Isolation and Properties of Phagocytic Vesicles from Polymorphonuclear Leukocytes

THOMAS P. STOSSEL, THOMAS D. POLLARD, ROBERT J. MASON, and MARTHA VAUGHAN

From the Molecular Disease Branch and Laboratory of Biochemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT A method for the isolation of intact phagocytic vesicles from guinea pig peritoneal-exudate granulocytes and human peripheral-blood leukocytes is presented. After leukocytes ingested the particles of a stable emulsion of paraffin oil, the uningested emulsion was washed away and the cells were homogenized. The homogenate was placed in the middle of a three-step discontinuous sucrose gradient and centrifuged for 1 hr at 100,000 *g*. The phagocytic vesicles, containing the low density paraffin-oil particles, were simultaneously washed and collected by floatation, while the other organelles, chiefly granules, sedimented through the lower wash layer, and the particle-free supernatant remained in the middle of the gradient.

Emulsion particles stained with Oil Red O were employed to assay the rate of phagocytosis and to mark the location of the particles in subcellular fractions. The dye was extracted from washed cells or cell fractions with dioxane and colorimetrically quantified. The purity of phagocytic vesicles obtained by this method was assessed by electron microscopy, chemical analysis, and assay of enzyme composition. Granule-associated enzymes, acid phosphatase, alkaline phosphatase, β-glucuronidase, and peroxidase were present in the phagocytic vesicles and originated from the granules. Cyanide-resistant NADH (reduced form of diphosphopyridine nucleotide) oxidase was also found. Enzymes associated with the vesicles exhibited latency to Triton X–100.

Uptake of particles and the transfer of total protein and phospholipid into phagocytic vesicles occurred simultaneously Accumulation of acid and alkaline phosphatase in the vesicles continued until phagocytosis ceased. Peroxidase, NADH oxidase, and β-glucuronidase activities in the phagocytic vesicles, on the other hand, were maximal by 30 min and increased little thereafter even when phagocytosis was still going on.

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INTRODUCTION

When a polymorphonuclear leukocyte (PMN)[1] engulfs a particle, the ingested material is encased within a vesicle derived from the plasma membrane (1). Microscopic and histochemical investigations have indicated that cytoplasmic granules, similar to liver lysosomes and rich in hydrolytic enzymes and bactericidal proteins (2), appear to fuse with the phagocytic vesicle (PV) and discharge their contents therein (1, 3–6). For want of a technique to isolate the PV, biochemical documentation of these events has been lacking, although in their original characterization of PMN granules, Cohn and Hirsch demonstrated release of granule enzymes into a supernatant fraction of PMN homogenates after phagocytosis (7). This finding implied that a change in granule enzyme distribution had occurred secondary to phagocytosis.

We have devised a procedure for the isolation of intact PVs in high purity and yield by means of a single centrifugation. PMNs were allowed to ingest the inert particles of emulsified paraffin oil. Paraffin oil, because of its indigestibility, is a suitable substrate for enzymatic studies of phagocytosis (8). The low density of these particles allowed the PV to be collected by floatation after homogenization of the cells that had ingested them. Emulsion particles stained with Oil Red O were used to assay the rate of phagocytosis and to locate the particles in subcellular fractions. In this paper we describe the method of isolation and the morphologic, chemical, and enzymatic composition of the PVs. With this technique we have been able to confirm biochemically that enzymes are transferred from granules to PVs and to obtain some data on the kinetics of enzyme translocation.

[1] *Abbreviations used in this paper:* PMN, polymorphonuclear leukocyte; POE, paraffin oil; POPP, p-bis 2-(5-phenyloxozoyl); PPO, 2,5-diphenyl oxazole; PV, phagocytic vesicle.

METHODS

Isolation of PMN. PMNs were obtained from two sources. Suspensions containing over 90% PMN were collected from guinea pig peritoneal exudates as previously described (9). Blood drawn from normal humans into plastic syringes was allowed to sediment for 45 min at 0°C in plastic centrifuge tubes after mixing with 0.2 volumes of a solution (pH 7.4) containing NaCl, 7 mg/ml, dextran (mol wt 500,000, Pharmacia, Uppsala, Sweden), 50 mg/ml, sodium citrate, 20 mg/ml, or sodium heparin (without preservative, Hynson, Wescott & Dunning, Inc., Baltimore, Md.), 2 mg/ml. Leukocytes were collected from the supernatant fluid by centrifugation at 100 *g* for 10 min. After one wash with 50 volumes of 15 mM phosphate buffer, pH 7.4, in 0.15 M NaCl, erythrocytes were lysed by addition of distilled water. Isotonicity was restored 60 sec later by adding NaCl, 36 mg/ml. The leukocytes were sedimented at 100 *g* (15 min) and suspended in Krebs-Ringer phosphate medium, pH 7.4. The final cell suspension consisted of approximately 85% PMN, 15% mononuclear cells, and contained a small number of erythrocytes and platelets.

Preparation of particles for phagocytosis. Emulsions of paraffin oil were prepared by sonic dispersion of 1 ml of heavy paraffin oil (Fisher Scientific Company, Pittsburgh, Pa.) in 3 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing bovine serum albumin (Fraction V from bovine serum, Lot D26304, Armour Pharmaceutical Company, Kankakee, Ill.), 20 mg/ml, for 90 sec at a setting of 2.8 amp (Branson Instruments, Co., Stamford, Conn.). The resulting stable emulsion contained droplets of paraffin oil (POE) approximately 0.5–5 μ in diameter (average about 3 μ). For studies in which rate of particle uptake was measured, Oil Red O (Allied Chemical Corporation, Morristown, N. J.) was dissolved in the paraffin oil by heating at 100° for 1 hr. After standing for several days at room temperature, the oil was centrifuged before use to remove undissolved dye.

Incubations. PMN suspended in Krebs-Ringer phosphate medium, pH 7.4, were incubated at 37°C with gentle shaking in siliconized glass Erlenmyer flasks. At zero time, 0.1 volume of freshly prepared POE, approximately 10^9 particles per milliliters final concentration, was added. The same volume of the albumin solution used to prepare the POE was added to paired control incubations. The final albumin concentration was 2 mg/ml. Incubations were terminated by the addition of 1 volume of ice-cold Krebs-Ringer phosphate buffer, pH 7.4. After centrifugation at 250 *g* for 10 min at 4°C, samples of the supernatant fluid (designated "medium") were saved. The cell pellets were washed three times with 20 volumes of ice-cold Krebs-Ringer phosphate buffer, pH 7.4, with centrifugations at 250 *g* for 10 min.

Rate of phagocytosis. To assay the rate of phagocytosis, samples of 0.5 ml were removed from the incubation mixtures at desired times and added to 6 ml of ice-cold 0.15 M NaCl containing 1 mM *N*-ethyl-maleimide in siliconized 15 ml glass centrifuge tubes. After centrifugation for 15 min at 150 *g*, the supernatant fluid was discarded, the cells were washed four times with similar medium, and the tubes drained by inversion. Oil Red O was extracted from the PMN as described below.

Oxidation of radioactive glucose by PMN. The procedures for incubation of PMN and collection and detection of $^{14}CO_2$ were exactly as previously described (9). The substrates, ^{14}C-1-glucose (47.8 mCi/mmole), ^{14}C-6-glucose (44.9 mCi/mmole), and ^{14}C-U-glucose (216 mCi/mmole), were purchased from New England Nuclear, Boston, Mass.

