KINETICS OF FUSION OF THE CYTOPLASMIC GRANULES

WITH PHAGOCYTIC VACUOLES IN HUMAN

POLYMORPHONUCLEAR LEUKOCYTES

Biochemical and Morphological Studies

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ABSTRACT

This study on human neutrophils was conducted to measure the kinetics of degranulation of the different cytoplasmic granules into phagocytic vacuoles, and to relate the timing of these events to the burst of respiration that accompanies phagocytosis by these cells. Purified neutrophils were incubated with latex particles opsonized with human immunoglobulin (Ig)G, and phagocytosis was stopped at timed intervals. The cells were examined by electron microscopy to document the sequence of degranulation of the cytoplasmic granules. The azurophil granules and lysosomes were identified by histochemical staining for peroxidase and acid phosphatase, respectively. Phagocytic vacuoles were separated from cell homogenates by floatation on sucrose gradients and assayed for contained lactoferrin, myeloperoxidase, and acid hydrolases. The conclusions drawn from the biochemical and morphological studies were in agreement and indicated: particle uptake and vacuole closure can be completed within 20 s; both the specific and azurophil granules fuse with the phagocytic vacuole much earlier than is generally appreciated, with half-saturation times of 39 s (99% confidence limits, 15–72); oxygen consumption has kinetics similar to those of the fusion of these granules with the phagosome; degranulation of the acid hydrolases β-glucuronidase, N-acetyl-β-glucosaminidase (biochemical assays), and acid phosphatase (biochemical assay and electron microscopic cytochemistry) have kinetics of degranulation that are similar to each other but totally different from and much slower than that of myeloperoxidase with half-saturation times of between 354 and 682 s (99% confidence limits, 246–883). This suggests that the acid hydrolases are not co-located with myeloperoxidase in the azurophil granule but are contained in distinct lysosomes, or "tertiary granules."

Phagocytosis by polymorphonuclear leukocytes is accomplished by an invagination of the plasma membrane which surrounds the engulfed particle, finally forming the limiting wall of the phagocytic

vacuole (8, 41). Phagocytosis is associated with a burst of oxygen consumption which is not due to mitochondrial respiration (32). This respiratory burst is important for the killing of certain organisms, whereas others are killed under anaerobic conditions (27). The killed organism is then digested (11).

Cytoplasmic granules contain various microbicidal substances and digestive enzymes which they release into the lumen of the vacuole (18, 26, 41) by fusion of their membranes with those of the vacuole wall (41).

It has been thought, on the basis of morphological (3-5) and subcellular fractionation studies (1, 7, 14, 24), that there are two main types of granule, the specific granule containing lactoferrin and some of the lysozyme, and the azurophil granule containing myeloperoxidase, the remainder of the lysozyme, and also the acid hydrolases. These two granules have been described as degranulating at different times after phagocytosis, with the specific granules releasing their contents first, within the 1st min, followed by the azurophils after about 3 min (2).

The kinetics of oxygen consumption by human neutrophils after the phagocytosis of latex particles coated with human immunoglobulin (Ig)G have recently been investigated (33). In this system it was found that latex particle uptake is very rapid, being largely complete within a minute, and that oxygen consumption demonstrates very similar kinetics after a lag phase of ~25 s. The reason for this delay between particle uptake and oxygen consumption has not been explained.

The present study was conducted to examine the timing of degranulation of the various granules into the phagocytic vacuole and to try to relate the kinetics of these processes to those of oxygen consumption, and to the biology of the cell as a whole. Human neutrophils were mixed with IgG-coated latex particles and rapidly stirred in a chamber. Phagocytosis and degranulation were stopped at timed intervals and evaluated both by electron microscopy and by analysis of the contents of granules that became associated with phagosomes and were separated from whole cell homogenates by floatation on sucrose gradients.

MATERIALS AND METHODS

Isolation of Cells

Neutrophils were isolated from human buffy coat residues. Each buffy coat (150 ml) was mixed with sodium chloride (300

ml of 0.15 mol/liter) containing sodium phosphate buffer (0.01 mol/liter, pH 7.4), Dextran 500 (50 ml of 10% by weight in 0.15 mol/liter NaCl, Sigma Chemical Co.) and heparin (5 IU/ml preservative free, Paynes & Byrne Ltd., Greenford, England). Erythrocytes were sedimented for 45 min, and then each 40 ml of the supernatant plasma was layered on 10 ml of ficoll/sodium metrizoate (Ficollpaque, Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and centrifuged at 400 g for 20 min. The neutrophils in the pellet were freed of contaminating erythrocytes by hemolysis with distilled water (20 ml) for 45 s before isotonicity was restored with an equal volume of hypertonic sodium chloride (0.3 mol/liter containing 10 IU/ml heparin). The cell suspension was centrifuged at 400 g for 5 min, and the neutrophils were then resuspended at a concentration of 1×10^8 cells/ml in RPMI 1640 medium (Flow Labs, Irvine, Scotland) containing heparin (5 IU/ml). The purity of neutrophils in these cell preparations is about 97% (34).

Biochemical Studies

ISOLATION OF PHAGOCYTIC VACUOLES: The major steps in the experimental protocol are outlined in Fig. 1. The neutrophil suspension (2.0 ml containing 2×10^8 cells) was rapidly stirred with a magnetic stirring rod in a thermostatically controlled (37°C) chamber above an oxygen electrode (33), and the temperature was allowed to equilibrate for 2 min. We then introduced into the chamber 2×10^{10} latex particles (0.81 μm diameter) coated with human IgG (33). Phagocytosis was stopped at varying intervals by adding this suspension to 50 ml of cold (0°C) Hanks' balanced salt solution containing tetrasodium EDTA (1 mmol/liter, pH 7.4, BDH Chemicals Ltd., Poole, England). The mixture was centrifuged at 75 g for 10 min at 4°C, and the pellet then suspended in 50 ml of 11.2% (by weight) sucrose and centrifuged again at 75 g for 10 min at 4°C. The
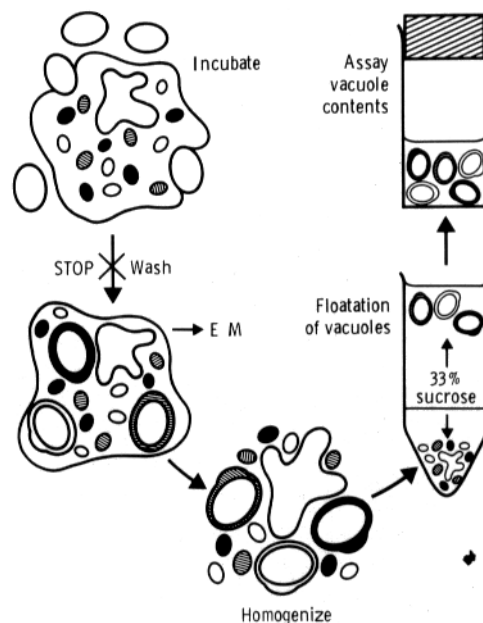

FIGURE 1 Schematic representation of the major steps in the experimental procedure for the isolation of phagocytic vacuoles. *EM*, electron microscopy.

pellet was then homogenized in 5.0 ml of 11.2% sucrose in a 7.0-ml Dounce homogenizer (Kontes Co., Vineland, N.J.) with 100 strokes of a tight-fitting (B) pestle. This homogenate was then mixed with 12.0 ml of 60% sucrose and divided into two aliquots. One aliquot (2.0 ml) was retained for determinations of the contents of the homogenate. Phagosomes were isolated from the remaining 15.0 ml. It was placed at the base of a 50-ml Sorvall centrifuge tube (DuPont Instruments-Sorvall, DuPont Co., New-town, Conn.), overlaid with 20.0 ml of 33% and then 5.0 ml of 11.2% sucrose, and centrifuged at 14,500 rpm for 20 min at 4°C in a Sorvall SS3 centrifuge with an SS-34 angle head rotor (radius, 4.25 in.; g max, 25,359; g min, 13,426). Latex particles at the interface between the 11.2% and 33% sucrose were then harvested. The concentration of sucrose was determined with an Abbé refractometer, and the sucrose was then diluted to a concentration of 11.2% with cold (4°C) H_2O containing EDTA (1.0 mmol/liter) and heparin (5 IU/ml). The suspension of latex was then centrifuged for 45 min at 4°C in a Sorvall SS3 centrifuge as described above. The pellet was finally suspended in 1.6 ml of 11.2% sucrose. All sucrose solutions contained EDTA (1.0 mmol/liter, pH 7.4) and heparin (5 IU/ml).

ASSAYS OF GRANULE CONTENTS: Myeloperoxidase was measured by a modification of the method of Bretz and Baggiol-ini (7). Just before spectrophotometry, the assay mixture was mixed with 1 ml of methanol and centrifuged for 2 min at 8,000 g in an Eppendorf 3200 centrifuge (Eppendorf Instruments Inc., Palo Alto, Calif.) to remove latex particles. Horseradish peroxi-dase (Sigma Chemical Co., type II 195 units/mg) was used as a standard.

Lactoferrin was measured by Laurell rocket immunoelectro-phoresis (23) with rabbit anti–human lactoferrin antiserum (Behring-Werke A. G., Marburg/Lahn, W. Germany) and hu-man colostrum lactoferrin (Calbiochem-Behring Corp., La Jolla, Calif.) as standard.

