INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106

FOWLER, ROSALIE AHUMADA LEON

CHARACTERIZATION OF HUMAN POLYMORPHONUCLEAR NEUTROPHIL PHAGOLYSOSOMES

THE UNIVERSITY OF ARIZONA M.S. 1983

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print ✓
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

CHARACTERIZATION OF HUMAN POLYMORPHONUCLEAR NEUTROPHIL PHAGOLYSOSOMES

by

Rosalie Ahumada León Fowler

A Thesis Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND MEDICAL MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 8 3

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Rosalie A. Fowler

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Richard Rest
R. F. REST
Assistant Professor of
Microbiology

3 May 1983
Date

ACKNOWLEDGEMENTS

I graciously thank Dr. Richard F. Rest for his help in planning and evaluating this research and Penny Moore for her assistance in the execution of this research.

I also sincerely thank my husband, Paul Fowler, and my children, Paul William, Richard Isaac and Thomas Zachery Fowler, for their support and patience in the completion of this thesis.

Finally I want to thank my mother, Maria León, for her assistance at home while I was busy completing my research.

TABLE OF CONTENTS

TABLE OF CONTENTS -- Continued

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS -- Continued

LIST OF TABLES

ABSTRACT

The kinetics of phagolysosomal formation in human polymorphonuclear leukocytes were studied. Normal human polymorphonuclear leukocytes (1 x 10^8 cells/ml) purified through Ficoll/Hypaque, were incubated with diisodecyl-phthalate-*E. coli* lipopolysaccharide 0111:B4 emulsions opsonized with fresh autologous serum. Phagocytosis was stopped at various times by the addition of cold medium 199 with 1 mM EDTA. These polymorphonuclear leukocytes were then washed and homogenized to 90-95% breakage. Phagolysosomes containing oil emulsion were isolated by flotation using discontinuous sucrose gradients and were collected for enzymatic analysis. Emulsion uptake by the leukocytes was measured using emulsions dyed with Oil-Red-0. Myeloperoxidase, lactic dehydrogenase, lactoferrin, lysozyme, and glycosidases, including β-D-glucuronidase and N-acetyl-β-D-glucosaminidase were assayed in all isolated (phagolysosome) fractions. Lactate dehydrogenase levels in the phagolysosomal fractions and in phagocytosis supernatants remained at zero time levels throughout the different assay times, indicating minimal lysis of phagocytosing polymorphonuclear leukocytes and minimal contamination of the phagolysosomes with cytoplasmic enzymes.

CHAPTER 1

INTRODUCTION

The primary function of the mature human polymorphonuclear cell is phagocytosis and destruction of microorganisms. Metchnikoff (1905) postulated and showed that this phagocytosis and destruction of microorganisms via host defenses is essential for well-being. Future studies by many investigators proved him correct in that decreased numbers or lack of neutrophils is characterized by an increased incidence of infection. Further, more occasionally, there occurs an individual with adequate numbers of neutrophils but functionally abnormal cells. In order to fully understand these disease states in which the neutrophils are functionally inadequate, it is necessary to look at normal cells and how they behave.

Morphology of the Human Neutrophil

Plasma Membrane

The plasma membrane of the mature human neutrophil is a trilaminar structure with an approximate total width of about 75-100 Å. Kitao, Hattori and Takeshita (1974), using sodium dodecyl sulfate polyacrylamide disc gel electrophoresis to analyze human neutrophil membrane, found five major

protein bands, a few minor protein bands and one carbohydrate component.

Pictures of neutrophils taken by scanning electron microscopes (Michaelis et al., 1971; Klainer and Betsch, 1973) reveal a ruffled appearance to the surface of the cell with pseudopods protruding out from it.

The membrane of mature leukocytes possesses receptors for several molecules. These receptors are for the Fc portion of the IgG molecule and for C_3 and/or one of its breakdown products (Messner and Jelinek, 1970; Ishizaka, Tomioka and Ishizaka, 1970).

Nucleus

The mature neutrophil is an end cell incapable of further division. Characteristically the nucleus of the mature neutrophil is multilobed, with the nuclear lobes being connected by thin strands of nuclear material. Nucleoli are completely absent and the chromatin material is condensed and well marginated.

Microtubules and Microfilaments

Microtubules forming a cytoskeleton are present in human polymorphonuclear leukocytes. They are approximately 250 Å in diameter and are often seen to radiate from the centriole. Treatment of human neutrophils with colchicine causes dissolution of microtubules and results in alteration

in cell function. Functions which are altered include inhibition of neutrophil mobilization (Fruhman, 1960), chemotaxis (Caner, 1965), phagosome lysosome fusion (Malwista and Bodel, 1967; Malwista, 1968, 1971), phagocytosis (Goldfinger, Howell and Seegmiller, 1965; Lehrer, 1973), post-phagocytic metabolic burst (Malwista and Bodel, 1967; Malwista, 1968, 1971), and adherence of PMNs to glass beads (Penny et al., 1966).

Microfilaments, although present in neutrophils, are not as abundant as they are in macrophages. They occur in peripheral locations in areas devoid of granules (DePetris, Karlsbad and Pernis, 1962).

Cytoplasmic Granules

Enzyme-rich cytoplasmic granules contained within a trilaminar membrane of 70-90 Å in width are a distinguishing feature of the mature neutrophil. Because of the role of the granules as the digestive apparatus of phagocytic cells, they have been widely studied using histochemical and chemical characterization.

Origin and Maturation of Polymorphonuclear Leukocytes

Myeloblast

The neutrophil originates in the bone marrow from a "pluripotential stem cell." This stem cell can become a

myeloblast which can develop into any cell of the myelocytic series. About 10 μm in diameter, this cell is very immature and restricted to the bone marrow. The myeloblast has a high nucleus-to-cytoplasm ratio and no granules (Capone, Weinreb and Chapman, 1964; Scott and Horn, 1970; Bainton, Ullyst and Farquhar, 1971).

Promyelocyte

If the myeloblast becomes committed to the neutrophilic series, it becomes a neutrophilic promyelocyte. It is about 15 μm in diameter and it is at this point that the azurophilic or 1^{o} granules begin to appear (Bainton and Farquhar, 1966). This cell is difficult to distinguish from the myeloblast except for the presence of granules and a slightly decreased nucleus-to-cytoplasm ratio. The enzyme content of the 1^{o} granules is summarized in Table 1.

Myelocyte

The promyelocytic neutrophil matures into the myelocyte. This cell has a diameter of 10 μm and a somewhat indented nucleus. It is during this portion of the neutrophil's development that the specific or secondary granule population appears (Scott and Horn, 1970; Bainton et al., 1971; Ackerman, 1971). The enzyme content of the specific granules is summarized in Table 2.

Table 1. Summary of the Enzyme Content of Primary Granules.

Azurophil (Primary) Granules
Acid hydrolases
Acid β-glycerophosphotase
β-D-glucuronidase
(n-acetyl-β-D-glucosaminidase)
α-mannosidase
Arylsulfatase
β-galactosidase
5'nucleotidase
α or β-fucosidase
Acid protease (cathepsin)
Neutral proteases
Chymotrypsin-like protease (Cathepsin G)
Elastase
Collagenase*
Myeloperoxidase
Lysozyme
Acid mucopolysaccharide

* Conflicting data exists on the exact location of this enzyme.

Table 2. Summary of the Enzyme Content of the Secondary Granules.

Specific (Secondary)
Lysozyme
Lactoferrin
Collagenase*
Alkaline phosphotase*
Vitamin B_{12}-binding proteins
α or β-D-glucosidase activity

* Conflicting data exist on the exact location of these enzymes.

Mature Polymorphonuclear Neutrophils

The mature neutrophil is the cell found circulating in the bloodstream of normal individuals. This cell is further subdivided into the metamyelocyte, band cell, and fully mature cell on the basis of the appearance of the nucleus.

A fully mature cell has a segmented nucleus, the metamyelocyte an indented nucleus, and the band cell has a horseshoe-shaped nucleus. From the myelocyte stage on, in the process of neutrophilic maturation, there is no longer any cell division. The mature cell possesses both the 1^{o} and 2^{o} granules in an approximate 2-3:1 ratio of specific-to-azurophil. Although still controversial, some investigators have reported the possibility of a third granule population in the mature neutrophil (Bretz and Baggiolini, 1974; West et al., 1974; Kane and Peters, 1975; Rest, Cooney and Spitznagel, 1978). These "tertiary" granules are thought to contain the enzymes known as acid hydrolases.

Polymorphonuclear Function

The primary function of the polymorphonuclear leukocyte is the ingestion and destruction of microorganisms, i.e., phagocytosis. Metchnikoff (1905) was the first to describe phagocytosis, and many reviews have been written on the subject (Mudd, McCutcheon and Lucke, 1934; Berry and

Spies, 1949; Wood et al., 1951; Wright and Dodd, 1955; Suter, 1956; Wood, 1960; Rabinovitch, 1968; Winkelstein, 1973; Stossel, 1974, 1975, 1976; Walter and Papadimitriou, 1978). For descriptive purposes, the act of phagocytosis can be divided into the five steps listed below.

Chemotaxis

Neutrophils are mobile cells, and their directed random movement along a concentration gradient of particular chemicals is called chemotaxis (Zimond and Hirsch, 1973; Keller, 1972; Keller, Hess and Cottier, 1975). Agents which are chemotactic for neutrophils are divided into two categories. Cytotoxins (Keller and Sorkin, 1967) comprise the first category, and these are agents which are directly chemotactic. They are water soluble, diffusible, and mostly polypeptide in nature. Cytotoxigens comprise the second category, and these include the formation of cytotoxins. Suffice it to say that elaborate methods have been used to study and measure chemotaxis. Increased chemotaxis then is seen in the presence of chemotactic agents such as bacteria and bacterial products, injured or damaged tissue and complement components and fractions, as well as other serum factors (Ward, Lepaw and Newman, 1968, Yoshida et al., 1975; Tempel et al., 1970; Shiffman et al., 1975; Lachman, Kay and Thompson, 1970; Ward, 1967; Gallin, Clark and Frank, 1975;

Stecher, Sorkin and Ryan, 1971; Kaplan, Kay and Austen, 1972; Weksler and Coupal, 1973; Kay and Kaplan, 1975; Kay, Pepper and Ewart, 1973; Kay, Pepper and McKenzie, 1974; and Stecher and Sorkin, 1972).