Preparation and fractionation of PMN homogenates. The washed PMN pellets were suspended in freshly prepared ice-cold 0.34 M sucrose buffered with 1 mM potassium phosphate, pH 7.0 (buffered sucrose). The same volume of buffered sucrose, approximately 5 ml/ml packed cells, was added to control cells and to cells which had ingested POE. Sodium heparin, 500 U/ml, final concentration, was added to the cell suspensions in sucrose to decrease the "stickiness" of subcellular particles and to enhance the efficiency of homogenization (10). The suspensions were homogenized in the cold room (2°C) in a chilled Dounce homogenizer with a tight pestle (Kontes Glass Co, Vineland, N. J.) until complete cell disruption was evident by phase contrast microscopy (about 25 strokes). In fact, when the cell suspension containing heparin was mixed vigorously, a viscous gel resulted which liquified during the homogenization. Liquification coincided with complete cell breakage observed microscopically.

Samples of homogenate were layered over an equal volume of buffered 0.4 M sucrose in cellulose nitrate tubes (Beckman Instrument Inc, Palo Alto, Calif.) and overlayered with an equal volume of buffered 0.25 M sucrose (Fig. 1). The tubes were centrifuged for 1 hr at 100,000 *g* in a Beckman Model L-1 ultracentrifuge (40 or 40.3 rotor). By this procedure, both floating and sedimenting fractions were washed during the separation procedure, while the particle-free supernatant fluid remained in the middle of the tube. The packed floating layer, the PVs, was lifted out of the tube with a spatula and dispersed gently in buffered 0.25 M sucrose with a Ten Broek homogenizer. The upper wash layer was aspirated from control preparation and saved for analysis (control "PV fraction"). The wash layers of the phagocytosis preparation were discarded. The supernatant fluid was aspirated and diluted to a sucrose concentration of 0.25 M by addition of 1 mM potassium phosphate, pH 7.0. The pellet was dispersed in buffered 0.25 M sucrose as described for the floating fraction. Fractions were immediately frozen in a dry-ice-ethanol bath and stored at −70°C until analyzed.

Chemical determinations. Protein was assayed by the method of Lowry, Rosebrough, Farr, and Randall, with bovine serum albumin as the standard (11). Turbidity of fractions containing POE was removed by filtering the final solution through 0.45 μ Millipore filters (Millipore Corp, Bedford, Mass.) Nucleic acids were precipitated, washed, delipidated, extracted into hot trichloroacetic acid, and measured in the extracts as described by Schneider (12). Herring sperm DNA (Calbiochem, Los Angeles, Calif.) and yeast RNA (Worthington Biochemical Corp, Freehold, N. J.) were used as standards. Glycogen was measured as previously described (9).

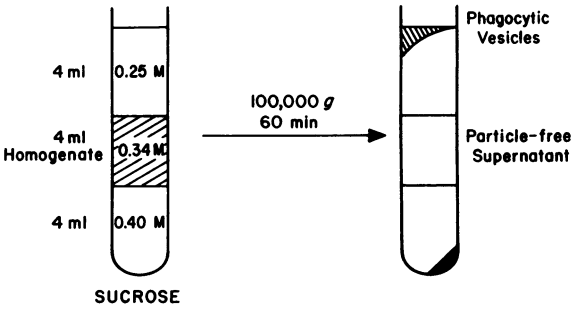


FIGURE 1 Procedure for the collection of phagocytic vesicles.

Lipids were extracted from the homogenates and subcellular fractions, partitioned, and washed by the procedure of Folch, Lees, and Sloan-Stanley (13). Lipid phosphorus was determined by the method of Bartlett (14). For the measurement of cholesterol, 10^5 dpm ^{3}H-cholesterol, about 1 pg (New England Nuclear) was added to lipid extracts in chloroform-methanol, 2:1. The extracts were evaporated to dryness under a stream of nitrogen and dissolved in 2 ml of ethanol. NaOH, 0.1 ml, 250 mg/ml, was added, and saponification was allowed to proceed at 100°C for 60 min. The nonsaponifiable lipids were extracted into hexane which was then evaporated to dryness under nitrogen. The samples were taken up in benzene and applied to 250 μ silica G plates (Analtech, Inc., Wilmington, Del.), prestained with 0.1% rhodamine-6-G in formic acid-acetic acid, 1:2 (v/v). The chromatogram was developed with benzene-ethyl acetate, 5:1, and areas corresponding to the known cholesterol (Applied Science Laboratories Inc., State College, Pa.) were eluted with benzene. Samples, after evaporation of benzene, were assayed for radioactivity in toluene containing 2,5-diphenyl-oxazole (PPO), 4 mg/ml, *p*-bis 2-(5-phenyloxazolyl) benzene (POPOP), 0.05 mg/ml, with a Packard Tri-Carb scintillation spectrometer with 30% efficiency. Cholesterol concentrations were determined by gas-liquid chromatography (Barber-Coleman Chromatography Div., Rockford, Ill.) with a 3% SE-30 column on Gas Chrom Q support (Applied Science Laboratories Inc.) at 250°C. The amounts measured were corrected for recovered radioactivity (72–98%). The paraffin oil did not interfere with the measurements of nucleic acids, phospholipid, or cholesterol.

Oil Red O was extracted from washed cell pellets or subcellular fractions for at least 1 hr at room temperature into *p*-dioxane (Matheson, Coleman & Bell, Norwood, Ohio). The extracts were centrifuged at 1000 *g* for 15 min and the optical density determined with a Coleman colorimeter (Coleman Instruments). The molar extinction coefficient, determined for Oil Red O in *p*-dioxane at 524 mμ (absorption peak) with a 1 cm light path, was 2.29×10^4 and was used in calculations. The absorbancy was not altered by paraffin oil or by water and was directly proportional to dye concentration. Although particle uptake was expressed in terms of micromoles of Oil Red O ingested in these experiments, such data can also be conveniently presented as the amount of paraffin oil taken up if the optical density of the oil containing the dye is determined.

Enzyme assays. For the measurement of activities of β-glucuronidase, acid phosphatase (to *p*-nitrophenyl phosphate and β-glycerophosphate), alkaline phosphatase, peroxidase and catalase, the assay conditions, analytical methods, and calculations of results were exactly as described by Michell, Karnovsky, and Karnovsky (15). Succinic dehydrogenase activity was measured by the method of King (16). NADH oxidase was assayed immediately after preparation of homogenates or cell fractions. The assay system contained 0.05 M Tris buffer, pH 7.5, KCN, 1 mM, 0.1% Triton X-100, 0.05–0.8 mg enzyme protein, and NADH, 1.5 mM, in a total volume of 0.2 ml. Incubations were for 5 min at 30°C. Samples of 0.03 ml of incubation mixture were removed at zero time and at 5 min into 3 ml of 0.1 N NaOH. This solution was centrifuged, and fluorescence was determined with a microfluorophotometer (American Instrument Co, Inc, Silver Spring, Md.). The change in NADH concentration was computed from a standard curve. Boiled enzyme blanks were used as controls. All enzymes with the exception of catalase, which demonstrates first order kinetics with respect to time, were assayed under conditions such that activity was constant with time and proportional to protein concentrations.