β-Glucuronidase, N-acetyl-β-glucosaminidase, lactate dehy-drogenase, and acid phosphatase in the homogenate and isolated vacuoles were assayed fluorometrically (20, 34). 4-Methyl um-belliferyl substrates were obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England.

Lactate dehydrogenase released into the medium was assayed by the rate of disappearance of NADH (nicotinamide adenine dinucleotide, reduced) in the presence of pyruvate (13). Latex particles were counted by phase contrast microscopy in an im-proved Neubauer counting chamber. (Gelman Hawksley Ltd., Northampton, England).

PHAGOCYTOSIS-INDUCED IODINATION OF VACUOLE CONTENTS: An attempt was made to measure the integrity of the phagosomes by determining a latent marker's association with them. Iodinated material was used, radioactivity being incorporated into the cells by phagocytosis in the presence of [125]I (29). Cells and latex were mixed under the standard experimental conditions in the presence of 3.0 μCi of [125]I (The Radiochemical Centre, Amersham, England, Code IMS. 30), which was added together with the latex particles. After incubation for 5 min, phagocytosis was stopped in the standard way, the cells were washed four times in the cold Hanks' balanced salt solution containing EDTA and then homogenized and the phagocytic vacuoles were recovered as described above. For latency studies, 1.0 ml of the vacuole preparations were mixed with 1.0 ml of water and sonicated for 30 s in a sonicator (Polaron Equipment Ltd., Finchley, England), and the latex particles were then sedi-mented by centrifugation at 8,000 g for 2 min in an Eppendorf 3200 centrifuge.

OXYGEN CONSUMPTION: Oxygen consumption was meas-ured with a Clark type of oxygen electrode situated in the base of a closed, thermostatically controlled (37°C) Perspex chamber (Rank Brothers, Bottisham, Cambridge, England) with a poten-tial of 0.8 V across the electrodes. The signal was recorded with a paper chart recorder set at an amplitude of 5 mV and a paper speed of 1 or 3 cm/min. The latex particles were introduced through a vent in the chamber stopper after the temperature had been allowed to equilibrate for 2 min. The oxygen electrode was calibrated with aqueous solutions of known oxygen tension meas-ured with a Corning-EEL pH/blood gas 165 analyzer (Corning Ltd., Halstead, Essex, England), and on each occasion of its use, the oxygen electrode was standardized by the addition of a few crystals of dithionite to water in the chamber, taking the normal oxygen tension in water as 230 nmol/ml at room temperature.

Under the standard incubation conditions, the oxygen within the incubation chamber is exhausted $\sim$1 min after the onset of respiratory burst. Measurements of the lag times before the burst of respiration began and of the initial linear rates of oxygen consumption were made with the usual concentration of cells and latex. For determinations of the kinetics of oxygen consump-tion, the concentrations of both the cells and latex particles were reduced 10-fold to allow measurements of oxygen consumption to proceed for longer time intervals.

Electron Microscopy

Phagocytosis was stopped at timed intervals by the addition of 5.0 ml of ice-cold glutaraldehyde (2.5%) in sodium cacodylate buffer (0.1 mol/liter, pH 7.4) to the incubation chamber. This mixture was then transferred to plastic centrifuge tubes contain-ing a further 10 ml of the fixative. After fixation for 45 min at room temperature, the cells were washed four times with sodium cacodylate (0.1 mol/liter, pH 7.4) containing sucrose (0.25 mol/liter) by resuspension and centrifugation (400 g × 5 min) between changes. A portion of each sample was processed for conventional electron microscopy (19) postfixation in osmium tetroxide, stain-ing en bloc with uranyl acetate (0.25%) in veronal acetate buffer (0.143 mol/liter, pH 7.4), and pelleting in agar before dehydra-tion and embedding in Araldite.

CYTOCHEMICAL STAINING FOR PEROXIDASE ACTIVITY: A portion of each sample was kept overnight at 4°C in the cacodylate sucrose solution, washed once in sucrose (5.0%) buffered at pH 7.4 with Tris (0.05 mol/liter, Sigma Chemical Co.) HCl, and stained for peroxidase by the diaminobenzidine (DAB) method (16). The cells were incubated in the substrate solution, which contained 5.0% sucrose, for 30 min at room temperature, after which they were washed twice in the buffered sucrose and fixed with 1% osmium tetroxide in sodium cacodylate buffer (0.1 mol/liter, pH 7.4) for 1 h. They were then rinsed five times in veronal acetate (0.143 mol/liter, pH 7.4), the third rinse containing 0.25% uranyl acetate. Processing for electron micros-copy was carried out as for the first portion of the cells.

Controls consisted of cells that had been incubated at 85°C for 15 min in the cacodylate sucrose solution before staining for peroxidase activity and of cells that were treated in the same way as the test cells except for exclusion of DAB or H_2O_2 from the incubation solution.

Silver sections were prepared and examined in an AEI Ltd. EM 6B electron microscope without heavy metal staining or after staining for 1 min in lead citrate (31).

CYTOCHEMICAL STAINING FOR ACID PHOSPHATASE ACTIVITY: Phagocytosis was arrested as described for the stud-ies on myeloperoxidase, except that 2% glutaraldehyde was used.

Total fixation time was 22 h, and the cells were then washed twice in sodium cacodylate buffer (0.01 mol/liter, pH 7.4) containing sucrose (0.25 mol/liter) and twice in Tris/maleate buffer (0.01 mol/liter, pH 5.2) containing sucrose (0.25 mol/liter) and dimethyl sulfoxide (10% by volume, Sigma Chemical Co.). The cells were allowed to remain in each washing solution for 5 min at 0°C before centrifugation.

Acid phosphatase was demonstrated by the lead method (15) as modified by Barka and Anderson (6) and Brunk and Ericsson (9). The substrate solution contained sodium β-glycerophosphate (0.008 mol/liter), lead nitrate (0.0024 mol/liter), Tris/maleate (0.01 mol/liter, pH 5.2), sucrose (0.3 mol/liter) and dimethyl sulfoxide (10%). Incubation was carried out at 37°C for 25 min with constant agitation, followed by centrifugation (400 g for 5 min) and two washes in ice-cold saline (0.15 mol/liter). The cells were then postfixed in 1% osmium tetroxide in sodium cacodylate (0.1 mol/liter, pH 7.4) for 1 h at room temperature and washed once with cold saline. Electron microscopy and counting were carried out as described for myeloperoxidase. Control cells were treated in the same manner as the test cells, except that sodium β-glycerophosphate was omitted from the substrate solution.

QUANTITATION OF THE KINETICS OF LATEX PHAGOCYTOSIS AND INTRAVACUOLAR PEROXIDASE ACTIVITY: The rate of phagocytosis was measured by counting the numbers of particles in sections of cells that had been fixed at timed intervals after the addition of the latex particles. Difficulty was experienced in the selection of suitable sections through cells for counting because of the different cross-sectional area of cells sectioned in different planes, the irregular shape of the cells, and the polymorphic nature of the nuclei that occupied varying proportions of the cross-sectional area of the cell. Latex particles were only counted in sections of cells that had been sectioned near their center (as indicated by diameter size) where the nucleus occupied less than approximately half the diameter of the cell. To avoid observer bias, the sections were inserted into the microscope in random order by one operator and counted by another who was unaware of the identity of the sample. We examined 25 cells in each of three different grids at each of the seven different time intervals. In addition, 25 cells from each time interval were selected at random for counts of the proportion of phagocytosed latex particles with associated peroxidase activity.

EXAMINATION OF PHAGOCYTIC VACUOLES: A sample of the phagocytic vacuoles was examined by electron microscopy. After the second centrifugation step, the supernatant fluid was removed and the fixative was gently layered over the pellet of phagocytic vacuoles. After fixation, fragments of the pellet were embedded in Araldite with careful regard to the orientation, enabling accurate sectioning through the cross section of the pellet and thereby facilitating electron microscopic examination of the pellet at different levels.

Expression of Results

To enable direct comparison of the kinetics of association of the granule markers with the latex particles within the same experiment and between experiments, the concentration of the granule markers at each time interval was expressed as a percentage of the final concentration of the markers that became associated with the phagocytic vacuoles. Rather than adopting an arbitrary value for this final concentration of the markers, we derived this final concentration of granule contents as the asymptote from a computerized curve-fitting program devised to get the least squares fit of an equation of the form $Y = C + B/(X$

+ A) (36). This program was first applied to the absolute and specific activity (as a function of the concentration of latex particles in each sample) values of the granule markers in each experiment to derive the separate asymptotic values. The absolute and specific activity of the granule contents at each time point were then expressed as a percentage of this asymptote. To correct for variations among samples, the activity of the granule contents associated with the latex particles was expressed as a percentage of the total activity in the particular homogenate from which they were obtained.

The statistical significance of the differences among the kinetics of the various processes was compared after linear regression analysis of the data, in which the activity of these processes was subjected to a linearizing transformation (log p/100-p) and related to log time. The statistical significance of the differences among the estimated log times at which the half-saturation point was reached (log p/100-p = 0) was examined by comparing the 99% confidence intervals within which this estimated time was calculated to occur (35).

Controls

The various controls employed will be discussed at appropriate points in Results.