Attachment

In order to have successful phagocytosis, the particle must be in close proximity to the neutrophil. The beginning of ingestion then is marked by a firm adhesion of the recognized particle to the surface of the leukocyte. There are certain serum proteins which enhance this recognition and adhesion and therefore the ingestion of particles by neutrophils. These serum proteins are called opsonins and include IgG (heat-stable Fc portion) (Messner et al., 1968; Ward and Zvaiflier, 1973) and the heat-labile fragments of the third component of complement (Shin, Smith and Wood, 1969; Stossel, 1973; Stossel, Alper and Rosen, 1973). On the surface of neutrophils there exist receptors for the Fc portion of the IgG molecule (Messner and Jelinek, 1970) and for fragments of the third component of complement (Henson, 1976; Logue, Rosse and Adams, 1973). Opsonization of the particle to be ingested with fresh serum greatly facilitates attachment and thus ingestion (Mantovani, 1975; Scribner and Fahrney, 1976; Ehlenberger and Nuzzenzweig, 1977).

Ingestion

Once attachment has taken place, the particle is internalized by the phagocyte. Engulfment occurs when pseudopods surround the particle and then fuse around it, forming a vacuole called a phagosome (Griffin, Leider and Silverstein, 1975). Many factors, including cell surface changes and interfacial tension, influence the ingestion process. A good review on the subject is by Klebanoff and Clark (1978). The phagosome is eventually drawn completely into the cell.

Degranulation

As the phagosome is being formed, the cytoplasmic granules converge, fuse, and discharge their contents into it. It is at this point that the phagosome becomes a phagolysosome. Previous literature indicates that it is the secondary granules which fuse with the phagosome first (Bainton, 1972, 1973; Bainton and Fraquhar, 1968). However, some investigators such as Segal, Dorling and Stephanie (1980) report that the specific and azurophil granules do not appear to degranulate in a sequential manner but rather degranulate at the same time. In the mature neutrophil the secondary granules outnumber the primary granules and contain lactoferrin, microbicidal proteins, vitamin B_{12}-binding protein, but generally lack the digestive enzymes (Olsson and Venge, 1972; Zeya and Spitznagel, 1968, 1969). The primary

granultes contain the lysosomal hydrolases and myeloperoxidase (Bainton, 1972, 1973; Bainton and Fraquhar, 1968).

Killing and Digestion

Once sequestered in the phagolysosome, the phatocytized particle is subjected to a lethal microenvironment. The antimicrobial systems are usually divided into those which require oxygen and those which are oxygen-independent (Klebanoff and Hamon, 1972; Lehrer and Cline, 1969; Lehrer, Hanifin and Cline, 1969, Lehrer, 1972; Handell, 1974; Quie et al., 1967; Holmes, Page and Good, 1967). The oxygen-dependent system can be further subdivided into those which are myeloperoxidate-mediated and those which are myeloperoxidase-independent. Several reviews have been written on these antimicrobial systems of the human polymorphonuclear neutrophil (Skarnes and Watson, 1957; Hirsh, 1960a, Klebanoff, 1975a, 1975b; DeChalet, 1975) and include factors such as lysosomal hydrolytic enzymes, acid pH, cationic proteins, lysosome, lactoferrin, superoxide anion (a highly reactive radical derived from the one electron reduction of O_2 at the cell surface, singlet oxygen and hydroxyl radical formation, and hydrogen peroxidase (particularly in association with myeloperoxidase and halide ions).

A summary of the events taking place during phagocytosis can be seen in Figure 1.

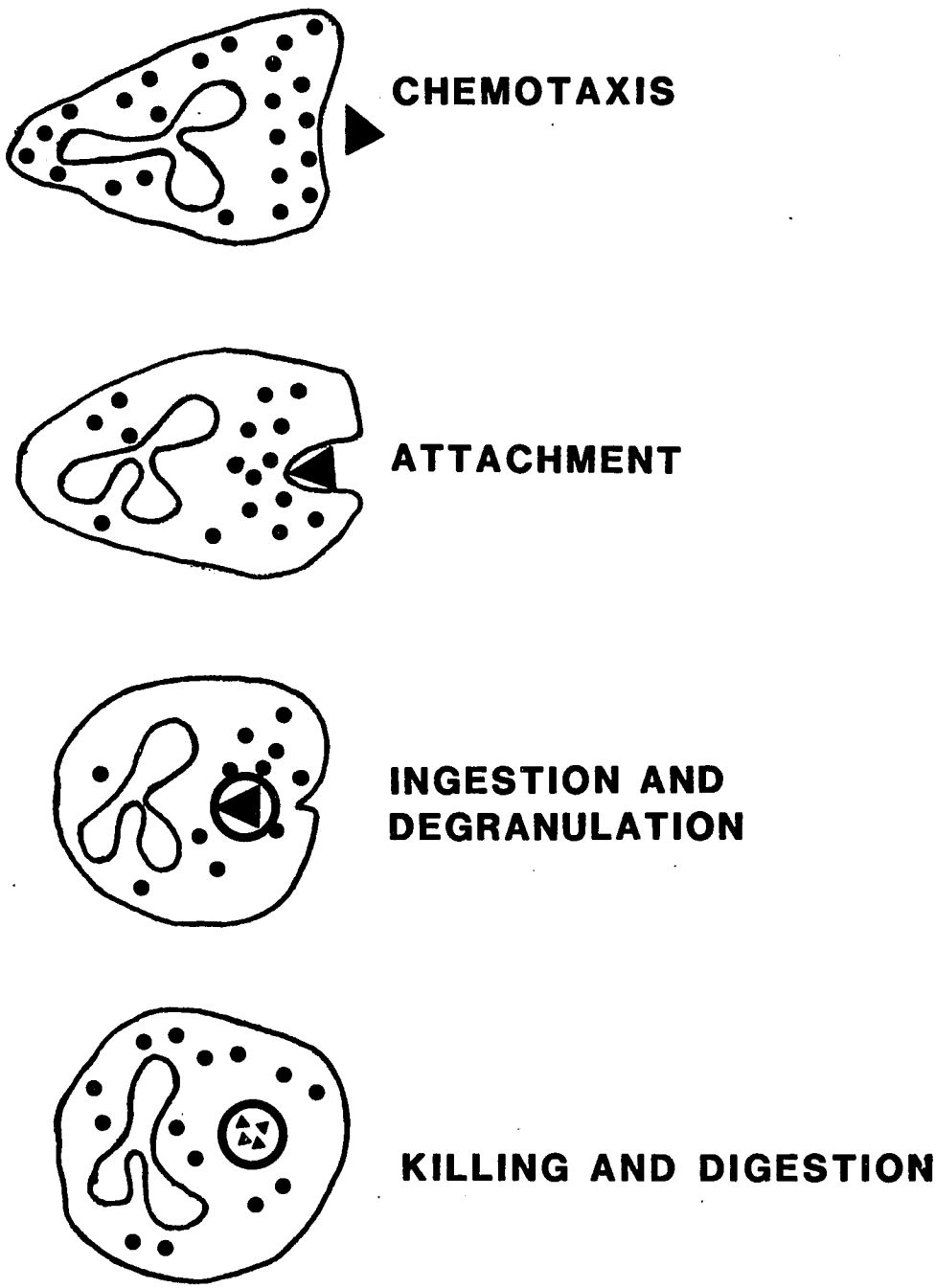

Figure 1. Summary of Events Taking Place During Phagocytosis.

Purpose of the Present Study: Isolation and Characterization of Human Phagolysosomes

During the process of phagocytosis there are a myriad of metabolic changes that are initiated within the neutrophil. These include increased glycolysis, increased hexosemonophosphate shunt activity, increased oxygen utilization, increased hydrogen peroxidase generation, increased lipid synthesis, increased RNA synthesis and degradation, a fall in the pH of the phagocytic vacuole, and activation of the acid active enzymes. Concentrating on changes occurring only within the phatocytic vacuole required a reliable method of isolation of these phagocytic vacuoles. Investigators in the literature have used several techniques. Stossel et al. (1971) employed a flotation method whereby buoyant emulsified paraffin oil droplets coated with protein served as the phagocytic particle. Once phatocytosis was completed, the cells were homogenized and the oil-containing phagolysosomes were collected by flotation using sucrose density gradients. Tagesson and Stendal (1973) used radiolabelled bacteria as their phagocytic particle. After disrupting the integrity of the whole neutrophil, they isolated the phagolysosomes by using zonal centrifugation. Finally in 1974 Leffell and Spitznagel used immune complex-coated latex beads as the phagocytic particle. Like Stossel, after homogenization of the whole neutrophils, phagolysosomes were collected on

sucrose gradients, this time by fractionation. Utilizing a system similar to Stossel's et al. (1971), the following work presents observations about the kinetics of phagolysosomal formation in human neutrophils. Biochemical documentation of degranulation into the phagolysosome was made by isolating phagolysosomes at different time intervals following phatocytosis and assaying for members of the different granule populations. By studying the kinetics of phagolysosomal formation, further insights into how normal neutrophils behave in healthy people can be achieved and thus provide a basis for the study and diagnosis of their behavior in diseased states of altered haemostasis.

CHAPTER 2

MATERIALS AND METHODS

Methods

Purification of Neutrophils

Neutrophils were isolated from freshly drawn units of blood from healthy male and female donors, as described previously by Leffel and Spitznagel (1974). Neutrophils were sedimented through hydroxyl-ethyl starch or Plasma-Gel (HTI Corp., Buffal, NY) to separate red blood cells from the neutrophils. Ficoll-Hypaque sedimentation was used to separate the neutrophils from mononuclear white blood cells. Any remaining contaminating red cells were then removed by hypotonic lysis with distilled water. Neutrophils were pelleted by a ten-minute centrifugation at 1000 rpm (in a Sorvall RC-3 rotor HL8) at $4^{o}C$, which also removed red blood cell debris. At this point the neutrophils were 90-96% pure. Viability of the purified neutrophils by trypan blue exclusion was 93-96%. The neutrophils were counted and adjusted to 2×10^{8} cells per ml in tissue culture Medium 199 and kept on ice until ready to use.