Preparation of samples for electron microscopy. The cells and the PV fraction (which was carefully lifted intact from the surface of the gradient) were fixed in 3% glutaraldehyde, 1 mM $CaCl_2$, 0.01 M phosphate buffer, pH 7.2, overnight at 4°C. After washing with buffer, they were treated with 1% osmium tetroxide in 0.01 M phosphate buffer, pH 7.2, at 22°C for 1 hr, dehydrated with ethanol, passed through two changes of propylene oxide, and embedded in Epon. Silver-gray thin sections were stained 30 min with 1% aqueous uranyl acetate followed by 15 min with lead citrate, and examined in a Siemens Elmiskop 101 (Siemens Corp. Iselin. N. J.).

Uptake of albumin-^{125}I and inulin-^{14}C. Albumin-^{125}I, 108 mCi/mmole, was prepared by the method of MacFarlane (17) using the same albumin that was employed for preparation of POE. Incubations with POE prepared from albumin-^{125}I contained 10^8 dpm/10 ml of final volume. The incubations, washing, and fractionations were conducted as described above. Samples of homogenates and subcellular fractions were assayed for gamma radioactivity with 80% efficiency with a Packard Auto-Gamma spectrometer (Packard Instrument Co.).

Inulin-^{14}C (inulin-carboxyl-^{14}C, 0.3 μCi/mg, New England Nuclear) was added to PMN suspensions just before the addition of POE. Samples (0.015 ml) of homogenates or subcellular fractions were dissolved in 1 ml NCS solubilizer (Amersham/Searle Corp, Arlington Heights, Ill.) to which 15 ml of toluene containing PPO (4 mg/ml) and POPOP (0.05 mg/ml) was added. Radioactivity was determined with the Packard Tri-Carb scintillation spectrometer.

TABLE I

Effect of Phagocytosis of Paraffin Oil Emulsion on Glucose Oxidation by Polymorphonuclear Leukocytes

	Guinea pig granulocytes		Human leukocytes	
Substrate	Control	Phagocytosis	Control	Phagocytosis
	$^{14}CO_2$: *cpm/mg cell protein/10^5 added cpm per 60 min*			
^{14}C-1-glucose	1,249 ±111*	12,734 ±711	281 ±17	959 ±35
^{14}C-6-glucose	26.8 ±0.1	64.0 ±2.9		
^{14}C-U-glucose	1,675 ±20	6,139 ±198		

* The means and the range of values obtained in three experiments are given.

RESULTS

Uptake of POE by granulocytes. Guinea pig and human PMN readily ingested POE, as shown morphologically by electron microscopy and chemically by the Oil Red O assay. Particle uptake quantified with the Oil Red O assay was proportional to cell concentration and could be saturated with respect to POE concentration. *N*-ethyl-maleimide, 1 mM, the concentration used to prevent further phagocytosis during the washing of cells before extraction, totally inhibited particle uptake. As shown in Table I, phagocytosis of POE stimulated oxidation of glucose through the hexose-monophosphate pathway to the same degree as has been reported to be associated with uptake of other types of particles (18).

Morphology of granulocytes ingesting POE. Electron micrographs of guinea pig PMN which had ingested POE showed that the cells contained numerous membrane-bounded vesicles (Fig. 2). These vesicles contained one or more spherical particles with amorphous electron-dense peripheries and clear centers, which were thought to represent the albumin-coated lipid droplets from which the lipid was extracted during preparation for electron microscopy. The vesicle membrane surrounding these particles was clearly trilaminar and identical in appearance and dimensions to the plasma membrane (Fig. 3). Many vesicles, particularly those containing multiple droplets, also contained granules or material similar to the granule matrix in consistency and density (Fig. 2B).

Morphology of isolated PV. PV fractions prepared for electron microscopy consisted primarily of membrane-bounded vesicles and some associated granules (Fig. 4). These isolated vesicles were identical to the vesicles seen in intact cells after POE ingestion. The inner spherical particles with their dense peripheries and lucent centers were surrounded by a trilaminar mem- appeared to be granule matrix, were frequently incorpobrane which was frequently intact, as shown in Fig. 5A, but often was broken by small gaps. Granules, or what rated within the vesicle membranes (Fig. 5B). Some granules were outside the vesicle membrane, but many of these external granules appeared tightly adherent to the outside of the PV (Fig. 5C).

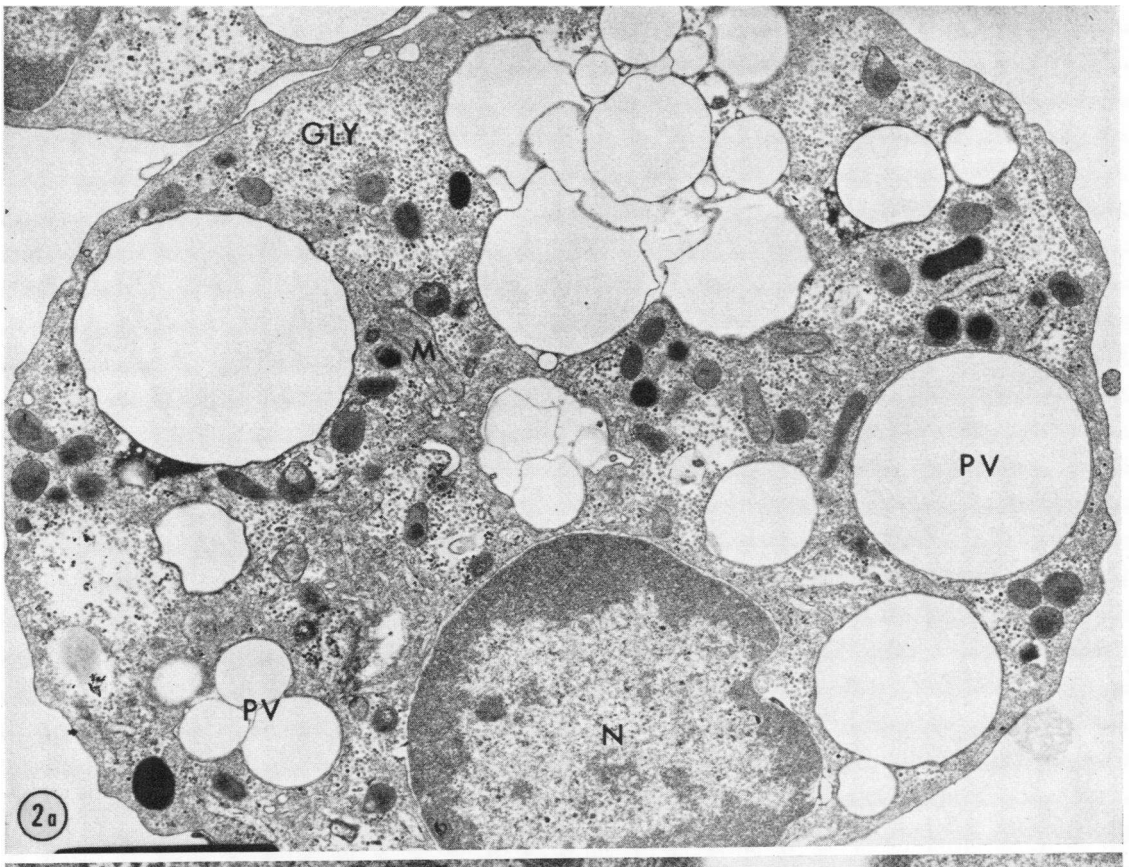
There was essentially no contamination of these isolated PV fractions by mitochondria, glycogen particles, nuclei, or other cytoplasmic matter.