RESULTS

Biochemistry

PHAGOCYTOSIS OF LATEX: The accuracy of the method of microscopically counting the latex particles was measured in two ways. Five repeated counts on the same sample gave an SD of 5.73%. Comparisons were also made between microscopic counts and turbidity, which was determined in a Thorp micronephelometer (Particle Data, Inc., Elmhurst, Ill.) on aliquots of the sample diluted 1:40 in water. The correlation between the microscopic count and turbidity in comparisons of 25 samples, five at timed intervals from each of five different experiments, was 0.89 (Student's t value = 10.70).
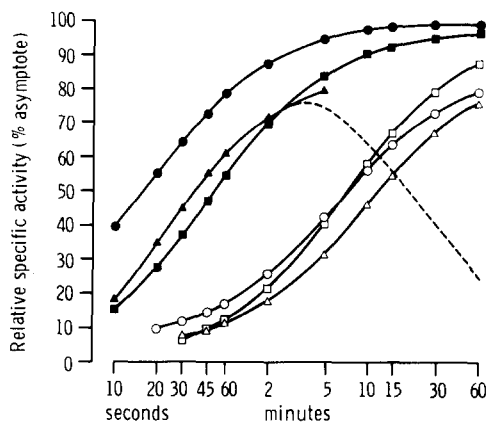
The mean ($\pm$ SE) number of particles taken up by each cell calculated from the number of particles in the homogenates of the three experiments at 30 and 60 min and the original number of cells was 91.5 $\pm$ 8.53 (n = 6). The proportion of the latex in the homogenate that was recovered by floatation from eight samples stopped at 0, 10, 20, 30, 45, 60, 120, and 300 s was 37.6, 27.7, 20.6, 28.1, 48.8, 27.7, 53.5, and 20.0%, respectively (33.0 $\pm$ 7.2 SEM).

The number of particles that remained with the cells when phagocytosis was arrested at 0 s was 14.0% (SE = 5.6, n = 8) of the asymptotic value. These particles were unlikely to have been taken up by phagocytosis, and were probably either adherent to the surface of the cell or incompletely
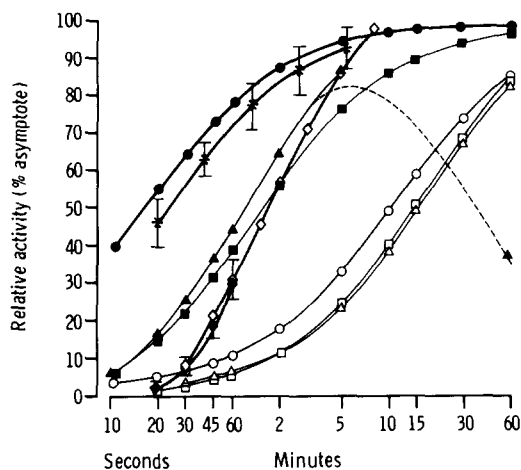
removed by the gentle centrifugation. This 0 s value was therefore regarded as a blank value and subtracted from the numbers of particles recovered at the later time intervals.

The latex particles recovered by floatation at the various time intervals are shown in Figs. 2–4 as a percentage of the asymptote. Uptake was rapid and was largely complete after 90 s.

KINETICS OF DEGRANULATION: The fitted curves of the kinetics of latex uptake and the specific activity of granule contents in relation to the latex concentration are shown in Fig. 2. The fitted curves of the kinetics of the increase in actual activity of the granule contents and latex uptake, the electron microscopic measurement of latex uptake, and measurements of oxygen consumption are shown in Fig. 3. The relationships between the fitted curves and the mean values for the actual activity and specific activity of each of the granule contents are shown in Fig. 4. The concentration of these granule contents in the homogenates and the percentage of these values represented by the asymptotic values in each of the three experiments are shown in Table I. The percentages of the total

FIGURE 3 Time dependence of the separation by floatation of latex particles and granule contents from neutrophil homogenates. Details are as for the legend to Fig. 2, except that the activity of the contents has been expressed in absolute terms rather than as a function of the number of latex particles. Electron microscopic measurement of the kinetics of latex phagocytosis showing the mean ± SEM. ᵼ, counts of the numbers of particles in the plane of the section of 75 cells expressed as a percentage of the derived asymptote. The kinetics of oxygen consumption are shown as the percentage of the derived asymptote at two different cell and particle concentrations. ◇, particle and cell concentrations at one-tenth of standard. ◆, mean ± SEM of six measurements at the standard cell concentration at which oxygen consumption could only be measured for the 1st min. The asymptotic value used at the higher cell concentration was extrapolated from that observed at the lower concentration.

cellular granule contents that became associated with the phagosomes were very similar to the values obtained by Stossel and co-workers (38) and Leffell and Spitznagel (25) in studies in which phagocytic vacuoles were also isolated by floatation from human neutrophils.

The curve-fitting program used in these studies to estimate the asymptote appears to fit accurately the time-course of latex phagocytosis and degranulation. For the numbers of latex particles and myeloperoxidase, the 60-min values are very close to the asymptotic values and at least 75% of this value for the acid hydrolases. Analysis of the kinetics of the association of lactoferrin with latex is complicated by the fact that the initial increase in the concentration of this protein was followed, after 5 min, by a considerable drop in the amount of immunoprecipitable material. Kinetics for this

FIGURE 2 Time dependence of the separation by floatation from neutrophil homogenates, of latex particles (●), lactoferrin (▲), myeloperoxidase (■), N-acetyl-β-glucosaminidase (○), β-glucuronidase (□), and acid phosphatase (△). The activity of these vacuole contents in each sample has been expressed as the specific activity per unit number of latex particles. The original measurements were expressed as the percentage of an asymptotic value derived from a curve-fitting program devised to get the least squares fit of an equation of the form $Y = C + B/(X + A)$ where A, B, and C are constants (36). The curves shown in this figure have been fitted to the averaged normalized values from three experiments (shown in Fig. 4).

material were measured up to the 5 min value, which represented between 80 and 90% of the asymptotic values.

Studies were conducted to ensure that the granule contents had actually become incorporated into phagocytic vacuoles rather than becoming

FIGURE 4 Time dependence of the separation by floatation of latex particles and granule contents from neutrophil homogenates. The mean (± SEM) of the normalized values of the actual $-\frac{\tau}{\perp}-$ and specific $-\frac{\tau}{\perp}-$ activities to which the curves shown in this figure and Figs. 2 and 3 were fitted. Details are described in the legend to Fig. 2.

associated with the latex particles because of nonspecific adsorption to their surface. Comparisons were made between a number of particles that had been phagocytosed and stopped after incubation for 20 min and an equal number of control particles that were mixed (5 strokes in a Dounce homogenizer [Kontes Co.]) with a homogenate of neutrophils and then separated by floatation. Values of the granule contents that became associated with the control particles were, respectively, 6.8, 7.0, 10.4, 0.8, and 0.1% of the concentration of the lactoferrin, myeloperoxidase, β-glucuronidase, N-acetyl-β-glucosaminidase, and acid phosphatase associated with the phagocytosed particles.

An attempt was then made to assess the integrity of the vacuoles by determining the latency of the radioactive iodine incorporated with the vacuoles. A mean of 3.67% (± 0.40 SEM, $n = 3$) of the 4.3 $\times$ 10^7 cpm added to the incubation chamber became associated with the cells, of which 28.5 ± 3.0% floated up with the latex. Sonication released 86.7% (± 1.08 SEM, $n = 6$) of this radioactivity, indicating that 24.8% of the total cell-associated activity was associated with the vacuoles and therefore latent. The recovery of vacuoles in this experiment was 51.8% (± 1.5 SEM, $n = 3$). This suggests that if all the cell-associated radioactivity were confined to the vacuoles, then 47.9% of the vacuoles had the integrity to retain latent iodinated material. The smaller the amount of radioactivity in the whole cell that is confined to the vacuoles, the greater this measure of apparent vacuolar integrity.

The results of three studies indicated that the latex particles are rapidly taken up by the cells. The engulfment of these particles was soon fol-
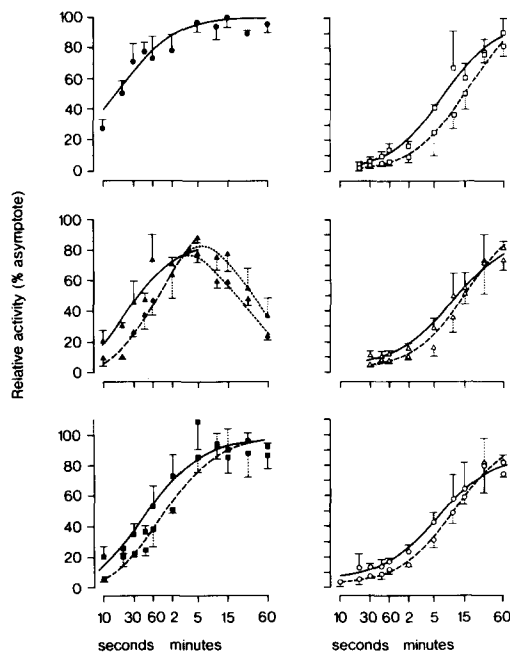
TABLE 1

Total Activity of Granule Contents

Experiment	Units	Latex-associated activity (specific activity)			Absolute activity (percent of activity in homogenate)			Total activity in control homogenate		
		1	2	3	1	2	3	1	2	3
		$U/10^9$ particles						U		
Myeloperoxidase	Purpurogallin Units	0.065	0.046	0.039	4.81	5.38	6.22	5.03	6.78	8.40
Lactoferrin	µg	9.02	2.57	5.45	4.08	4.26	2.31	1,448	1,163	1,035
Acid phosphatase	mU	121.79	93.31	42.94	1.54	1.32	1.71	53,667	53,600	68,067
β-Glucuronidase	mU	2.18	5.49	2.29	3.70	3.26	2.15	6,417	4,350	6,033
N-Acetyl-β-glucosaminidase	mU	5.58	6.00	2.99	2.44	4.98	4.90	987	1,164	1,300
Latex particles recovered	($\times 10^9$)	2.88	7.41	12.80						

The table shows the total activity of granule contents in the neutrophil homogenates and the amount of this total activity that finally becomes associated with the latex particles. This final or asymptotic value has been derived from a computerized curve-fitting program (see text and legend to Fig. 2 for details).

lowed by the accumulation of myeloperoxidase and lactoferrin in the vacuoles. These contents of the azurophil and specific granules, respectively, appeared to have similar rapid rates of degranulation, but the kinetics of association of these two proteins are not directly comparable because the concentration of immunoprecipitable lactoferrin falls after reaching a peak of activity after ~ 5 min, and therefore they were analyzed over different time-courses.