Preparation of Oil Emulsions

Diisodecylphthalate oil emulsions were made with or without a dye, oil-red-0, in the oil. For enzyme measurements no dye was used. These emulsions were prepared by adding 3 mls of tissue culture medium 199 to 40 mgms E. coli lipopolysaccharide 0111:B4 (Difco Chemical Co.) and sonicated for 5 seconds at a 7 setting on a Ultrasonic Inc. Model W-225R sonicator. The prepared lipopolysaccharide was then mixed with 1.0 ml of diisodecylphthalate oil and sonicated for 90 seconds at a 7 setting on a Ultrasonic Inc. Model W-225R sonicator. If measurement of oil uptake by whole cells or by phagolysosomes was desired, the oil-red-0 dye was included in the lipopolysaccharide emulsion mixture. The dye was dissolved in the diisodecylphalate mixture by heating at 100^{o}C for 1 hour followed by centrifugation to remove any undissolved dye. Experiments used for enzyme analysis contained no dye.

Opsonization of the Oil Emulsion

At the time the units of blood were drawn from the donors, a sample was also taken for autologous serum. Just prior to phagocytosis the lipopolysaccharide-oil emulsion was opsonized with the autologous serum. This was done by placing the serum and oil emulsion in a 1:1 ratio on a Scientific Instruments Model 151 rotator at 37^{o}C for 25 minutes.

Phagocytosis

Phagocytosis was conducted in a 37°C New Brunswick Gyratory Waterbath Shaker Model G76 shaking water bath. Plastic round bottom 50 milliliter centrifuge tubes were used as assay containers for all the phagocytosis experiments. Each assay mixture had a total volume of 2 mls. First, 1 ml of 2 x 10^8 neutrophils and 0.6 ml tissue culture Medium 199 plus 25 mM Hepes was prewarmed for 10 minutes in a 37°C water bath. Then, 0.4 ml of opsonized oil emulsion was added to each assay tube and incubated for the appropriate time. Phagocytosis was stopped by adding 25 mls of cold tissue culture Medium 199 plus 1.0 mM EDTA. The tubes were kept on ice until all the times were complete. The phagocytosis mixtures were then centrifuged for 10 minutes in a Sorvall HL-8 rotor, 1000 rpm, at 4°C.

Isolation of Oil-Containing Phagolysosomes

The supernatant was removed, measured and saved from the phagocytosis assay tubes. The cellular pellet was resuspended in a 2.0 ml M sucrose. This was homogenized using a chilled Dounce homogenizer with a tight pestle at 4°C until cells exhibited 90-95% breakage. Homogenization time to achieve adequate breakage was usually between 3 to 5 minutes. The whole cell homogenate was then made up to 4.5 mls with 0.4 M sucrose, and 0.5 ml of it was saved frozen for enzyme

assays. Phagloysosomes were separated by flotation using the remaining 4 mls of whole cell homogenate which were placed on discontinuous sucrose gradients, described as follows.

First, 1 ml of 60% sucrose was placed at the bottom of a 15 ml Corex tube. This was overlaid with the 4 mls of whole cell homogenate in 0.4 M sucrose. Nine mls of 0.25 M sucrose was placed over the whole cell homogenate and the gradients were spun in the cold at 16,000 rpm in a J21B Beckman centrifuge. The gradients (before centrifugation) appeared as in Figure 2.

After centrifugation the gradient was divided into three fractions as in Figure 3. These were very carefully removed, measured and saved for further analysis. Fraction #1 contained the oil-rich purified phagolysosomes while Fraction 3 contained the remainder of cellular components. A pictoral summary of the isolation of oil-containing phagolysosomes is seen in Figure 4.

Oil-Red-0 Uptake--
Whole Cell Analysis

Phagocytosis was performed as previously described except that cells were not homogenized. After the cells were pelleted by centrifugation in a Sorvall RC-3 rotor HL8 at 1000 rpm (4^{o}C), the supernatants were removed and discarded. The pellet was resuspended by vortexing in 3 mls

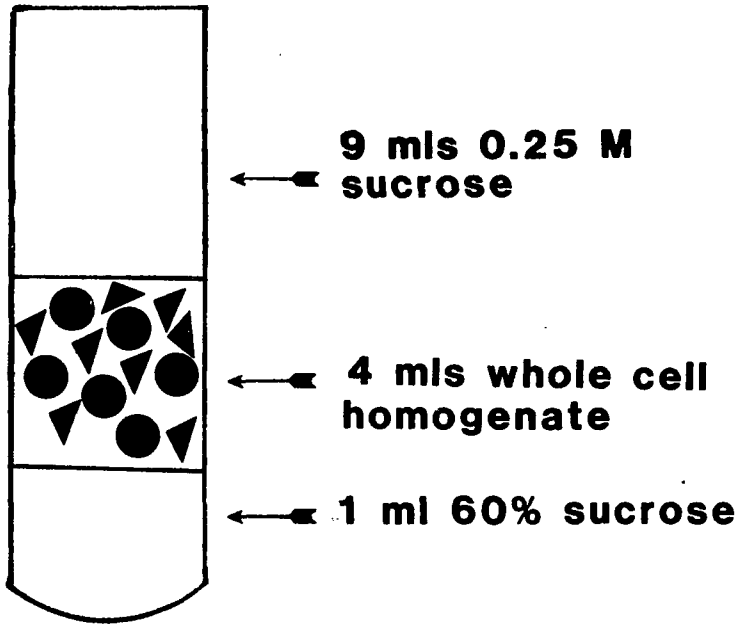

Figure 2. Discontinuous Sucrose Gradients as They Appeared Before Centrifugation.

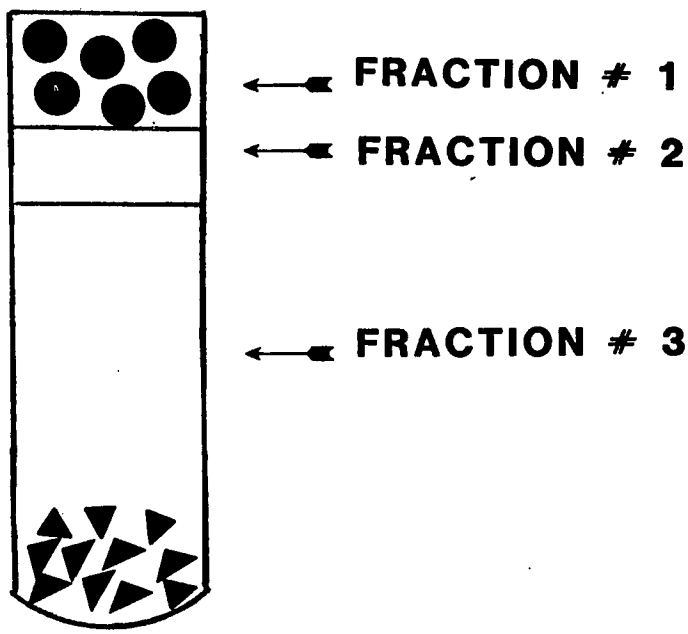

Figure 3. Discontinuous Sucrose Gradient after Centrifugation. -- Fraction #1 contained purified phagolysosomes, fraction #2 had little if any enzymatic activity, and fraction #3 contained the majority of the cellular debris.

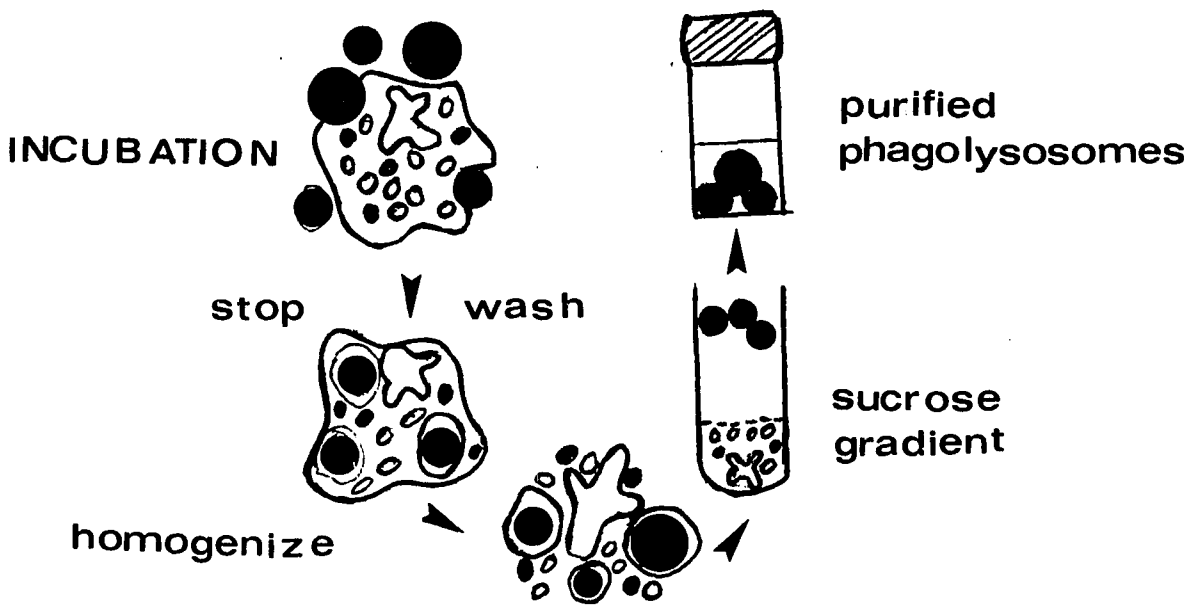

Figure 4. Pictoral Summary of the Isolation of Oil-Containing Phagolysosomes.

Dioxane and centrifuged for 12 minutes at 1200 rpm at 4°C in a Sorvall RC-3 rotor HL8. The supernatant was removed and oil-red-0 content was measured by direct spectrophotometric reading at 525 nm.