Distribution of chemical components among subcellular fractions. Homogenates prepared from phagocytosing cells invariably contained less protein than homogenates derived from paired incubations of resting cells ($P < 0.01$). This difference presumably represents, at least in part, loss of cellular protein into the incubation medium during phagocytosis (19, 20), although the albumin in the medium precluded accurate measurements to prove this point. Table II shows that the concentrations of phospholipid, DNA, and RNA in PMN homogenates, expressed per milligrams of homogenate protein, did not change due to phagocytosis. Thus, the loss of phospholipid, DNA, and RNA secondary to phagocytosis was of the same magnitude as the loss of protein. The studies of PMN enzymes described below gave similar results. The most likely explanation for the decrease in all cell components is that some PMN are broken during the phagocytic process.

Essentially all of the cell-associated Oil Red O was found in the PV fraction (Table III). This fraction was virtually free of contamination by nucleic acids. Much of the nucleic acid was found in the supernatant fraction, probably due to the efficiency of homogenization afforded by heparin which appeared to have facilitated the destruction of nuclei as well as of the cells. When heparin was omitted from the homogenizing medium in two experiments, 88 and 89% of the DNA was in the pellet. The use of heparin also increased the fraction of cell protein that was recovered in the fluid fraction but did not alter the distribution of enzymes described below. No glycogen was found in the PV fraction.

Less than 0.1% of inulin-^{14}C was taken into the PMN during phagocytosis of POE, and this was principally found in the supernatant fraction. Thus, the incubation medium was effectively excluded from the PMN during phagocytosis of POE. When the POE was prepared with albumin-^{125}I, however, it was found that 4.9% of the radioactivity in the incubation medium was taken up by the cells during 45 min of phagocytosis. During the same period, the PMN ingested 17.5% of the Oil Red O, i.e., 17.5% of the POE. These results suggest that 28% of the albumin in the medium was bound to the POE. Of the radioactive albumin incorporated into the cells, 91% was associated with the PV, 8% with the supernatant, and 1% with the pellet. From these values and the amounts of protein measured in the PV and originally present in the incubation medium, it could be

FIGURE 2 Electron micrographs of thin sections of guinea pig PMN which have ingested droplets of paraffin oil emulsion for 30 min. The mineral oil has been extracted during the preparation for electron microscopy so the droplets appear as clear holes in the cell. The droplets are contained in membrane bounded phagocytic vesicles (PV). Some PV contain more than one droplet and/or some dense material thought to be granule contents (arrows). Also shown are the nucleus (N), mitochondria (M), glycogen (GLY). Magnification: (A) × 13,000; (B) × 47,000.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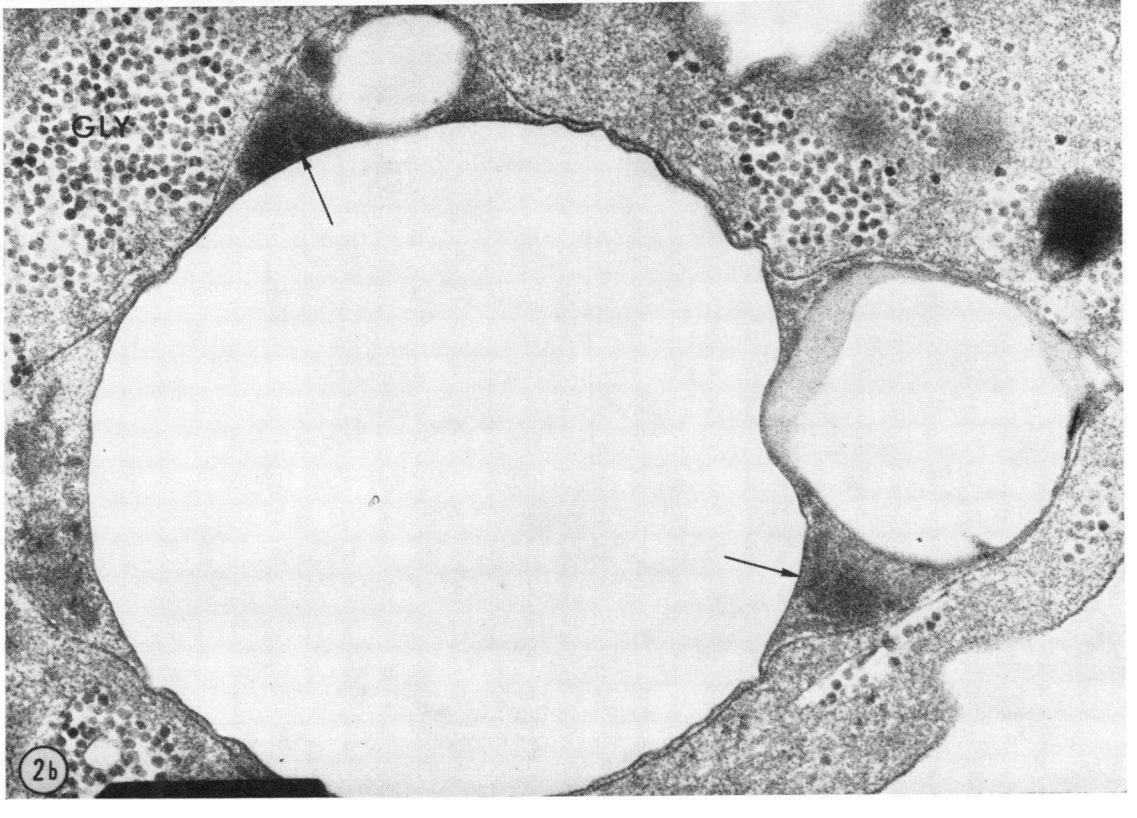
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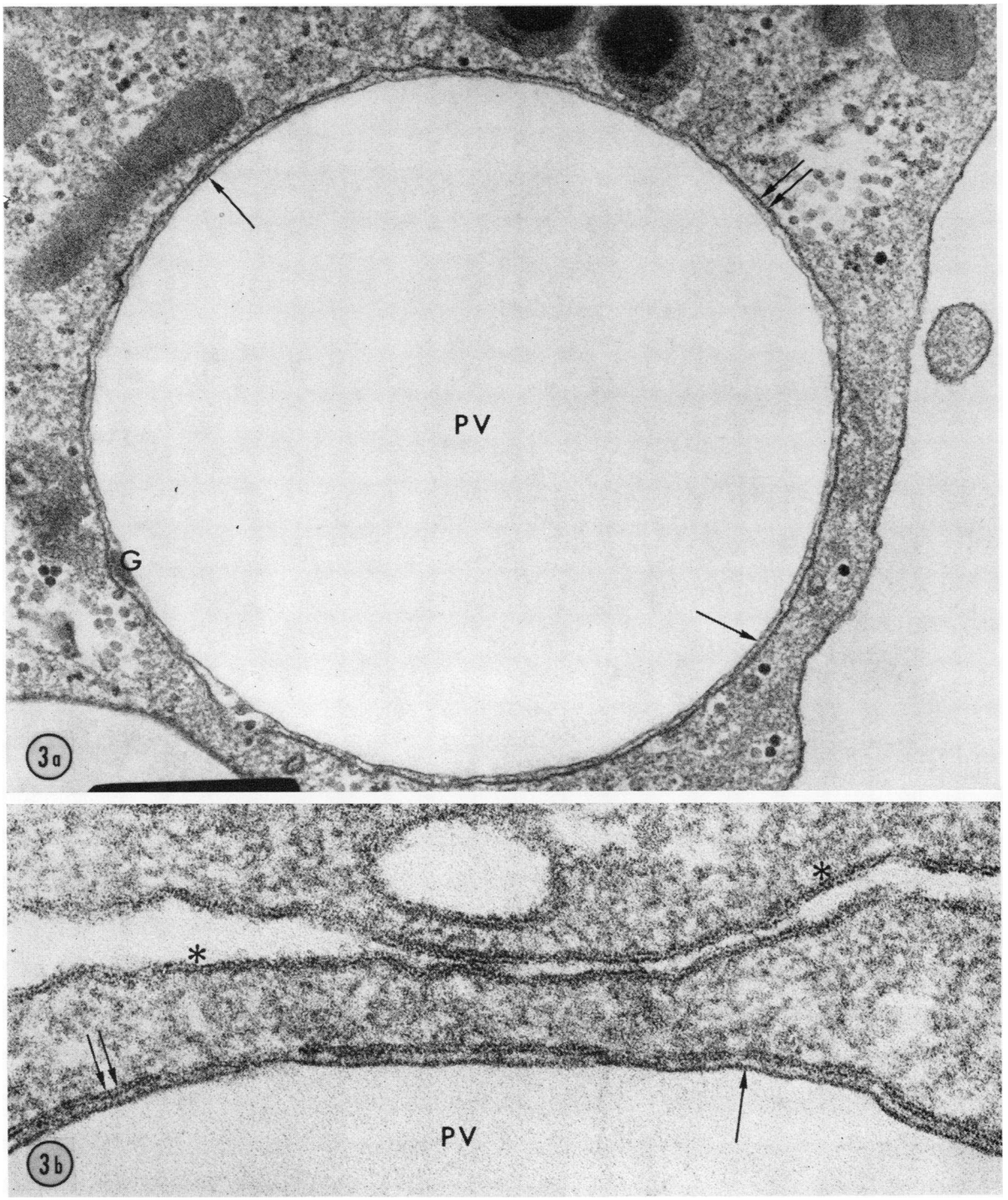