The kinetics of association of the three acid hydrolases were similar to each other (Figs. 2–4) and appeared to commence later and progress much more slowly than those of lactoferrin and myeloperoxidase.

The results of the relationship of the activity of the various vacuole-associated materials with time when compared by linear regression analysis are shown in Fig. 5 and Table II. There was no significant difference between the slopes of the regression lines of the different granule contents. However, there was a significant difference at the 99% confidence level between the times taken for some of the granule contents to reach the half-saturation point (Table II). It took lactoferrin 39.2 s and myeloperoxiodase 39.3 s, whereas β-glucuronidase, N-acetyl-β-glucosaminidase, and acid phosphatase took 419, 354, and 682, respectively, to reach the half-saturation point of the specific activity.

The activity of these granule-associated proteins in the whole cell homogenate changed with time.

To simplify expression and calculation, the figures at each time interval were expressed as a percentage of the value in the control cells at 0 s, and these values were pooled for the times from 15 to 60 min. The mean ($\pm$ SEM, $n = 9$) values of the
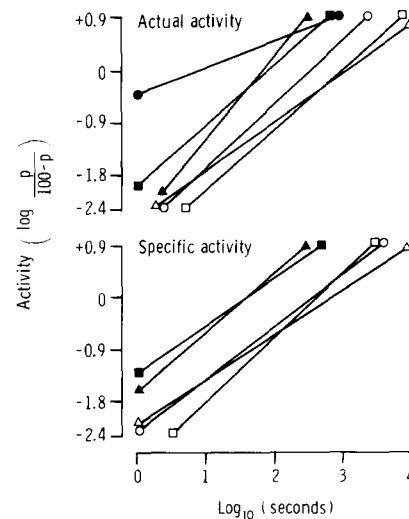

FIGURE 5 Linear regression analysis of the time dependence of the separation by floatation of latex particles and granule contents from neutrophil homogenates. Activity represents the normalized value of the individual results (p) after a linearizing transformation. Data points from which the regression lines were drawn are not shown. Results are expressed in Table II. Symbols have the same significance as in the legend to Fig. 2.

TABLE II

Linear Regression Relationships

	Specific activity				Actual activity			
			Half-saturation time (Log time)	Half-saturation time (De-transformed to seconds)			Half-saturation time (Log time)	Half-saturation time (De-transformed to seconds)
Parameter	Regression coefficient ($\pm$ SEM)	Correlation coefficient	(with 99% confidence intervals)		Regression coefficient ($\pm$ SEM)	Correlation coefficient	(with 99% confidence intervals)	
Latex (27)*					0.460 $\pm$ 0.120	0.607	0.855 (0.039–1.312)	7.16 (1.09–20.53)
Lactoferrin (18)	1.051 $\pm$ 0.119	0.910	1.592 (1.563–1.620)	39.18 (36.56–41.69)	1.425 $\pm$ 0.158	0.905	1.836 (1.788–1.887)	68.54 (61.37–77.10)
Myeloperoxidase (33)	0.829 $\pm$ 0.141	0.726	1.594 (1.202–1.858)	39.26 (15.92–72.11)	1.027 $\pm$ 0.122	0.835	1.928 (1.758–2.078)	84.71 (57.28–119.7)
β-Glucuronidase (25)	1.092 $\pm$ 0.102	0.913	2.622 (2.536–2.711)	418.8 (343.5–514.0)	1.040 $\pm$ 0.083	0.926	2.968 (2.895–3.049)	929.0 (785.1–1012)
N-Acetyl-β-glucosaminidase (31)	0.908 $\pm$ 0.132	0.788	2.549 (2.390–2.729)	354.0 (245.5–535.7)	0.915 $\pm$ 0.065	0.936	2.830 (2.811–2.853)	676.1 (647.1–712.8)
Acid phosphatase (28)	0.774 $\pm$ 0.089	0.862	2.834 (2.735–2.946)	682.3 (543.2–883.0)	0.842 $\pm$ 0.053	0.951	3.051 (3.026–3.097)	1123 (1062–1250)

The table shows the linear regression relationships between the number of latex particles and associated granule contents (as a percentage of the calculated final concentration; $y = p/(100 - p)$ see text for details) and time (x = log time in seconds). See Fig. 5.

* Number of observations in parentheses.

various granule contents over this time period were myeloperoxidase, 83.0 ± 4.8; lactoferrin 54.4 ± 6.6; β-glucuronidase, 61.2 ± 4.0; N-acetyl-β-glucosaminidase, 62.0 ± 4.5; and acid phosphatase, 58.7 ± 9.3%; whereas that of lactate dehydrogenase was 94.4 ± 3.3%. In a few pilot studies these granule contents were assayed in the suspending medium at various timed intervals, and it was found that the decrease in the activity of these markers in the cell homogenates was compatible with their release into the surrounding medium by secretion (25) (data not shown). Stirring the cells in the chamber did not damage them extensively. Lactate dehydrogenase released from the cells was assayed in the medium at 10-min intervals for 1 h: it did not obviously increase with prolongation of the incubation time and was never greater than 3.92% of that in the whole cells (mean ± SEM, 3.25 ± 0.22).

Electron Microscopy

KINETICS OF LATEX UPTAKE: The rate of increase of the numbers of intracellular particles observed in sections of neutrophils by electron microscopy was very similar to that observed by floatation (Fig. 3). No intracellular particles were seen in sections from cells where phagocytosis was stopped at 0 or after 5 s, and few after 10 s. The mean (± SEM) number of particles per cell observed in the plane of the section in each of 75 cells at timed intervals between 20 and 320 s were: 20 s, 3.00 ± 0.42; 40 s, 5.39 ± 0.39; 80 s, 5.87 ± 0.48; 160 s, 5.96 ± 0.48; and 320 s, 7.96 ± 0.46. The significance of the difference among these observations was examined by Student's *t* test, and significant differences were obtained between the values at 20 s and other time intervals ($P < 0.001$) but differences between the other intervals were not significant at the 10% confidence level. An analysis of variance of the regression of count on time was then undertaken to assess whether there was a time-dependent increase in particle uptake. Ratios of variance (F) of 54.5 and 61.6 were obtained for the absolute numbers and their square roots, respectively; such high values indicate that it is highly probable that there is a time-dependent increase in the number of latex particles observed.

EXAMINATION OF ISOLATED PHAGOCYTIC VACUOLES: Electron microscopy of a sample of latex particles, isolated in the standard way after phagocytosis had been arrested 60 s after the addition of the particles to the cells, indicated that phagocytic vacuoles were purified by this technique (Fig. 6). Most of the latex was removed by the organic solvents used in the preparations of the specimens for microscopy, leaving a rim of protein, probably the opsonizing IgG, to identify the outer margins of the particles. This ring of protein was generally surrounded by a membrane, the wall of the phagocytic vacuole, and granule contents could be clearly seen in the lumen between this membrane and the particle. There was no morphological evidence of contamination of the vacuole preparations with other cellular components. To ensure that the apparent morphological purity of these vacuoles did not result from differential layering of the vacuoles and other organelles during the final pelleting, one of these pellets was fixed *in situ*, cross sectioned, and examined at all levels. The vacuoles appeared to be homogeneously distributed and largely free of contaminating organelles throughout. A few isolated granules were present in the lowest 5 μm of the pellet whose total thickness was ~300 μm. Some of these apparently isolated granules may be associated with a phagocytic vacuole outside the plane of the section (Fig. 7).

KINETICS OF DEGRANULATION: Electron microscopic cytochemical examination of cell sections for peroxidase activity (Figs. 8 and 9) confirmed the biochemical evidence of early association of peroxidatic activity with the vacuoles (Table III). Both peroxidase-negative and -positive granules were seen to fuse with the vacuoles very soon after phagocytosis, and sometimes even before complete ingestion of the particle (Fig. 8). In some cases, peroxidase-negative and -positive granules could be seen fusing with vacuoles simultaneously in the same cell (Fig. 10 *a*, *b*).