Oil-Red-0 Content of Phagolysosomes

Phagocytosis and homogenization of human neutrophils was performed as described previously. The purified phagolysosomes were then resuspended in 1 ml Dioxane by vortex mixing and centrifuged for 12 minutes at 1200 rpm at 4°C in a Sorvall RC-3 rotor HL8. The supernatant was removed and oil-red-0 content was measured by direct spectrophotometric reading at 525 nm.

Myeloperoxidase Assay

Myeloperoxidase was measured by observing the reduction of 0-dianisidine as described by Worthington (1972). The substrate was 30% H_2O_2 diluted 1:10,000 in pH 6.0 phosphate buffer. A unit of myeloperoxidase was defined as that amount of enzyme causing an increase in absorbance at 460 nm of 0.001 in a 1 ml reaction with O-dianisidine as the hydrogen donor. Human leukemic neutrophil myeloperoxidase purified by a modification of Olsson and Venge (1972) was used as a standard. All determinations were done in duplicate and 0.01% Triton X-100 was added to all assay mixtures, except those done the same day, to check for latency.

Lactoferrin

Lactoferrin was measured using radial immunidiffusion by the method of Mancini, Carbonara and Heremans (1965). Human milk lactoferrin purified by the method of Querinjean, Chandan and Shahani (1971) was used for standards. Antibodies to lactoferrin were made in rabbits. All determinations were done in duplicate and 0.03% mixed alkyltrimethyl bromide was in all the assay mixtures.

Lysozyme

Lysozyme was measured spectrophotometrically as described by Welsh and Spitznagel (1971). The substrate for lysozyme assays was a commercially available (Difco Chemical Co.) ultraviolet-killed dried culture of Micrococcus lysodeikticus. Egg white lysozyme (500 µg/ml) was used to prepare standards. A 0.01% solution of Triton X-100 was present in all assays which were read for a decrease in absorbance at a wavelength of 450 nm.

Lactate Dehydrogenase

Lactate dehydrogenase was measured spectrophotometrically as described by Bergmeyer, Bernt and Hess (1963). The substrate consisting of NADH and sodium pyruvate was made up in phosphate buffer (pH 7.0) with 1% bovine serum albumin. Standards for the assay were made from dilutions of commercially available (Sigma) lactate dehydrogenase,

11 mgm/ml. A 0.01% solution of Triton X-100 was present in all assay mixtures which were read at 340 nm with a blue filter.

Glycosidase Assays

Five glycosidases were examined spectrofluorometrically using 4-methyl umbelliferyl glycoside substrates, as described by Rest et al. (1978). The five enzymes examined were beta-D-glucuronidase, N-acetyl-beta-D glucoaminidase, alpha-D-mannosidase, alpha-D-galactosidase, and alpha-D-glucosidase. Glycosidases were assayed in a total volume of 100 µl in 12 x 75 mn glass tubes containing the appropriate 4-methyl umbelliferyl glycoside substrate (Research Products International Corporation) and buffer listed in Table 3.

The 4-methyl umbelliferyl glycoside substrates were dissolved in 100 ul dimethyl sulphoxide (Sigma Chemical Co., St. Louis, MO) and then 3.9 ml of 0.1 M sodium acetate buffer of appropriate pH plus 40 µl 1.0% Triton X-100 was added. Each assay tube contained 80 ul of substrate plus 20 µl of sample and was then incubated at 37°C for correct time. At the end of the incubation period the reactions were stopped by the addition of stopping buffer, i.e., 20 ml of 20 mM glycine/NaOH buffer containing 5 mM EDTA (sodium salt). Fluorescence was read within one hour with a Aminco Spec-

Table 3. 4-Methyl Umbelliferyl Substrates and Buffers Used in Glycosidase Assays.

4-Methyl Umbelliferyl Glucoside	Incubation Time (minutes)	Buffer
4 µg β-D-glucuronide	30	0.1 M acetate/NaOH, pH 3.5-4.3
4 µg Acid amido-2-deoxy-β-D-glucopyranoside	30	0.1 M acetate/NaOH, pH 5.7-5.8
4 µg α-Dmannopyranoside	60	0.1 M acetate/NaOH, pH 4.5
4 µg α-D-galactopyranoside	60	0.1 M acetate/NaOH, pH 4.0-4.1
4 µg α-D-glucopyranoside	60	0.1 M PO_4, pH 6.9-8.0

trofluorometer SPF-125 model spectrofluorometer, at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. A standard curve was constructed using dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} of 1 mM 4-methyl umbelliferone sodium salt in stopping buffer. Results were expressed as nmol of substrate hydrolyzed per minute.

Materials

Hypaque (50% sodium)was obtained from Winthrop Laboratories, Menlo Park, CA. Plasma gel was purchased from HTI Corporation, Buffalo, NY. p-Dioxane was obtained from Matheson, Coleman and Bell, Norwood, OH. Diisodecylphalate oil was supplied by Fisher Scientific Company, Pittsburgh, PA, and oil-red-O came from Allied Chemical Corporation, Morristown, NJ. 4-Methyl umbelliferyl glycosides all were purchased from Research Products International Corp., Elk Grove Village, IL. All chemicals were of analytical reagent grade if available, and other materials were of the highest quality commercially available.

CHAPTER 3

RESULTS

Oil Uptake Whole Cell Analysis

Stossel et al. (1971), Segal et al. (1980) and Leffel and Spitznagel (1974) have shown that polymorphonuclear cells readily ingest solubilized particles, i.e., oil emulsions and latex beads. In order to measure the kinetics and ascertain the uptake of normal human neutrophils of lipopolysaccharide-diisodecyphthalate oil emulsions opsonized with autologous serum, the uptake by whole cells was first investigated. Oil-red-O dye was added to these emulsions so that their uptake could be measured by direct spectrophotometric readings. After addition of dioxane the oil and cells were dissolved, releasing the dye into suspension, allowing for direct spectrophotometric reading at 252 nm. A sample of 8 experiments measuring oil-red-O uptake by whole cells over time, revealed that uptake of the lipopolysaccharide-coated oil emulsion began immediately and increased over time. These experiments did not differentiate between attachment and actual ingestion but instead measured the combination of both. See Table 4 and Figure 5.

Table 4. Oil-Red-O Uptake Whole Cell Analysis.

Time	Experiment Number								N	$\bar{X}$	Std. Dev.
	1	2	3	4	5	6	7	8			
0 sec	0.036	0.033	0.261	0.021	0.069	0.066	0.018	0.0335	8	0.045	0.018
5 sec			0.789	0.519					2	0.65	0.19
10 sec			1.290	0.636					2	0.96	0.46
30 sec			1.026	2.004	1.581				3	1.54	0.49
1 min		3.234	2.592	2.463					3	2.76	0.41
3 min	3.636		3.336	5.253	4.5405 4.6005				5	4.29	0.78
7 min			6.057	8.337	7.77 7.341				4	7.38	0.97
15 min					9.381 10.116	7.464			3	8.99	1.37
30 min		7.464			10.8	9.066			3	9.11	1.67
45 min					11.397		8.982		2	10.19	1.71
60 min					12.096	10.125	10.956	0.3245	4	10.88	0.89
75 min							9.846	0.4595 0.0575	3	10.29	0.39

Table 4. -- Continued

Time	Experiment Number								N	$\bar{X}$	Std. Dev.
	1	2	3	4	5	6	7	8			
90 min							9.951	11.0745 11.4045	3	10.81	0.76
105 min								11.0355 11.43434	2	11.23	0.28

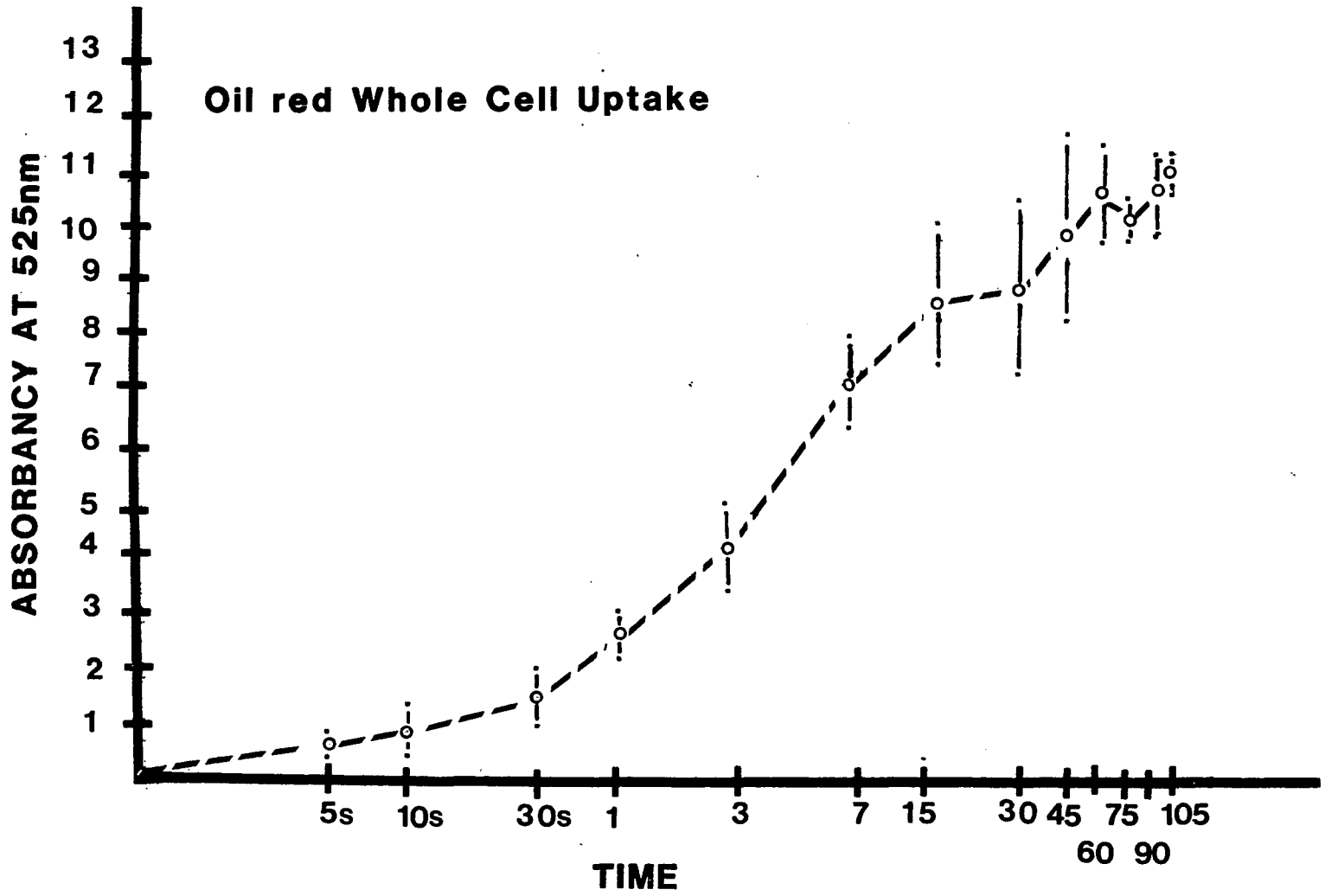

Figure 5. Oil-Red-0 Uptake Over Time--Uptake Shows an Increase Beginning Immediately.