FIGURE 3 Same preparations as Fig. 2. (A) This electron micrograph shows a PV containing a droplet. The center of the droplet is clear and is surrounded by a thin dense rim (arrow). This dense material coating the droplet is thought to be the albumin which coats the paraffin oil in the emulsion. The PV membrane (double arrows) encloses the droplet and some dense material which may be granule contents (G). (B) At high magnification, the PV membrane appears trilaminar (double arrows) and is identical in appearance to the plasma membranes of the two adjacent cells shown (*). The albumin coat on the paraffin oil droplet is seen as a single dense line (arrow). Magnification: (A) × 55,000; (B) × 180,000.

calculated that at least 27% of the protein in the PV was albumin.

The appearance of protein and phospholipid in the PV fraction was matched by a decrease in these components in the pellet fraction, indicating a shift from the pellets to the vesicles. The average protein-phospholipid weight ratio of guinea pig PV was 2.90 compared with 8.68 for whole homogenates. Correction for the

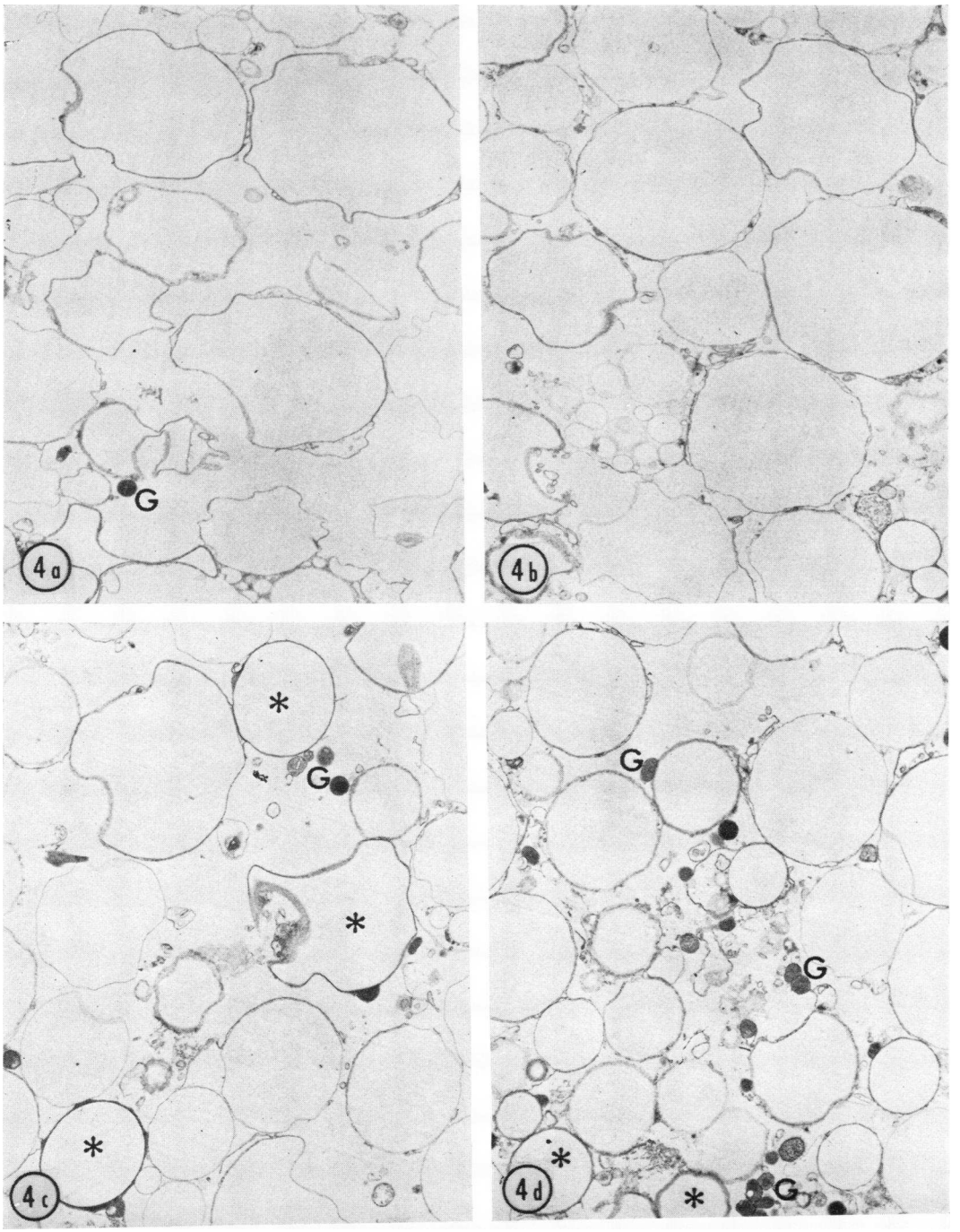

FIGURE 4 A series of electron micrographs of thin sections of a guinea pig PV fraction taken at several levels, proceeding from (A) at the top to (D) at the bottom of the floating fraction. The number of granules (G) and of PV's containing dense, granule-like material (*) increase toward the bottom of the fraction (C and D). Magnification: × 9,500.