Electron microscopic cytochemistry for acid phosphatase confirmed the delay in the degranulation of this enzyme. Granules staining for acid phosphatase reaction product were much less numerous than those staining for myeloperoxidase (Fig. 11 *a*), and the appearance of reaction product in the vacuole was much slower (Table III). Counting of ingested latex particles with associated phosphatase activity was done on the phosphorescent screen of the electron microscope. All particles with evident accumulation of reaction product were recorded as acid phosphatase positive. Because of the difficulty of avoiding nonspecific deposition of lead, especially when staining whole cells ([9], Fig. 11 *b*), it is possible that the number of vacuoles recorded as containing the reaction
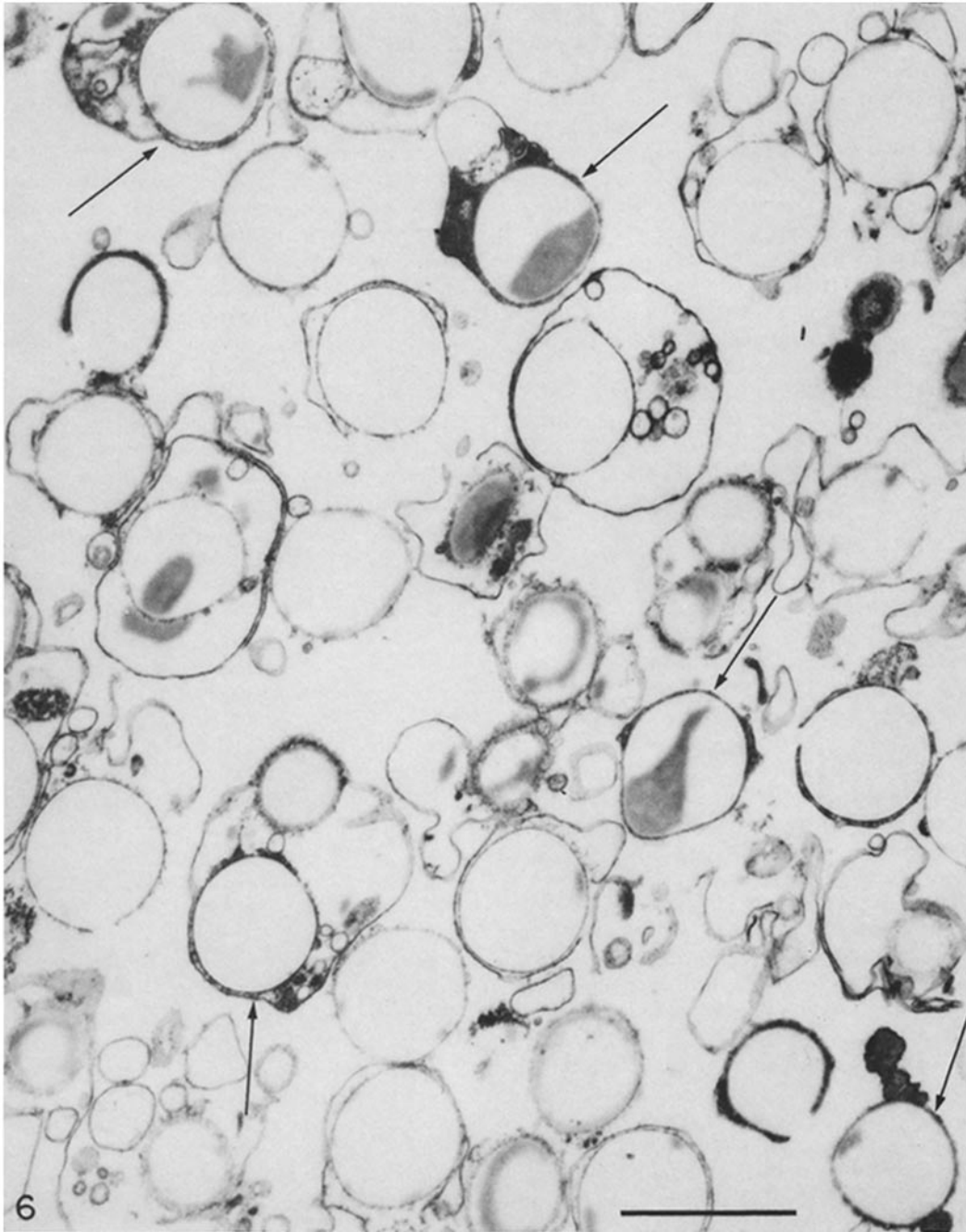
FIGURE 6   Phagocytic vacuoles containing latex particles prepared from polymorphonuclear leukocytes by floatation and fixed in suspension. Phagocytosis was stopped after 60 s. Contents of granules (arrows) can be seen to surround the membrane-bounded particles which have been partially extracted by processing for electron microscopy. Bar, 1.0 μm. × 25,000.

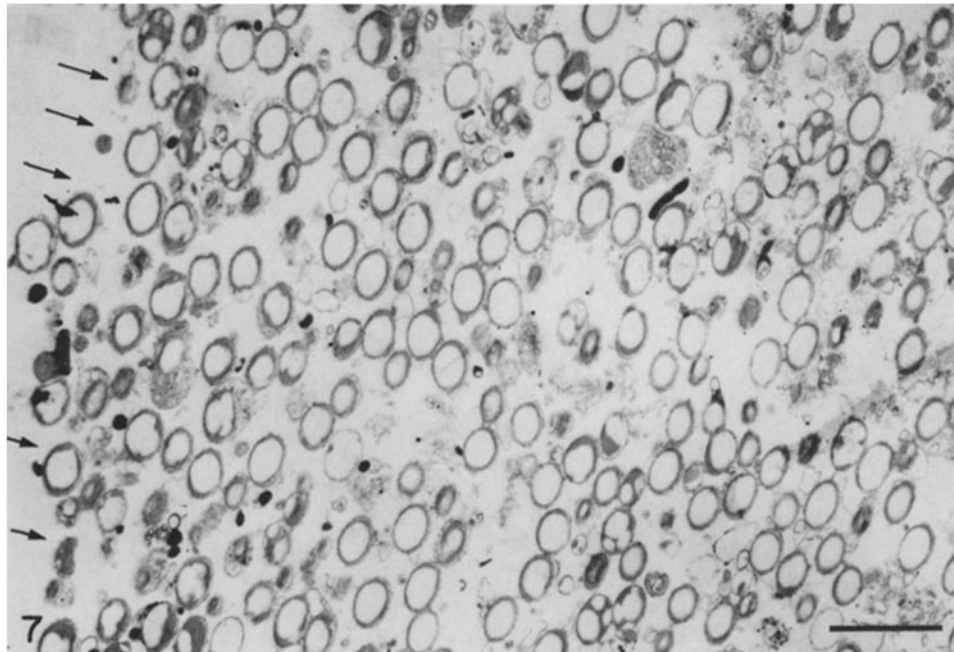FIGURE 7 Phagocytic vacuoles prepared as in Fig. 6 but fixed as a pellet after the second centrifugation. The section was taken through the full depth of the pellet (300 μm), and this micrograph shows the lower surface of the pellet (arrows) that contained the highest proportion of discrete granules. Bar, 2.0 μm. × 7,500.
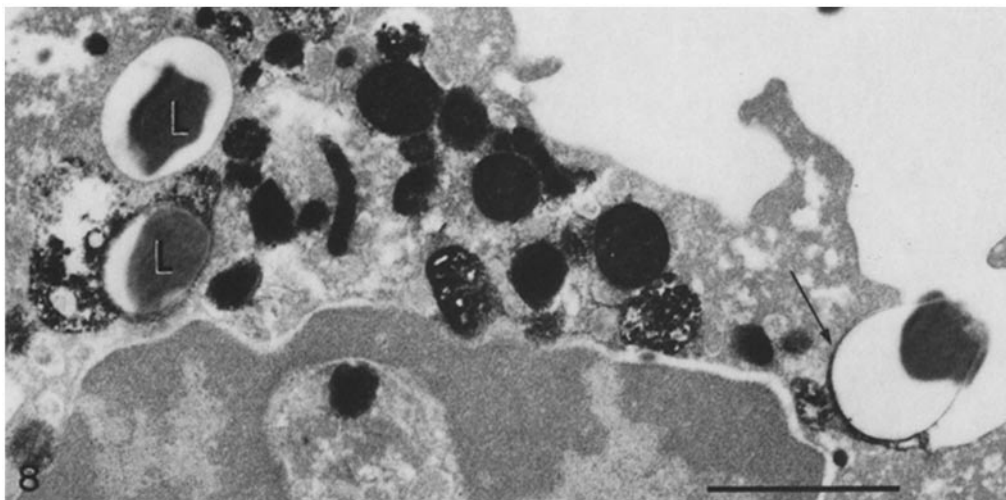