Oil Content of Phagolysosomes

Once the uptake of the diisodecylphthalate lipopolysaccharide emulsions by normal human neotrophils was established, the next step was to look at the oil content of isolated phagolysosomes. Again, oil-red-O dye was added to the diisodecylphthalate lipopolysaccharide oil emulsions so that oil content of the purified phagolysosomes could be measured by direct spectrophotometric readings after addition of dioxane. The oil content in isolated phagolysosomes over time was slightly different than that of whole cells. In five experiments, membrane-bound oil droplets began to be measurable at about 15 seconds (Table 5). A graph of experiment 4 can be seen in Figure 6.

Enzyme Analysis of Oil-Containing Phagolysosomes

(All values are expressed as percent of whole cell homogenate.)

Myeloperoxidase

Myeloperoxidase, the marker enzyme for the azurophil or primary granules, was measured in all purified phagolysosome fractions. The presence of myeloperoxidase in these preparations was taken as an indication of the degranulation of the primary granules into the phagocytic vacuole. A sampling of three experiments revealed that the presence of myeloperoxidase in the phagocytic vacuoles was insignificant

Table 5. Oil-Red-O Content of Purified Phagolysosomes.

Time	Experiment Number					N	$\bar{X}$	Standard Deviation
	1	2	3	4	5			
0 sec	0.0813	0.09	0.0175	0.0168	0.063	5	0.054	0.031
5 sec			0.015			1		
10 sec		0.255	0.0775			2	0.166	0.089
15 sec				0.1632 0.1496	0.616	3	0.3096	0.22
20 sec		0.375				1		
30 sec	1.0476		0.2825	0.1848 0.220	0.989	5	0.545	0.388
40 sec		0.365						
45 sec				0.4956 0.4032	1.225	3	0.708	0.37
60 sec	2.384	0.460		0.5796 0.440	1.6588	5	1.105	0.784
108 sec				0.6888	3.548	2	2.118	1.43

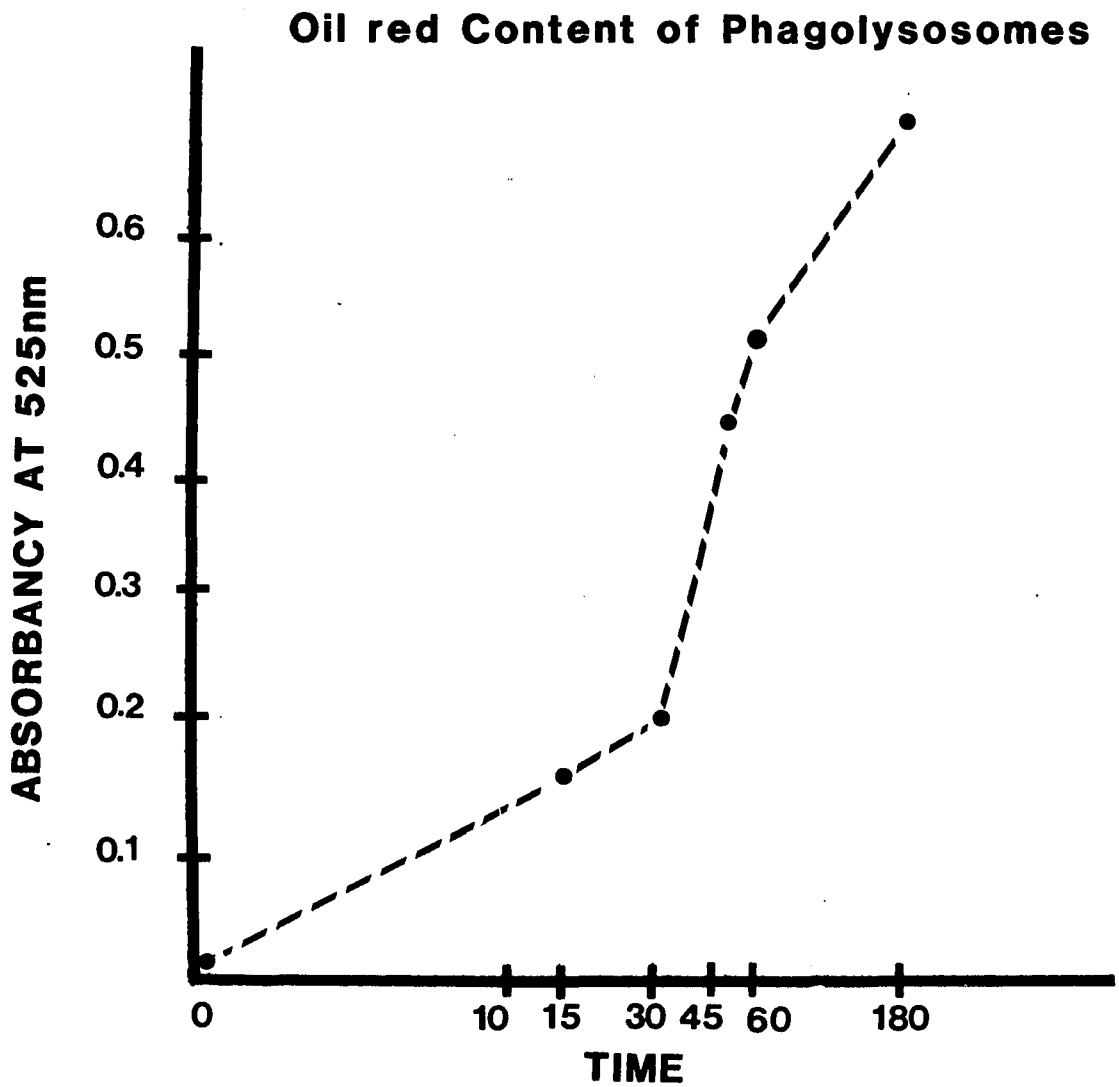

Figure 6. Oil-red-O Content of Purified Phagolysosomes, Experiment 4.

until approximately 180 seconds, at which point it increased dramatically to 2-3% of the whole cell homogenate (Figure 7). To prove that assays were indeed of membrane-bound phagolysosomes, same-day myeloperoxidase assays were done on samples, first without Triton X100 and then with Triton X100. In all cases, myeloperoxidase content increased after the addition of Triton, confirming the isolation of membrane-bound phagolysosomes with the release of myeloperoxidase only after the addition of membrane-destroying detergent.

Lactoferrin

Lactoferrin, the market enzyme for the secondary granule population, was measured. It reversibly binds two atoms of iron per molecule and shows microbiostatic properties when not fully saturated with iron. In addition to being present in leukocytes, lactoferrin is present in body secretions such as milk, tears, saliva, nasal and bronchial secretions, bile, urine, seminal fluid, and cervical mucus. Measurement of lactoferrin in purified phagolysosomes by immunidiffusion proved to be quite difficult. Some experiments revealed no increase in phagolysosome lactoferrin content, while others revealed some increase over time. Two of the experiments showing an increase over time seemed to start increasing at about 3 to 5 minutes, showing 4-5% of the whole-cell homogenate activity (Figure 8).