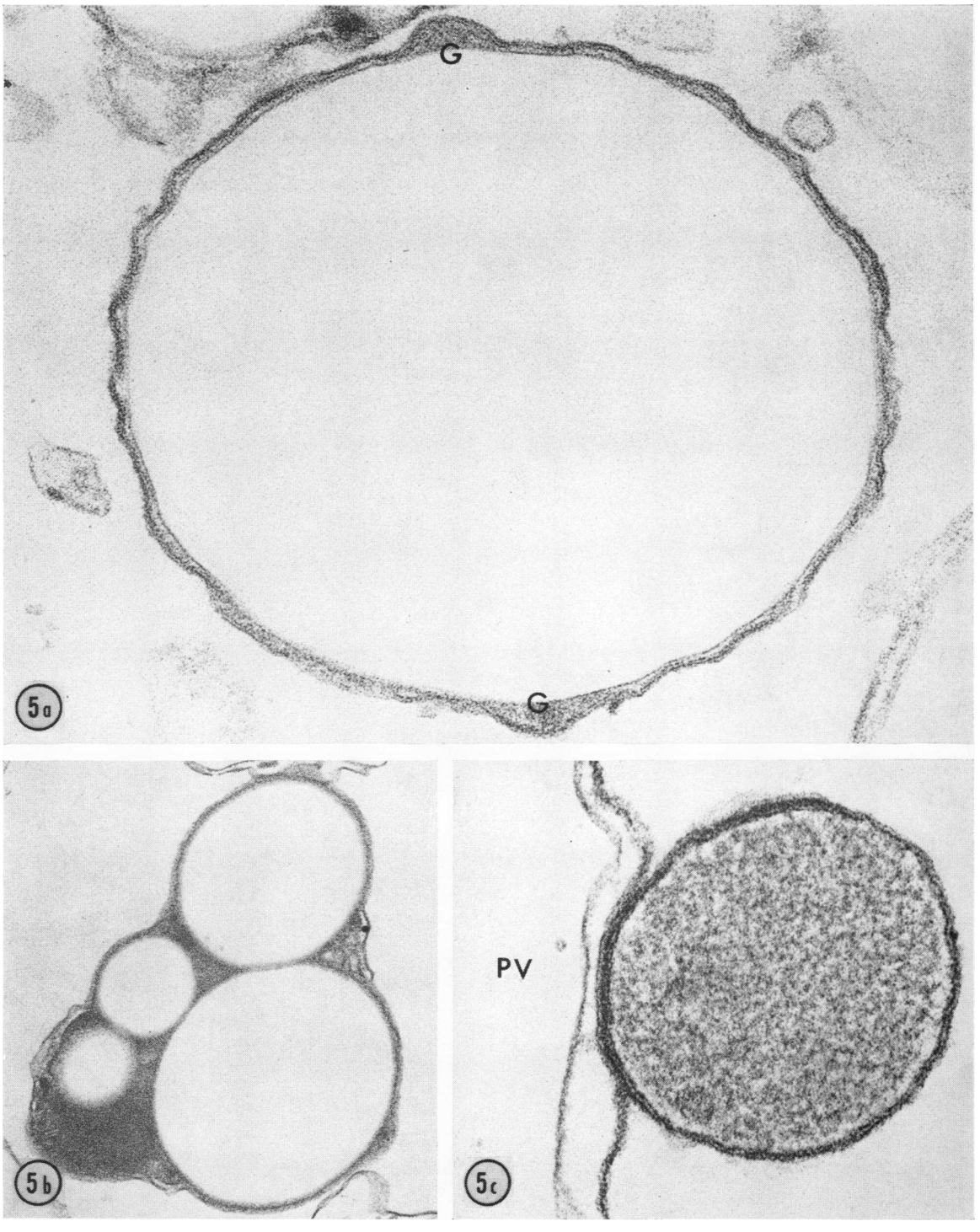

FIGURE 5 Same preparation as in Fig. 4. (A) This electron micrograph of a single isolated PV shows the trilaminar membrane surrounding the lipid-extracted paraffin oil droplet. The appearance of the membrane and the inclusion of presumed granule material (G) are identical to the PV within the cells, as shown in Fig. 3. (B) This PV with multiple mineral oil droplets and extensive dense material, similar in appearance to granule matrix, resembles some of the PVs seen in intact cells, as shown in Fig. 2(B). (C) A number of the granules in the PV fraction are closely apposed to the PV. Here the trilaminar membranes of a granule and a PV appear to be fused, forming a pentalaminar structure similar to a "tight junction." Magnification: (A) × 95,000; (B) × 45,000; (C) × 140,000.

TABLE II

Chemical Composition and Enzyme Specific Activities of Guinea Pig Granulocyte Homogenates

	Control	Phagocytosis	No. of preparations
Protein	7.80 ±0.62*	7.11 ±1.25	6
Phospholipid	4.59 ±0.12	4.63 ±0.06	3
DNA	168.50 ±12.2	165.30 ±13.1	5
RNA	32.0 ±1.1	34.40 ±7.9	4
Oil Red 0	0	19.50 ±1.5	4
Acid phosphatase	0.197 ±0.008	0.205 ±0.009	6
Alkaline phosphatase	0.235 ±0.013	0.215 ±0.012	6
β-glucuronidase	3.90 ±0.12	3.87 ±0.13	5
Peroxidase	0.340 ±0.087	0.318 ±0.075	3
NADH oxidase	2.21 ±0.07	2.33 ±0.08	3
Catalase	0.117 ±0.023	0.150 ±0.018	3

* Means ±SEM are given.

The homogenates were prepared from granulocytes incubated for 30 min with or without paraffin oil emulsion. Protein content is expressed in milligrams per milliliters of homogenate; phospholipid in micrograms lipid phosphorus per milligram of protein; DNA and RNA in micrograms per milligram of protein; Oil Red 0 in micromoles per milligram of protein; acid phosphatase and alkaline phosphatase in micromoles of p-nitrophenol released per minute/milligram of protein; β-glucuronidase in nanomoles of product produced per minute/milligram of protein; peroxidase in micromoles of tetraguaiacol synthesized per minute/milligram of protein; catalase in units per milligram of protein; NADH oxidase in nanomoles of NADH oxidized per minute/milligram of protein.

albumin content of the PV changed the protein-phospholipid weight ratio of the PV to 2.12. The molar cholesterol-phospholipid ratio of two preparations of guinea pig PV was reproducibly 0.69, which was slightly higher than the ratio of the whole PMN homogenates (0.50) or of the pellets (0.54).

Intracellular distribution of PMN enzymes. The specific activities of the enzymes assayed in PMN homogenates were not changed by phagocytosis of POE (Table II). Variable decreases, however, were observed for the total activities of the enzymes due to phagocytosis (up to 16%), which could be accounted for by the appearance of the enzymes in the extracellular incubation medium (data not shown). These findings are in agreement with those of previous investigations of phagocytosis of other particle types by human granulocytes and by macrophages (19, 20).