FIGURE 8 Section through a human polymorphonuclear leukocyte (*PMN*) where phagocytosis was stopped 20 s after the addition of IgG-coated latex particles. Internalized particles (*L*) can be seen within closed vacuoles. Cytochemical staining for peroxidase activity demonstrates reaction product in azurophil granules and in phagocytic vacuoles, one of which is still in the process of closing (arrows). Bar, 5.0 μm. × 25,000.
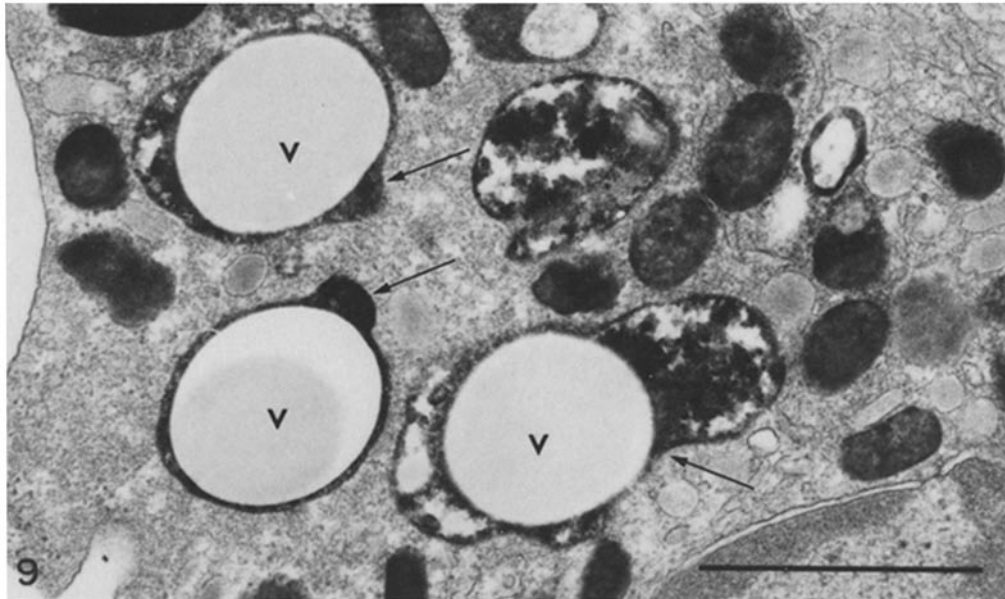
FIGURE 9 A PMN where phagocytosis was stopped 30 s after the addition of latex particles. Peroxidase staining shows reaction product (arrows) within phagocytic vacuoles (V). Bar, 1.0 μm. $\times$ 37,500.

TABLE III

Electron Microscopic Cytochemistry of Neutrophils

	Time	Intracellular particles/cell section	Reaction product positive vacuoles	
			Number/sec-tion	Percent-age of total
	min			
Myeloper-	0.33	3.44 ± 0.86	1.88 ± 0.60	54.7
oxidase	0.66	5.04 ± 0.76	3.44 ± 0.57	68.3
	1.33	5.36 ± 0.50	3.72 ± 0.42	69.4
	2.66	7.08 ± 0.58	5.96 ± 0.52	84.2
	5.33	6.76 ± 0.91	5.88 ± 0.81	87.0
Acid phos-	1	11.44 ± 1.00	0.44 ± 0.13	3.9
phatase	20	23.28 ± 1.50	2.88 ± 0.45	12.4
	60	22.84 ± 2.20	6.00 ± 0.79	26.3

Results show the numbers and percentages of phagocytic vacuoles that contain latex particles and reaction product for myeloperoxidase or acid phosphatase at the various time intervals. Results are expressed as the mean (± SEM) number of particles or vacuoles in the plane of the section of each of the 25 cells examined.

product of acid phosphatase was slightly greater than the true number.

Oxygen Consumption Studies

Under the standard experimental conditions, oxygen consumption commenced 9.3 s (SE = 0.40,

n = 6) after the addition of latex and became linear after 21.1 s (SE = 0.68, n = 6), which is very similar to the initial kinetics of the association of myeloperoxidase and lactoferrin with latex.

It is impossible to measure the prolonged kinetics of respiration of the neutrophils under these precise experimental circumstances because of the rapidity of the exhaustion of the oxygen within the chamber. The kinetics of this process at times later than 90 s after the addition of particles can thus only be determined with lower concentrations of cells. The early kinetics of respiration at different concentrations of cells and particles were thus compared to determine whether it was likely that the kinetics at high cell concentrations could be accurately extrapolated from those observed at the lower cell concentrations. Comparisons were made between the linear rate of oxygen consumption and cell density at concentrations of 2.0, 7.0, and 10.0 $\times$ 10^7 cells/ml. Seven observations were made at each time, and the coefficient of correlation between these two parameters was 0.95 (t = 12.82). This indicates that at least over the early time intervals, the kinetics of oxygen consumption are similar at different cell concentrations, and that those at the lower cell concentrations are at least a fair indication of the probable kinetics at high cell concentrations. The early kinetics at the standard cell concentrations and over a more pro-
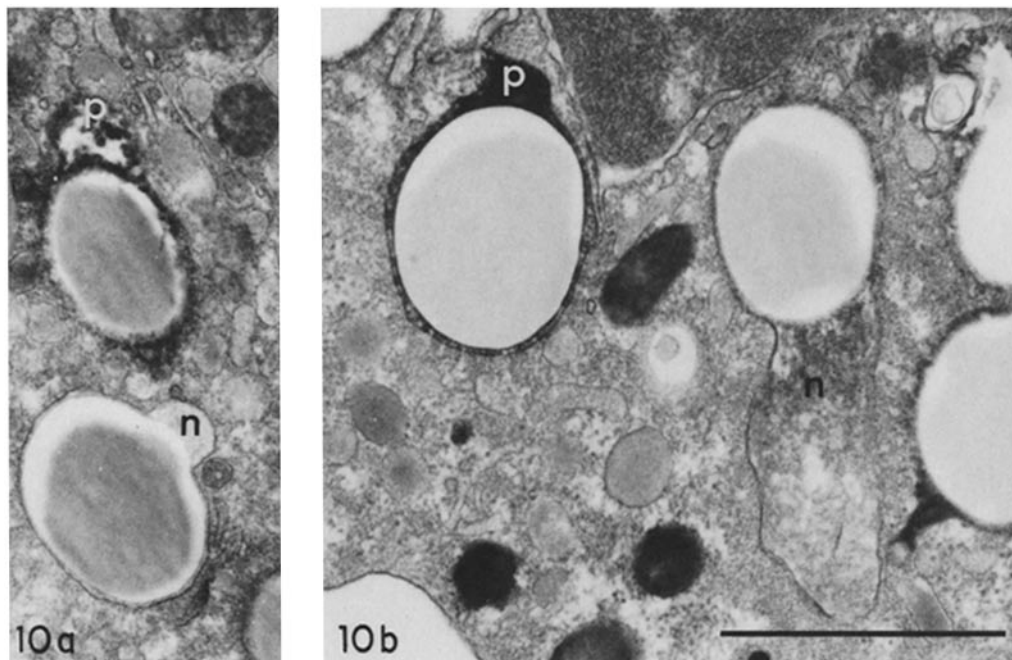
FIGURE 10 (a and b) Section through PMN in which phagocytosis was stopped 30 s after the addition of latex particles. Peroxidase staining indicates the simultaneous fusion of both peroxidase-positive (p) and -negative (n) granules with phagocytic vacuoles in the same cells. Bars, 1.0 μm. × 37,500.

longed period with a 10-fold reduction of the concentration of cells are shown in Fig. 3. The onset and kinetics of degranulation of the specific and azurophil granules appear to be similar to those of oxygen consumption. The maximal linear rate of oxygen consumption at the lower cell concentration was 41.85 ($\pm$ 1.98 SEM, $n = 3$) nmol/10^7 cells per min.

DISCUSSION

The respiratory burst that is associated with phagocytosis results in oxidizing conditions within the phagocytic vacuole (21) that are important for the killing of certain bacteria (27). It is likely that the contents of the cytoplasmic granules form an integral part of the oxidase system responsible for this oxygen consumption, or are important in the metabolism of the products thereof. For example, myeloperoxidase is thought to promote the hydrogen peroxide-dependent halogenation of the ingested bacteria (21), and it definitely seems to interact with the reduced oxygen products as evidenced by a decrease in the amount of iodination (29) and the accumulation of hydrogen peroxide (22) in the cells of myeloperoxidase-deficient sub-

jects. The respiratory burst has recently been shown to be more rapid and brief than was previously thought and is largely complete within 90 s of exposing the cells to latex particles (33). However, the results of previous studies indicated that myeloperoxidase is only released into the vacuole 3 min after phagocytosis (2), which appeared to be an inordinately long time after the respiratory burst, in view of the instability of hydrogen peroxide and oxygen radicals (17). The present studies were thus conducted in an attempt to relate the kinetics of degranulation of the cytoplasmic granules directly to those of the respiratory burst.