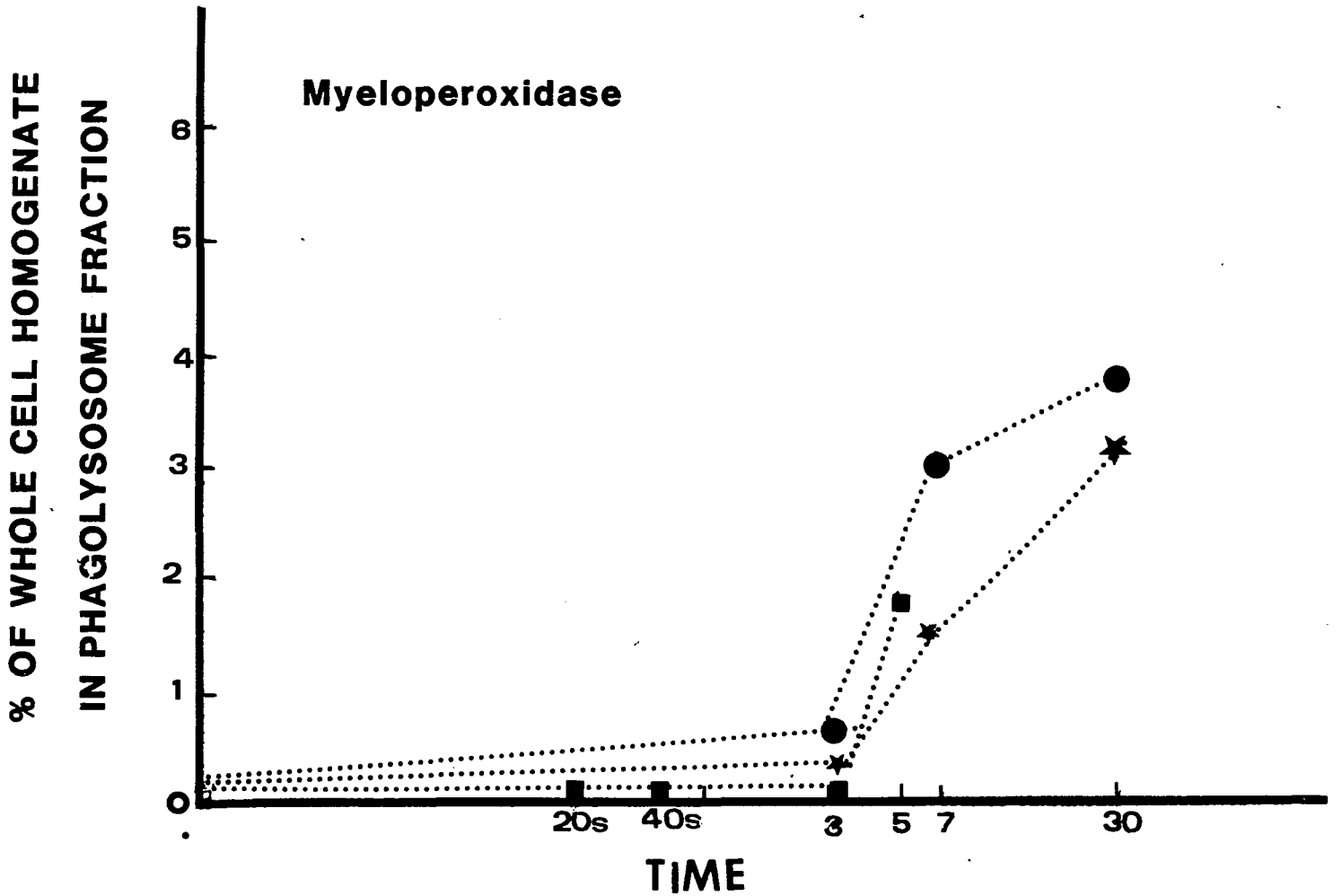

Figure 7. Myeloperoxidase Content of Isolated Phagolysosomes--
Increase Over Time Beginning at About Three Minutes.

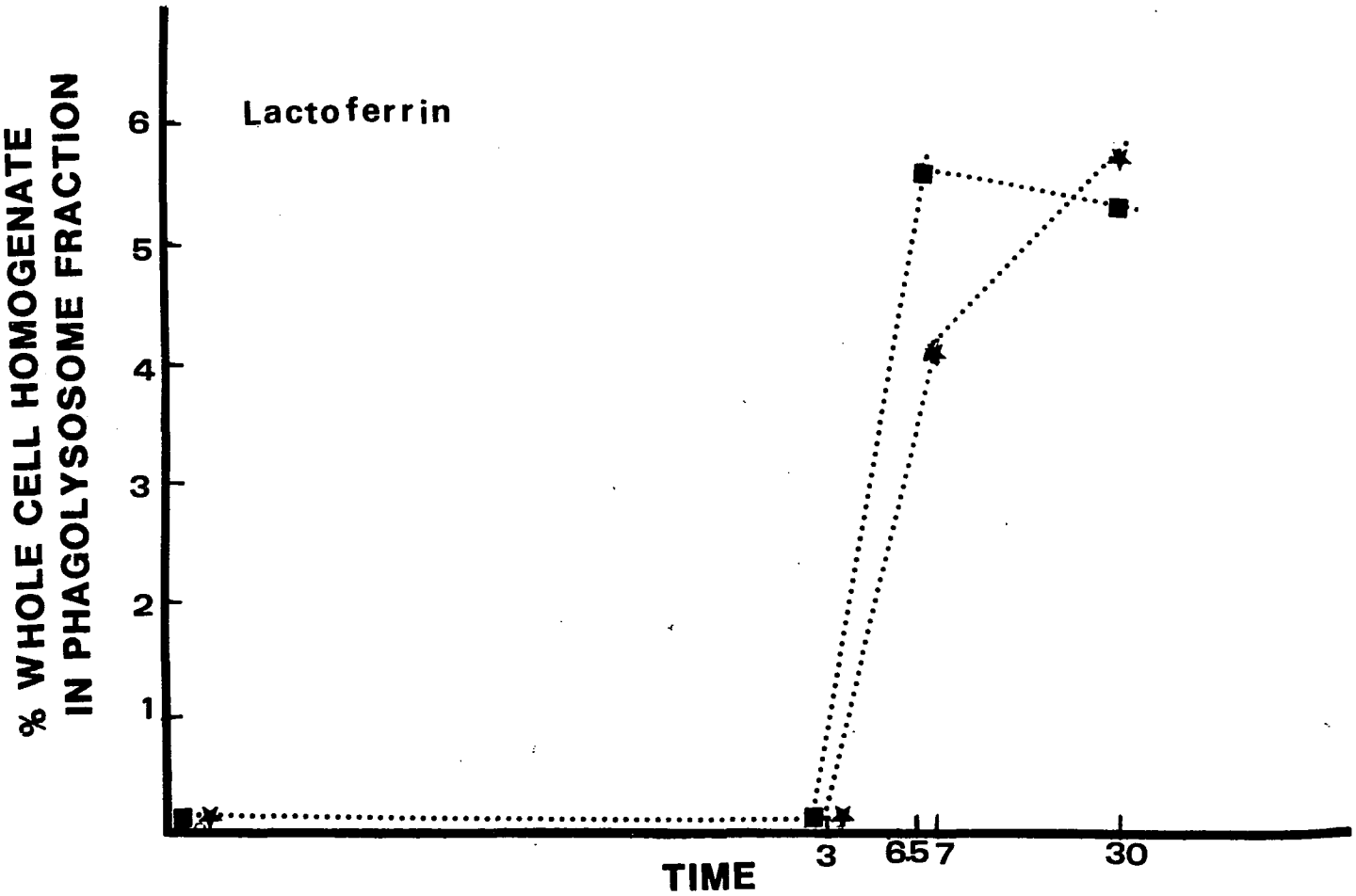

Figure 8. Lactoferrin Content of Isolated Phagolysosomes--
Lactoferrin Content Increased Over Time.

Since it has been postulated by Leffel and Spitznagel (1972, 1975) that lactoferrin subserves a major portion of its role outside the polymorphonuclear cell, the phagocytosis supernatant was also examined for lactoferrin content. It was found that between 40-80% of the lactoferrin content of the whole cell homogenate was found in the phagocytosis supernatant by 5 minutes. A sample of three experiments which showed this phenomenon can be seen in Figure 9.

Lysozyme

Lysozyme is not a marker enzyme for either granule population, but rather appears to be distributed somewhat equally in the azurophil and specific granule populations, as well as also being found in other body secretions. Lysozyme is a bacteriolytic enzyme that causes lysis of certain lysozyme-sensitive bacteria by hydrolyzing bacterial cell wall components. Since not all organisms are sensitive to lysozyme, its function within the neutrophil is thought to be digestion of glycopeptide debris from bacteria killed in other ways (Chipman and Sharon, 1969). The lysozyme content and kinetics of its appearance in isolated phagolysosomes did not appear to be like that of either myeloperoxidase or lactoferrin. Lysozyme showed a slight increase over time in the phagolysosomal fraction. The increase seemed to begin between 1 and 3 minutes and never reached more than 1.5% of

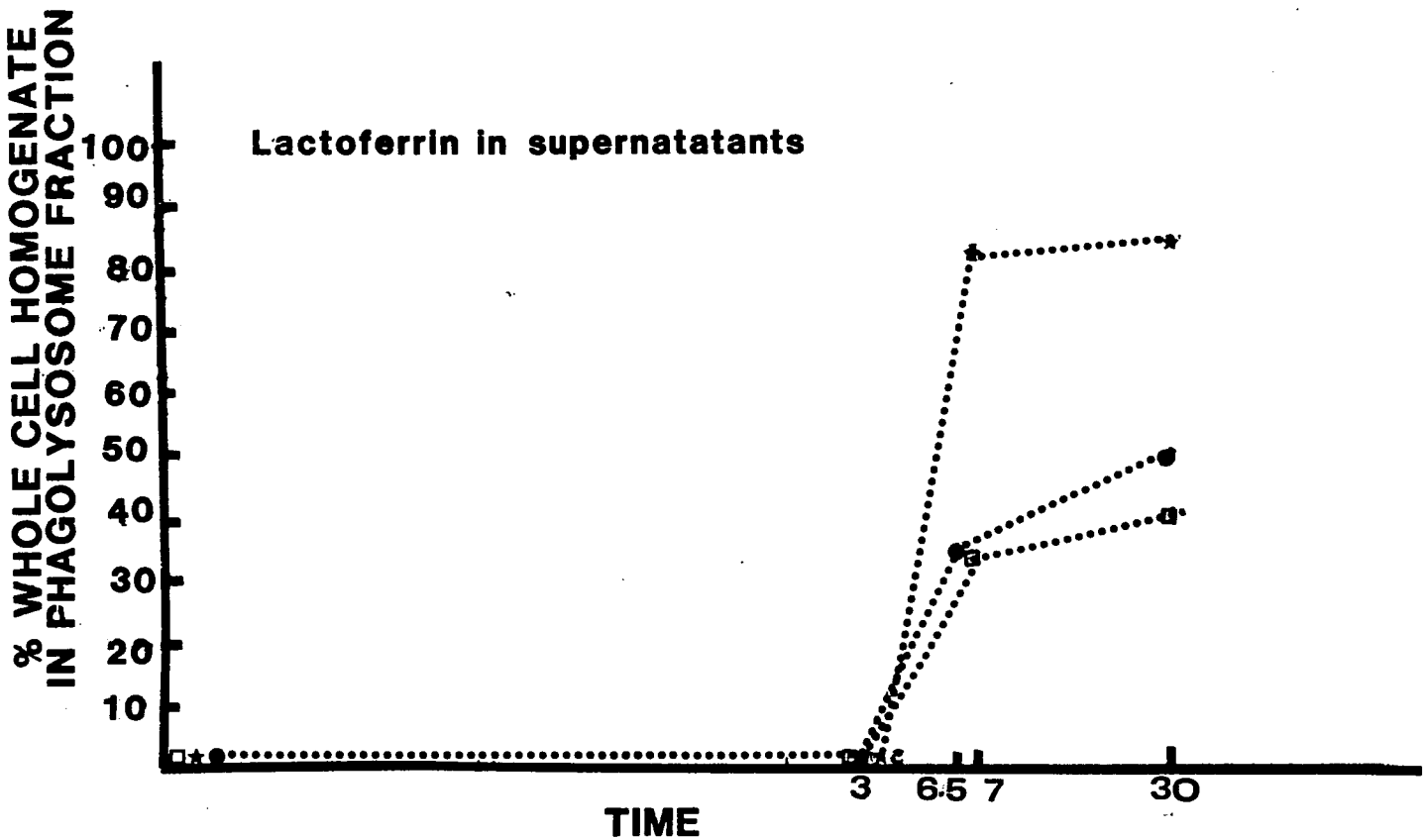

Figure 9. Lactoferrin Content of Phatocytosis Supernatant--Lactoferrin Content Increased Over Time and Thus Indicated Great Quantities of Extracellular Degranulation.

whole cell homogenate. A sample of two experiments is seen in Figure 10.