The PV contained the granule-associated enzymes, acid phosphatase, alkaline phosphatase, β-glucuronidase, and peroxidase (Table IV). Acid phosphatase activity

TABLE III

Distribution of Chemical Components in Guinea Pig Granulocyte Subcellular Fractions

	Phagocytic vesicles		Supernatant		Pellet		Total recovered		No. of Preparations
	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	
	% of total recovered						%‡		
Protein	0.5 ±0.3*	8.5 ±0.8	63.2 ±0.8	64.5 ±4.8	36.3 ±1.2	27.0 ±7.0	89.3 ±3.3	91.9 ±4.8	6
Phospholipid	0.9 ±0.9	18.0 ±2.7	0	0	99.1 ±1.4	82.0 ±2.4	72.5 ±9.7	73.6 ±7.4	3
DNA	0	0.6 ±0.4	80.2 ±14.3	81.4 ±14.4	19.8 ±13.7	18.0 ±17.0	79.9 ±2.4	75.0 ±2.5	5
RNA	0	1.4 ±0.9	62.4 ±1.3	72.5 ±16.6	37.6 ±2.4	26.1 ±2.4	82.3 ±3.3	86.2 ±4.2	4
Oil Red 0	0	97.3 ±0.2	0	0.1 ±0.1	0	2.6 ±0.3	0	94.6 ±1.3	4

* Means ±SEM are given.

‡ Percentage recovered = $\frac{\text{Amount in vesicle plus fluid plus pellet fractions}}{\text{Amount in whole homogenate}} \times 100.$

The fractions were prepared from granulocytes incubated for 30 min with or without paraffin oil emulsion.

TABLE IV

Distribution of Enzymes in Guinea Pig Granulocyte Subcellular Fractions

	Phagocytic vesicles		Supernatant		Pellet		Total recovered		No. of preparations
	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	
	% of total recovered activity						%		
Acid phosphatase	0.1 ±0.1*	32.5 ±3.7	15.4 ±1.6	14.3 ±1.6	84.5 ±2.0	53.2 ±3.7	92.2 ±8.5	99.3 ±8.5	6
Alkaline phosphatase	0.6 ±0.6	22.9 ±2.4	14.2 ±4.7	18.3 ±1.3	85.2 ±5.4	58.8 ±0.4	85.7 ±7.6	88.7 ±4.5	6
β-glucuronidase	0.7 ±0.6	25.7 ±5.2	19.4 ±6.2	24.7 ±5.4	79.9 ±5.3	49.6 ±6.6	100.8 ±8.8	101.1 ±4.4	5
Peroxidase	0	16.9 ±7.9	16.5 ±9.1	20.9 ±7.8	83.5 ±10.0	62.2 ±10.3	95.6 ±1.0	94.7 ±1.9	3
NADH oxidase	0	23.6 ±0.3	55.3 ±15.8	58.2 ±1.6	44.7 ±16.4	18.2 ±1.3	94.0 ±3.2	129.0 ±16.8	3
Catalase	0	0	95.3 ±11.8	98.0 ±2.2	4.7 ±4.8	2.0 ±2.1	98.3 ±4.5	95.3 ±4.4	3

* Means ±SEM are given.

The data are presented as in Table III.

The fractions were prepared from granulocytes incubated for 30 min with or without paraffin oil emulsion.

in all fractions toward *p*-nitrophenylphosphate was essentially identical to activity toward β-glycerophosphate. Cyanide-resistant NADH-oxidase activity was also detected in the PV. The appearance of these enzymes in the PV fractions was accompanied by a concomitant decrease in pellet activities of the enzymes compared with the pellets obtained from resting cells. Small variable increases in the supernatant activities of β-glucuronidase, peroxidase, and alkaline phosphatase were noted in supernatant fractions from phagocytosing cells (when compared with the fluid fraction from paired control incubations). No increase in supernatant fraction acid phosphatase activity was observed, however. The subcellular distribution of catalase activity was not altered by phagocytosis of POE, and the absence of this soluble enzyme from the PV and near absence from the pellet fractions is further evidence for the completeness of the separation procedure. Essentially no succinic dehydrogenase activity was found in the PV although the total activity in the PMN homogenates was quite low.

The distribution of enzymes of human leukocytes was qualitatively similar to that in the guinea pig exudate PMN although the uptake of POE per total cell protein was less than with guinea pig PMN (Table V). Perhaps this difference was due to contamination of human leukocytes preparations by other cell types, such as platelets, which by being less avidly phagocytic would contribute enzyme activities to the pellet alone.

Latency of PV enzymes. When the acid phosphatase assay was performed in buffered 0.25 M sucrose instead of in the standard hypotonic medium, the measured activity of the PV was doubled by the addition of 0.1% Triton X–100. The latent enzyme content was released by low concentrations (less than 0.01%) of Triton X–100.

Time course studies. As shown in Fig. 6, uptake of POE and accumulation in the PV of protein, alkaline phosphatase, and acid phosphatase proceeded in parallel throughout the incubation period (up to 2 hr in other experiments). The time course of transfer of phospholipid to the PV was similar to that for total protein and the phosphatases (data not shown). In this as in other studies, acid-phosphatase activity was the same whether measured with *p*-nitrophenyl phosphate or β-glycerophosphate as substrate. Most of the transfer of β-glucuronidase, peroxidase, and NADH-oxidase activities on the other hand occurred early, and in the experiment

TABLE V

The Distribution of Components in Human Leukocyte Subcellular Fractions

	Phagocytic vesicles		Supernatant		Pellet		Total recovered		No. of Preparations
	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	
	% of total recovered component						%		
Protein	0	2.6 ±0.6*	59.3 ±0.4	53.8 ±13.1	40.7 ±0.4	43.6 ±13.6	97.8 ±9.5	95.6 ±8.8	4
Acid phosphatase	0	7.1 ±1.2	15.2 ±1.6	18.3 ±1.3	84.8 ±1.6	74.6 ±1.9	94.2 ±5.5	94.1 ±7.8	4
Alkaline phosphatase	0	6.9 ±2.0	12.8 ±2.6	18.9 ±8.7	87.2 ±2.6	74.2 ±7.9	88.1 ±2.2	89.6 ±3.2	4
β-glucuronidase	0	8.9 ±2.1	9.9 ±1.3	11.3 ±4.0	90.1 ±1.5	79.8 ±2.1	93.4 ±5.2	90.8 ±1.2	4
Peroxidase	0	10.4 ±7.1	3.7 ±0.5	4.9 ±1.2	96.3 ±0.5	84.7 ±8.9	77.8 ±7.3	92.9 ±12.3	2

* Means ±SEM are given where more than two preparations were analyzed; otherwise means and ranges are given.

The data are presented as in Table III.

The fractions were prepared from leukocytes incubated for 45 min with or without paraffin oil emulsion.

shown in Fig. 6 had essentially ceased after 15 min despite continued uptake of POE.

When phagocytosis was abruptly terminated and POE removed by washing the PMN with ice-cold buffer, a small amount of additional protein, phospholipid, and enzymes entered the PV fraction during subsequent incubation of the cells without POE (Fig. 7). These findings are presumably a manifestation of at least a brief time lag between formation of new vesicles and transfer of granule components into them. The experiment summarized in Fig. 7B illustrates that cells washed in this manner were viable and capable of further phagocytosis.

DISCUSSION

The method described in this paper for the isolation of PV is simple and rapid, and permits measurement of the rate of phagocytosis. Wetzel and Korn have described a procedure for obtaining PV from *Acanthamoeba castellanii* after phagocytosis of polystyrene spheres, by centrifuging homogenates through a four-step discontinuous sucrose gradient (21). That method has the advantages of the technique reported here, but homogenization of cells containing rigid latex spheres may damage the PV, as discussed below.