It is obviously impossible to quantitate the processes of oxygen consumption and degranulation in each vacuole, or to synchronize the phagocytosis of particles by cells. In these experiments, a large number of cells were mixed with latex particles opsonized with human IgG and rapidly stirred in a chamber—a system that results in the rapid uptake of the particles (33). The phagocytic vacuoles were then isolated by floatation, and the rate of degranulation was measured by determining the rate of association of the granule contents with these vacuoles. The phagocytosis of particles in
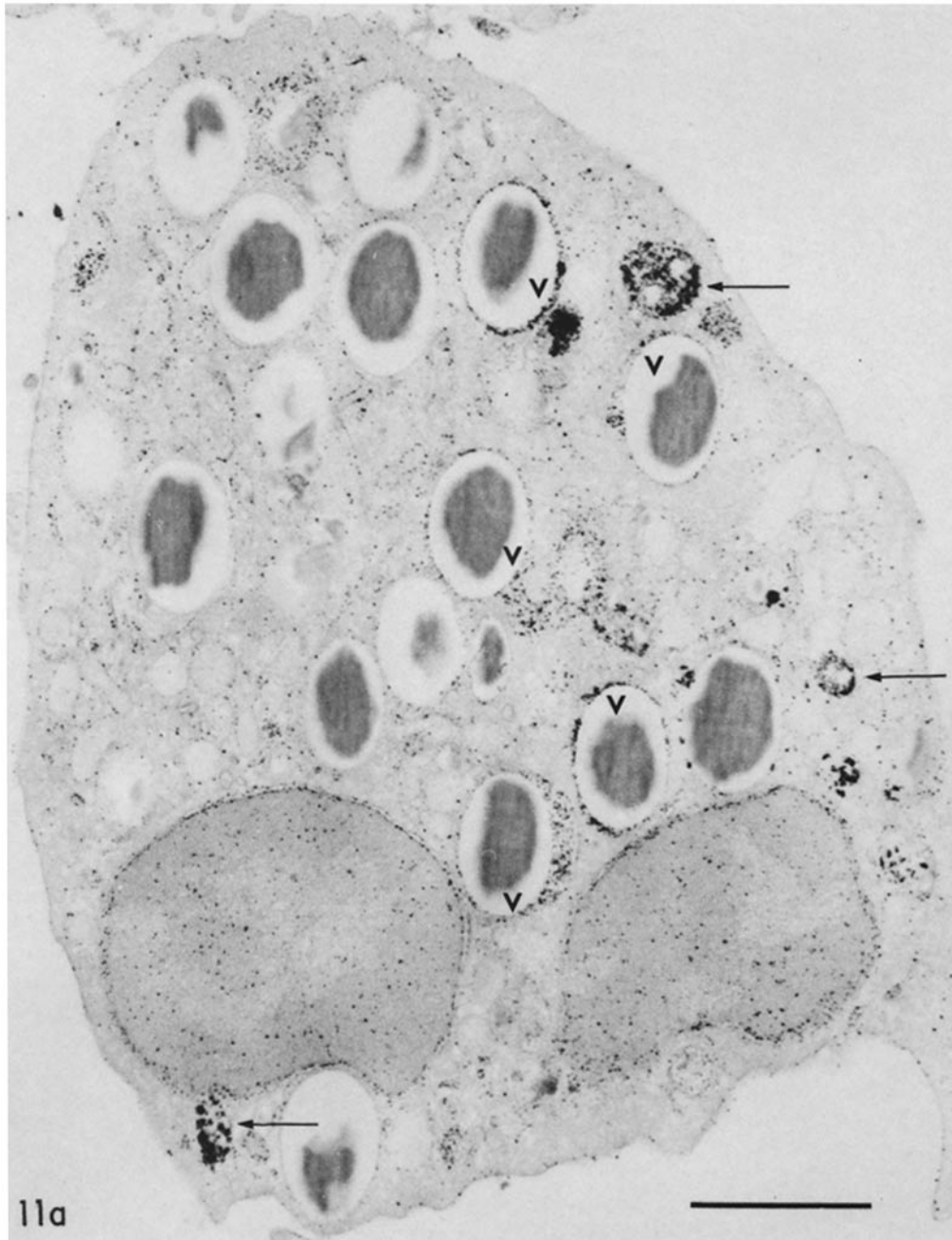
FIGURE 11 Section through a PMN in which phagocytosis was stopped 5 min after the addition of particles to the cells. When the section was stained for acid phosphatase (a), only a relatively small proportion of the granules demonstrated the reaction product (arrows) also observed in only a minority of phagocytic vacuoles (V). Cells incubated in an identical manner in the absence of the substrate β-glycerophosphate (b) show only diffuse nonspecific staining. Bar, 1.0 μm. × 25,000.

11b
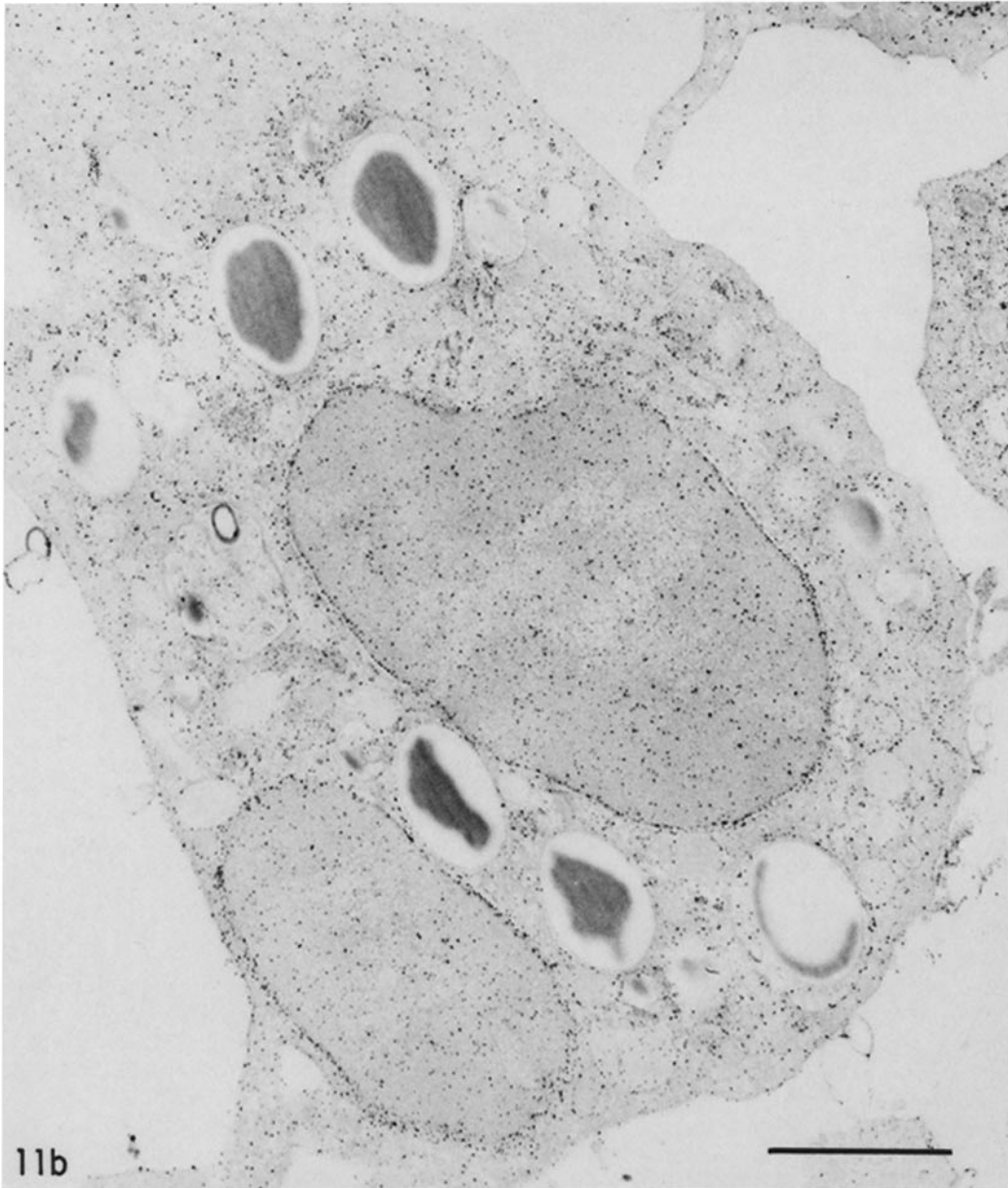
this system was sufficiently fast that a large proportion of the particles were taken up before degranulation commenced. This gave a parameter—the amount of the granule content that had become associated with the latex particles—by which the rate of degranulation could be assessed. Thus, at the earlier time intervals the vacuoles only contained the latex particles, and the quantity of granule contents that floated up with each particle

was much less than at later time intervals when the granule contents had had time to accumulate within the vacuole. The rate of increase of the concentration of granule contents per unit number of latex particles, here described as the specific activity, gives a measure of the rate of association of these contents with the phagocytic vacuole.

The results of these experiments were surprisingly different from the current views on the sub-

ject. Degranulation of the specific granules and azurophil granules has been described as occurring in a sequential manner, with the specific granules degranulating at about 90 s after phagocytosis and preceding the azurophil granules, which released their contents after about 3 min (2). We found that lactoferrin from the specific granule and myeloperoxidase from the azurophil granule became associated with the vacuole much earlier and that we were unable to distinguish between the timing of the association of these two markers with the vacuoles. Degranulation commenced about 20 s after phagocytosis, and half-saturation times were about 40 s for both myeloperoxidase and lactoferrin.

The other point of difference between the results of these studies and current concepts was the subcellular localization of the acid hydrolase enzymes. These were thought to be largely contained, together with myeloperoxidase, within the azurophil granule (1, 3–5, 7, 14), although certain subpopulations of granules were also thought to house these enzymes (1, 20, 30, 37, 39). However, the present studies revealed a marked difference between the rate of association of myeloperoxidase and that of the three acid hydrolases (Fig. 2–4). The span of time that reflected the 99% confidence limits around the mean saturation times was calculated and shown to differ widely for myeloperoxidase and each of the acid hydrolases (Table II).