Lactate Dehydrogenase

Lactate dehydrogenase is a widely distributed cytoplastmic enzyme of mammalian cells. It was detectable in all phagolysosomal fractions, but unlike myeloperoxidase, its concentration did not increase over time. Levels of lactate dehydrogenase in isolated phagolysosomal fractions remained at zero time levels, indicating minimal lysis of phagocytosing PMN and minimal contamination of phagolysosomes with cytoplasmic enzymes.

Glycosidases

The existence of a third granule population containing the group of enzymes known as acid hydrolases is still not completely resolved (Rest et al., 1978). Segal et al. (1980) found that with his system of phagocytosis, utilizing immunoglobulin-coated latex beads, the acid hydrolases β-D-glucuronidase and N-acetyl-β-D-glucosaminidase had kinetics of degranulation that were similar to each other but totally different from myeloperoxidase. He says 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."

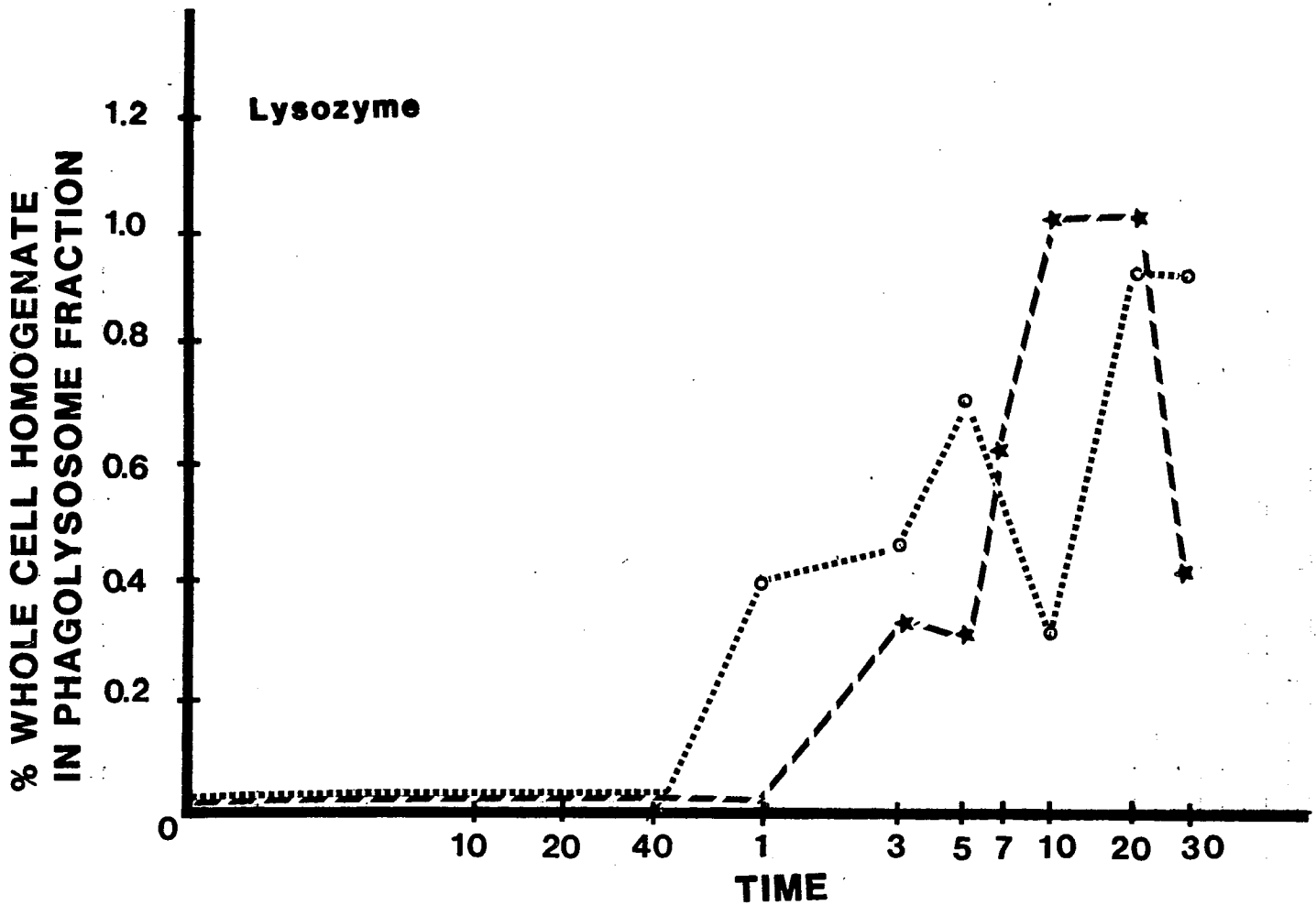

Figure 10. Lysozyme Content of Isolated Phagolysosomes--Lysozyme Showed Only Slight Increase Over Time.

In experiments presented here of the five glycosidases examined, i.e., beta-D-glucuronidase, N-acetyl-β-D-glucosidase, alpha-D-mannosidase, alpha-D-galactosidase, and alpha-D-glucosidase, only two showed a consistent increase over time in the phagolysosomal fraction. These two were N-acetyl-β-D-glucosaminidase and beta-D-glucuronidase. A sample of an experiment showing this increase can be seen in Figure 11. The other three glycosidases in some phagolysomal fractions showed an increase over time, but this was not a consistent finding. The percent of whole-cell homogenate of N-acetyl-β-D-glucosaminidase and beta-D-glucuronidase in the phagolysosomal fraction seemed to increase slightly at 1-5 minutes, with a larger increase taking place at about 10 minutes.

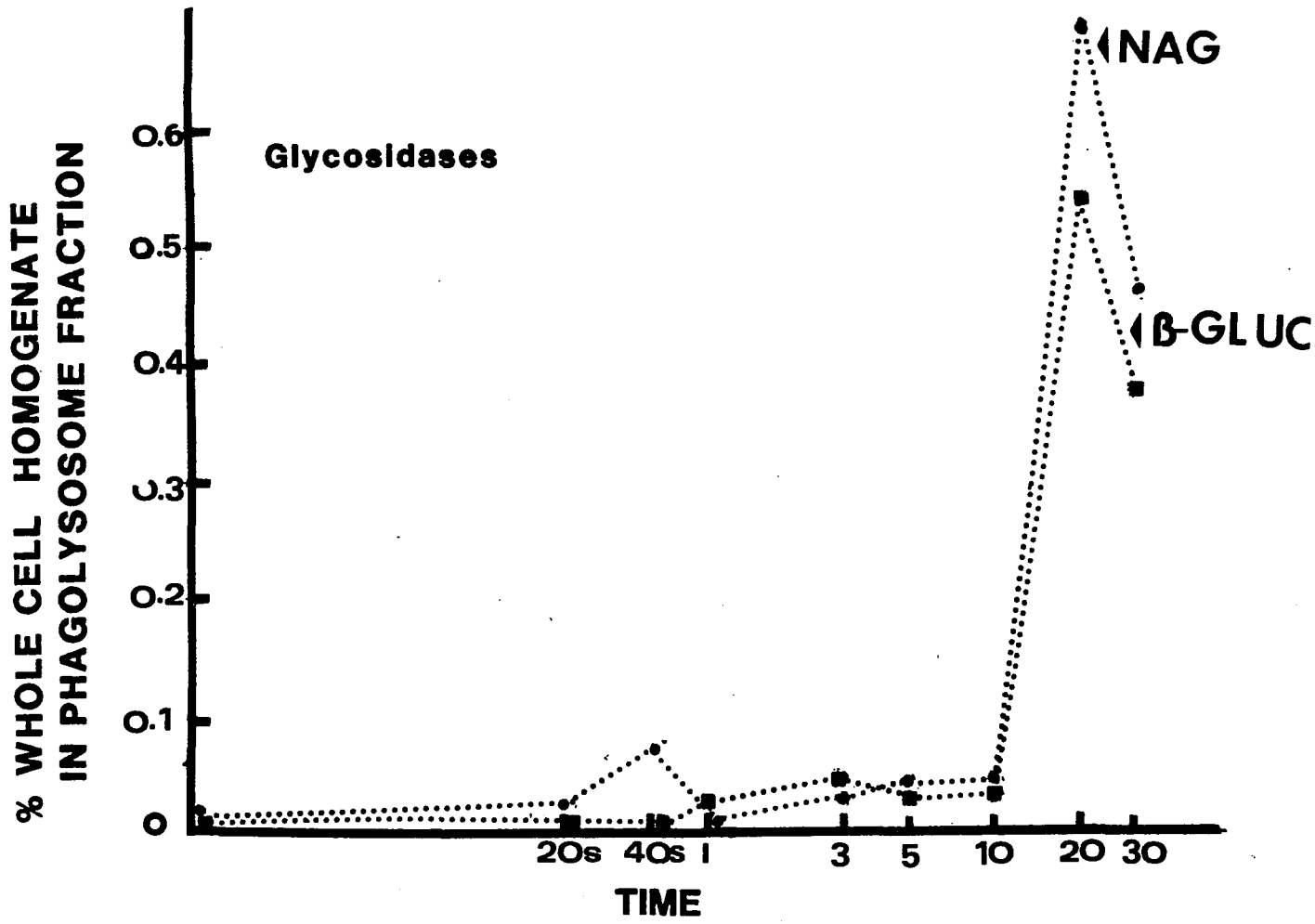

Figure 11. N-acetyl-β-D-glucosaminidase (NAG) and beta-D-glucuronidase (β-GLUC) Content of Isolated Phagolysosomes--Glycosidases Showed a Somewhat Delayed Increase Over Time as Compared to Myeloperoxidase.

