathology. J. T. Dingle and R. T. Deas, editors.
North-Holland Publishing Co., Amsterdam. 99-126.

3. Palade, G. E. 1973. Intracellular aspects of the process of protein secretion. Science (Wash.
DC). 189:347-358.

4. Brown, W. J. D., and G. E. Palade. 1977. Production of secretory proteins in animal cells.
In International Cell Biology, 1976-1977. B. B. Brinkley and K. R. Porter, editors. The
Rockefeller University Press, New York. 308-317.

5. Brown, W. J. D., W. A. Shannon, Jr., and W. J. Snell. 1982. Specific and azurophilic granules
from rabbit polymorphonuclear leukocytes. II. Cell surface localization of granule mem-
brane and content proteins before and after degranulation. J. Cell Biol. 96:1040-1046.

6. Brown, W. J. D., W. A. Shannon, Jr. 1980. Characterization of membranes of specific and
azurophilic granules from rabbit polymorphonuclear (PMN) leukocytes. J. Cell Biol. 87:208a.

7. Hirsch, J. G. 1956. Phagocytosis: a bactericidal substance from polymorphonuclear leuko-
cytes. J. Exp. Med. 103:589-611.

8. Takamori, K., and T. Yamauchi. 1980. Biochemical properties of polymorphonuclear
macrophages from venous blood and peritoneal exudates of rabbits. Infect. Immun. 29:395-400.

9. Arziman, P. C., and T. P. Stossel. 1980. Prevention of degradation of human polymorpho-
nuclear leukocyte proteins by diisopropylphosphorothioate. Blood 56:442-447.

10. Zeya, H. J., and J. K. Spitznagel. 1971. Characterization of cytoplasmic protein-bearing granules
of polymorphonuclear leukocytes. Lab. Invest. 24:229-236.

11. Beaufay, H., P. Jacques, P. Baudhuin, O. Z. Sellinger, J. Berthet, and C. de Duve. 1964.
Resolution of mitochondria from rat liver into three distinct populations of cytoplasmic
particles by means of density equilibration in various gradients. Biochem. J. 92:184-295.

12. Hubbard, A. L., and A. C. Cobb. 1972. The enzymatic iodination of the red cell membrane.
J. Cell Biol. 55:390-405.

13. Hubbard, A. L., and Z. A. Cobb. 1975. Externally exposed plasma membrane proteins.
1. Enzymatic iodination of I. L. cells. J. Cell Biol. 64:436-440.

14. Jamieson, J. D. 1976. Secretion of lysosomal enzymes induced by immune complexes and
complement. J. Lymph. Res. 14:695-697.

15. Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrastructural stain for
proteins in polysaccharide gels shows regional variation in cerebrational fluid proteins.
Science (Wash. DC). 211:1437-1438.

16. Stoffler, G., T. Stein, and D. Wallach. 1971. Electrophoretic analysis of the major
polypeptides of the human erythrocyte membrane. Biochemistry. 10:2602-2616.

17. Bretz, U., and M. Baggioili. 1974. Biochemical and morphological characterization of azuro-
philic and specific granules of human neutrophil polymorphonuclear leukocytes. J. Cell Biol.
63:251-269.

18. Hoyer, H. W., E. C. Ullman, and J. J. Connolly. 1969. Microfilaments and microtubules in calcium
regulated secretory granules of rabbit parotid gland. Isolation, subdivision, and characterization of
membrane and content proteins before and after degranulation. J. Cell Biol. 76:323-340.