Thus the conclusions drawn from these results are: the phagocytosis of latex opsonized with IgG is rapidly followed by the degranulation of both the specific and azurophil granules, which do not appear to degranulate in an obviously sequential manner; degranulation of the acid hydrolases is much slower than that of myeloperoxidase, indicating that these different enzymes are unlikely to be located within the same organelle. Because these results were so different from those of previous studies, supportive evidence was sought by electron microscopic examination of the cells and by cytochemical staining techniques for the localization of myeloperoxidase and acid phosphatase. These morphological studies were conducted specifically to examine possible weaknesses in the biochemical techniques. Examination of preparations of latex particles indicated that a large proportion of them were contained within phagocytic vacuoles, and that there was little contamination by other cellular components (Figs. 6 and 7). The rate of phagocytosis in the biochemical experi-

ments was determined by counting the numbers of these latex particles that were recovered by floatation after phagocytosis was arrested at timed intervals. The counts of particles in the plane of the sections examined morphologically confirmed that the kinetics of the recovery of latex particles by floatation accurately reflected the kinetics of internalization of the latex particles (Fig. 3). It is highly probable that the rapid cooling of the cells to 0°C by plunging them into ice-cold medium arrests degranulation almost immediately; however, because one of the points at issue is the determination of the earliest time at which the granules fuse with the phagocytic vacuoles, the stopping solution in the electron microscopic studies contained cold glutaraldehyde, which has been used by others (41) to fix cells rapidly in studies of the kinetics of phagocytosis and degranulation. Although different methods were used to arrest degranulation, they produced the same answer: the electron micrographs showed that the cytoplasmic granules fuse with the phagosome very soon after and, on occasion, before completion of ingestion of the particle, which occurs between 10 and 20 s after exposure to the cells. The early fusion of the azurophil granule was confirmed by the demonstration of the electron-dense product of the peroxidase reaction in more than half the vacuoles when phagocytosis was arrested as early as 20 s after the addition of the particles to the cells (Table III, Figs. 8 and 9). Lactoferrin, as a marker of the specific granule, was not specifically identified, but granules that stained negatively for peroxidase and were therefore probably specific granules were observed to fuse with the phagocytic vacuole soon after engulfment, at the same time as peroxidase-positive granules (Fig. 10). Granules containing the reaction product for acid phosphatase were far less numerous than those containing myeloperoxidase (compare Figs. 8 and 11 a, b), and in comparison with peroxidase, a much smaller proportion of the vacuoles contained reaction product of the phosphatase at comparable times after phagocytosis (Table III, Fig. 11), confirming the localization of these two enzymes to different organelles within the cells. These results strongly support the findings in the floatation experiments of the different rates of degranulation of the myeloperoxidase-containing azurophil granule and the lysosomes that contain the acid hydrolases. They also indicate that different ratios of contents assayed in the phagocytic vacuoles isolated at different times after particle uptake did not vary

because of differential recovery by floatation.

The complementary techniques of quantitative biochemical assay of a large representative sample and semi-quantitative electron microscopic cytochemical study with a greater attention to detail in fewer cells have given mutually supportive results. Previous researchers, however, have described slower and sequential degranulation of the specific and azurophil granules, concluding that myeloperoxidase and the acid hydrolases are co-located in the azurophil granules. Sequential degranulation of the specific and azurophil granules was described by Bainton (2) who examined this process in rabbit neutrophils engulfing bacteria. The discrepancies between her findings and those in the present study could have several explanations including differences in the source of cells, the object of phagocytosis, the markers used for the different granules, or the experimental protocol. The source of cells is unlikely to be the responsible factor. Zucker-Franklin and Hirsch (41) also investigated the uptake of bacteria by rabbit neutrophils isolated from peritoneal exudates and found that degranulation was often very rapid, sometimes commencing before ingestion of the entire microorganism had been completed, and that there did not seem to be a predilection for the discharge of any particular type of granule. The fact that they also used bacteria as the object of phagocytosis suggests that this factor is unlikely to be responsible for the variation. The use by Bainton of alkaline phosphatase as the marker of the specific granule, the accuracy of which does not seem to be in doubt in the rabbit cell (1, 4 5), would not explain the relative delay in degranulation of myeloperoxidase, the common azurophil marker. A possible reason for the discrepancy lies in the design of the experiments. In this study and in that of Zucker-Franklin and Hirsch, the neutrophils were first isolated and then thoroughly mixed with the particles in vitro, whereas in the experiments of Bainton the timed sequence of events commenced upon injection of the bacteria into the peritoneal cavity, and the kinetics of phagocytosis were not established. As this was an in vivo study, as opposed to the other in vitro studies, the rate of phagocytosis is likely to have been slower because the peritoneal cavity is large and nonhomogeneous and because the absence of a vigorous mixing system will result in a lower frequency of collisions between particles and cells. A slower rate of phagocytosis could explain the apparent delay in degranulation in comparison with that observed in

our study, in which the kinetics of degranulation were related to kinetics of phagocytosis, but not the difference that was observed between the rates of degranulation of the specific and azurophil granules.

It is generally thought that myeloperoxidase and the lysosomal enzymes, the acid hydrolases (1, 3–5, 7, 14), are located in the same azurophil granules. However, the existence of a separate group of granules containing acid hydrolases has been hotly disputed. A group of small, dense, pleomorphic tertiary granules in rabbit neutrophils that stained positively for acid phosphatase were first described by Wetzel and colleagues (28, 40). These tertiary granules may have corresponded to a group of lysosomal particles discovered in rabbit neutrophils by Baggiolini, Hirsch, and de Duve (1) through zonal differential centrifugation; these particles were later thought to represent lysosomes derived from contaminating monocytes (14). In human cells, a distinct group of small peroxidase-negative granules were identified by Daems and van der Ploeg (12) through electron microscopy. Subcellular fractionation studies (7, 20, 30, 37, 39) have shown different density distributions for myeloperoxidase and the acid hydrolases, which indicated that descriptions of the localization of the total content of these enzymes within the same granule were oversimplifying the position. The major peaks of distribution corresponded fairly closely, but in all these studies there was a lack of homogeneity on the grounds of either different shapes of the density distribution profile of the main bands of enzyme activity or the presence of various minor bands. The conclusions of these studies have generally been that the acid hydrolases have a dual localization, with most of the activity being associated with myeloperoxidase in the azurophil granules and with minor amounts of these enzymes being in other particulate organelles. Our study gives strong support to the identification, in human neutrophils, of a distinct group of lysosomes, or tertiary granules. This distinction is made on the grounds of very different kinetics of degranulation of myeloperoxidase as compared with that of the acid hydrolases. Both biochemical (Figs. 2–4) and electron microscopic cytochemical evidence (Figs. 8–11, Table III) showed that myeloperoxidase becomes associated with endocytosed latex much earlier and faster than the lysosomal enzymes. There was no evidence to support the codistribution of even a proportion of the acid hydrolases with myelo-

peroxidase in the azurophil granule.

Having determined the kinetics of degranulation of the different populations of granules, we attempted to relate these observations to the kinetics of oxygen consumption. Direct comparisons are difficult because, with the high concentrations of cells that are required for accurate measurement of the granule contents within the phagosomes, the oxygen in the incubation chamber is rapidly exhausted, and prolonged kinetic measurements are impossible. Despite this problem, several methods were employed to relate oxygen consumption to degranulation. A 10-fold reduction in the number of latex particles and cells enabled a prolonged assessment of the kinetics of oxygen consumption, which, under these circumstances, were found to be very similar to those of degranulation of lactoferrin and myeloperoxidase (Fig. 3). Although we would have preferred to measure the prolonged kinetics of oxygen consumption at the higher cell concentrations, the results of studies with dilute cell suspensions appear to be relevant. Oxygen consumption commences at the same time after exposure of the different concentrations of cells to latex; there is a close correlation between the number of cells and the linear rate of oxygen consumption, the amount of which is stoichiometrically related to the numbers of particles engulfed (33). At the standard higher concentrations of latex particles, oxygen consumption could be measured over the 1st min. It commenced ~10 s after the addition of the latex particles and became linear after ~10 s, which was very similar to the initial kinetics of the degranulation of myeloperoxidase and lactoferrin (Fig. 3).

These results indicate that the kinetics of oxygen consumption are very similar to those of degranulation of the specific and azurophil granules, which is compatible with the direct involvement of the contents of these granules in the respiratory burst, or subsequent interaction with the products thereof. The intravacuolar concentration of immunoprecipitable lactoferrin begins to fall after about 2 min (Figs. 2–4). This could reflect either digestion by the lysosome enzymes starting to enter the vacuole at about this time or secretion from within the vacuole. Either way, the reduction in the effective intravacuolar concentration of this protein suggests that its function is likely to be related to the early events after phagocytosis and is unlikely to be simply a bacteriostatic effect of depriving the organism of iron through high-affinity binding (10).

These studies indicate a close temporal relationship between degranulation of the specific and azurophil granules and the burst of respiration that is associated with phagocytosis, which are subsequently followed by degranulation of the lysosomes. They also provide a logical physiological basis for the observed delay between early oxidative bacterial killing and the subsequent degradation of bacterial constituents (11).

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