The demonstration of transfer of granule enzymes into the PV confirms the hypothesis based on electron microscopic and cytochemical studies that granule enzymes enter the PV during phagocytosis (1, 3–5). These results differ from earlier studies with the rabbit, horse, and human PMN, in which transfer of granule enzymes into the soluble fraction of the homogenized cells was observed after phagocytosis of bacteria, yeast, or polystyrene particles (7, 20, 22). Phagocytosis of POE did not appreciably increase the activity of granule-associated enzymes in the supernatant fraction of the homogenate. It seems probable that the PV containing paraffin oil, because of their deformability, may be less susceptible to breakage than are vesicles containing more rigid particles. The integrity of the isolated PV was documented by the latency of the enzymes contained therein to Triton X–100. Furthermore, since over 90% of the cell-associated albumin-^{125}I was found with the PV, although the albumin could easily be washed

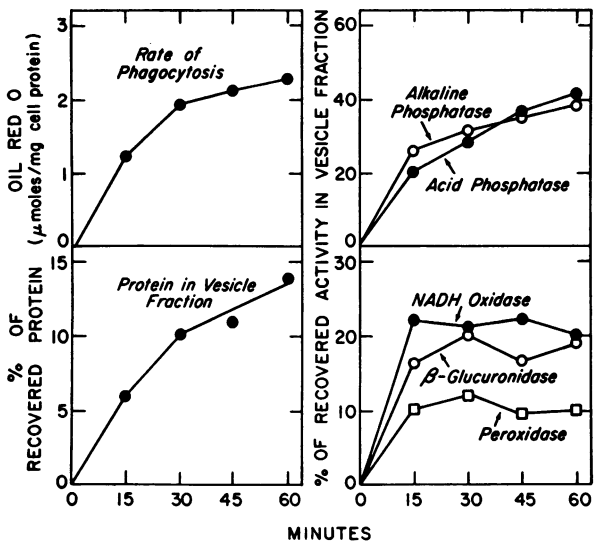

FIGURE 6 Composition of phagocytic vesicles as a function of time of phagocytosis. Guinea pig polymorphonuclear leukocytes were incubated with paraffin oil emulsion in Krebs-Ringer phosphate medium, pH 7.4 at 37°C. Samples were removed at the indicated times and fractions prepared as described in the text. The recoveries of components were not affected by duration of phagocytosis. β-Glycerophosphate and *p*-nitrophenyl phosphate were used as substrates for acid phosphatase and gave identical results.

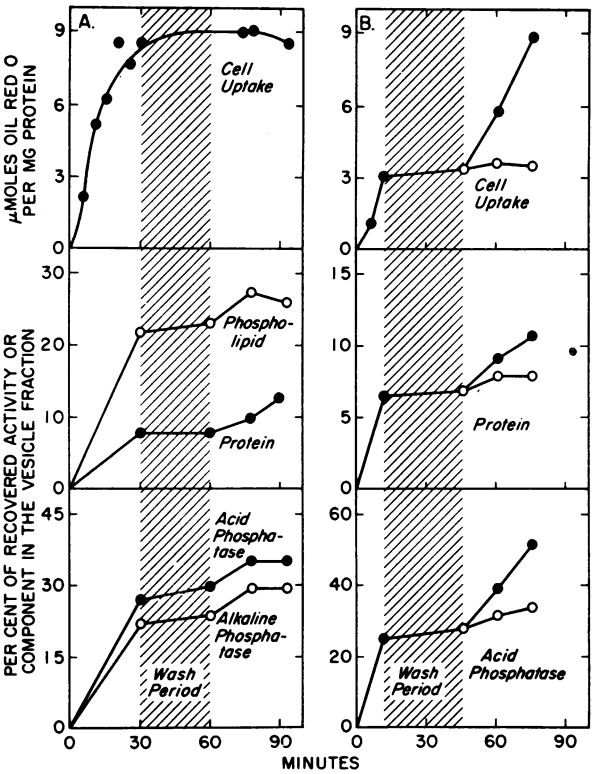

FIGURE 7 The effect of abrupt termination of phagocytosis on composition of phagocytic vesicles. Phagocytosis by guinea pig granulocytes was allowed to proceed in Krebs-Ringer phosphate medium at 37°C for 30 min (Exp. A) or 10 min (Exp. B), at which time the emulsion was removed from the cells by exhaustive washing with ice-cold Krebs-Ringer phosphate medium (centrifugations at 100 *g* for 10 min; total duration of wash period 30 min). In Exp. A, the incubation was then resumed at 37°C in Krebs-Ringer phosphate medium without paraffin oil emulsion. In Exp. B, the PMN suspension was divided into two equal portions. One portion was reincubated at 37°C with paraffin oil emulsion (●) and the other without paraffin oil emulsion (○).

off of the POE by centrifugation in salt solution or by passing the POE over a Sepharose 2B column, we infer that the vesicles were not permeable to albumin. It is likely that much of the reported solubilization of granule enzymes, apparently secondary to phagocytosis, is an artifact resulting from damage to the PV in the course of homogenization and fractionation.

The observed differences between rates of transfer to the PV of acid and alkaline phosphatases and those of the other enzymes presumably arise from the fact that these enzymes originate in different types of granules. The granules are distinguishable morphologically and separable on the basis of differing density. Analysis of granules from guinea pig PMN has shown the β-glucuronidase and peroxidase are contained in the most dense granules, while alkaline phosphatase and acid phosphatase are found respectively in granules of intermediate and low density (15).[2] The differences between the percentage of peroxidase and of β-glucuronidase appearing in the PV could result if the population of dense granules that interacts with the PV contains these enzymes in relative proportions that are different from those in the bulk of the granules of this type.

The distribution of cyanide–resistant NADH oxidase differs from that of the granule-associated enzymes discussed above (25). In homogenates of both resting and phagocytosing cells, about half of the total activity (which was the same in both types of homogenates) was in the supernatant fluid fraction. NADH-oxidase activity was present in the PV isolated from both guinea pig and human PMN, and in these homogenates the percentage of total activity recovered in the pellet was decreased relative to that in homogenates from the resting cells. Since no catalase (a soluble enzyme) or succinic dehydrogenase (a mitochondrial enzyme) was found in the PV, and the NADH-oxidase activity exhibited latency, it seems unlikely that its presence in the PV fraction resulted from artifactual adsorption or association with PV in the course of fractionation of homogenates. It has been suggested that cyanide–resistant NADH oxidase may play a part in the reactions through which hydrogen peroxide is generated during phagocytosis (25). Perhaps it is the portion of the enzyme that is transferred to the PV that is specifically involved.

All of our findings are consistent with the view that particle uptake and granule interaction with the PV are closely integrated events. Once the formation of PV has been completed, little further degranulation occurs, i.e., the mere presence of PV in the cytoplasm is not sufficient to induce granule fusion and enzyme transfer. We infer that the process of fusion with granules is an integral part of or dependent on the molecular events associated with vesicle formation. Alternatively, a regulatory mechanism may limit the enzyme content of the PV.

[2] A similar distribution of enzyme activities was noted among fractions of granules separated from rabbit PMN (23, 24). An acid phosphatase which was associated with slowly sedimenting particles from the rabbit cells, however, hydrolyzed p-nitrophenyl phosphate much more readily than it did β-glycerophosphate, whereas the enzyme from guinea pig cells exhibited little preference (15). In our experiments, acid phosphatase activity in whole homogenates or in fractions was the same with both substrates.

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