CHAPTER 4

DISCUSSION

One of the most important functions of human neutrophils is phagocytosis, phagocytosis being the physical act of ingestion by which microorganisms, being recognized as foreign by normal polymorphonuclear white cells, are taken up into the cells. Once inside, the bacteria are sequestered in an intracellular vacuole or phagosome whereby the cell can deliver granule components converting the phagosome into a phagolysosome. What is unique and of life-sustaining importance is that without these normally functioning neutrophils, a host is much more susceptible to microbial infections. This is well documented, in that people suffering from Chediak-Higashi syndrome (a congenital disorder marked by an impairment in the process of degranulation that results in a deficiency in the ability of neutrophils to kill ingested bacteria) suffer from repeated bacterial infection. Densen and Mandel (1978) found bactericidal activity occurring within the phagolysosome. The physical act of sequestration of the microorganisms from the nutrient-rich plasma is deleterious to some bacteria. Along with this, a rapid drop in phagolososomal pH has been reported by Jacques and

Bainton (1978). This could have a two-fold effect. First, acidic conditions are harmful to some bacteria such as pneumococcus, which are very susceptible to acid pH. Second, the acidic conditions within the phagolososome may lead the way for activation of the arsenal of granule enzymes.

To study (1) phagolososomal formation and (2) degranulation, the system reported here used normal human neutrophils and serum-opsonized lipopolysaccharide-coated oil particles. The following discussion will be divided into these two areas.

Phagolysosome Formation

In the experiments presented, phagocytosis was conducted over multiple time periods following initial contact between the serum-optonized lipopolysaccharide-coated oil particles and neutrophils. From this work, it appeared that attachment of the opsonized lipopolysaccharide oil emulsions begins immediately. As early as 5 seconds after contact with the oil emulsions there appeared a measurable increase in oil uptake by whole cell analysis. In measuring the amount of membrane-bound oil emulsions, it was found that it too increased rapidly after initial contact. A detectable increase of phagolysosome-bound oil emulsion was found to begin at 10 seconds. In this respect the results agree with those of Segal et al. (1980), who used IgG-coated latex beads

instead of oil emulsions. There was a discrepancy, however, in the amount of time which the cells continued to show an increase in uptake. Segal reported that with his system of IgG-coated latex beads that the uptake reaches a maximum at about 5 minutes. In the system reported here, the cells continue to take up increasing amounts of oil for 15 minutes. There are two possible reasons for this. First, Segal uses latex beads which are very uniform in size, approximately 0.8 μm. The oil emulsions were anything but uniform in size. Photographs taken of phagocytizing cells reveal a great variety in the size of oil droplets (Figure 12).

Another possible explanation between these experiments and Segal's work is that the LPS-coated oil emulsions were opsonized with fresh autologous human serum so that complement was present and probably tripped via the alternate pathway. Segal, on the other hand, used IgG-coated latex beads without complement being present.

Degranulation

Once particle ingestion has taken place, the granules near the vacuole fuse with it and discharge their contents into it. It is in this way that bacteria are sequestered and then killed by neutrophils.

Documentation of just when the specific and azurophil granule contents appear in the phagolysosome was monitored

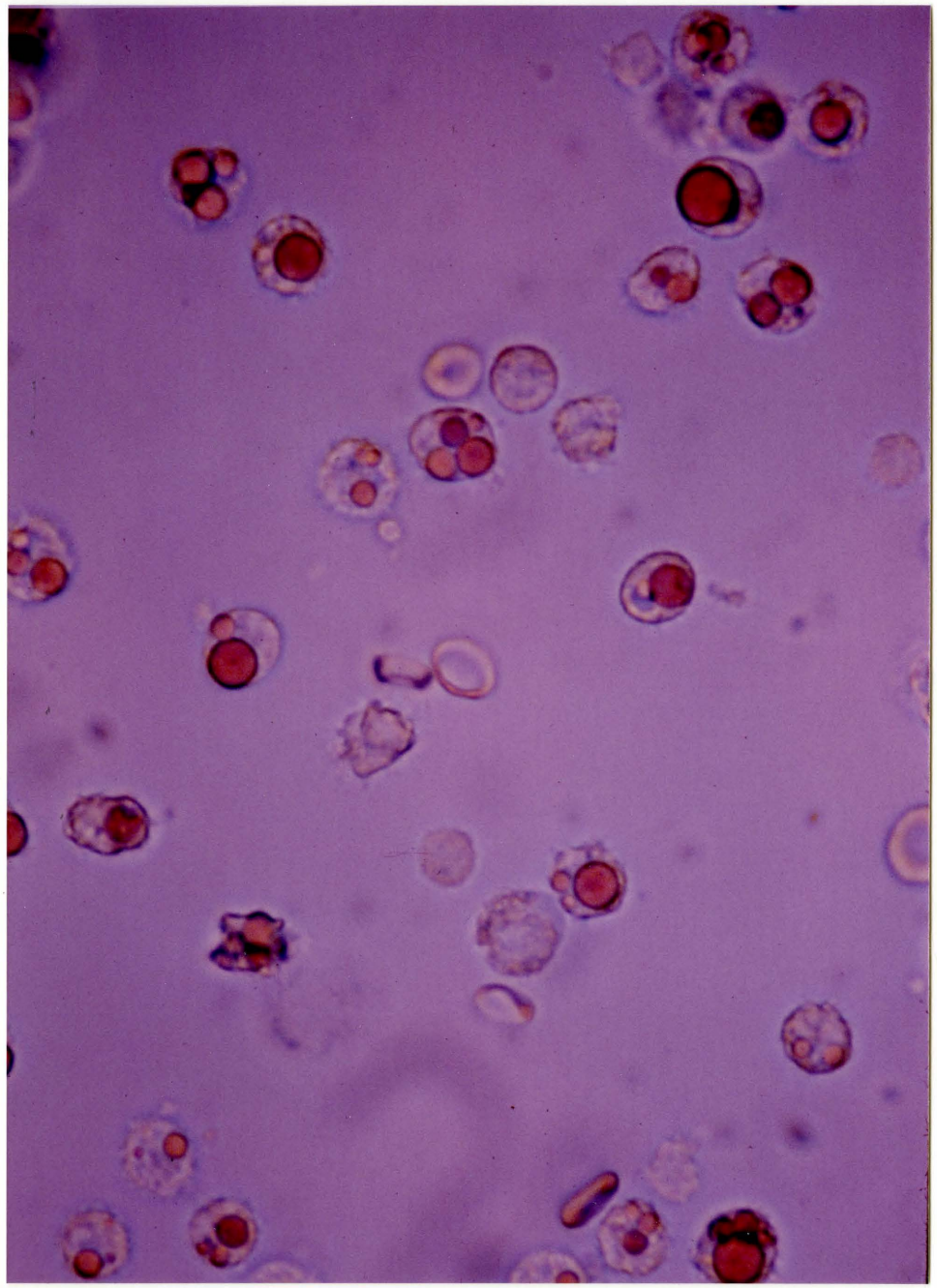

Figure 12. Photograph of human polymorphonuclear neutrophils phagocytizing opsonized oil emulsions. -- Note the great variety in size of the opsonized oil droplets (450x).

because of their possible role in damaging or actually killing phagocytized bacteria. Myeloperoxidase, the marker enzyme for the azurophil granules, first appeared at 3 minutes following phagocytosis. Its importance for the bactericidal activity is well documented, in that humans with a hereditary deficiency of myeloperoxidase suffer repeated infection due to the inability of their neutrophils to kill bacteria in an expeditious manner (Lehrer, Hanifin and Cline, 1969). Lactoferrin (the marker for the specific granules) was difficult to measure in isolated phagolysosomes, but it, like myeloperoxidase, was first detectable at 3 minutes following phagocytosis. It has been reported by Arnold, Ide and McGee (1977) that apolactoferrin, the iron unsaturated form of lactoferrin, is bactericidal for Vibrio cholorae and Streptococcus mutans. Lysozyme is an enzyme which is found in both primary and secondary granule populations and belongs to a class of enzymes capable of hydrolyzing bacterial cell walls. While most bacteria of clinical significance are resistant to lysozyme itself, it may be that the major function of lysozyme is its action in combination with other neutrophil components. Lysozyme's function could also be that of digesting phagocytized bacteria after they are killed instead of acting as a primary bactericidal component (Elsbach, 1980). In experiments presented here, lysozyme was detectable in the phagolysosome

fractions between 1 and 3 minutes. It, like myeloperoxidase and lactoferrin, showed an increase over time in the phagolysosomal fraction but its concentration never reached the levels of myeloperoxidase and lactoferrin.

Animal studies examining rabbit and chicken leukocytes (Hirsch, 1960) showed that the sequence of phagocytosis followed by degranulation is identical in humans and other animal species, such as rabbits and guinea pigs. The kinetics of degranulation between the different granule populations of animal leukocytes versus human leukocytes is still being investigated. Bainton (1973) and Henson (1971) reported that rabbit leukocytes show a definite sequential degranulation, with the specific granules degranulating first followed by the azurophilic granules. The exact sequence of degranulation in human neutrophils has been described in the past as sequential, but more recently this observation has been questioned by Segal et al. (1980) and by the work presented here. Spitznagel et al. (1974) reported that the release of constituents from the two granule classes during phagocytosis as in animal studies represents independent phenomenon. The specific degranulation generally preceeds that of the azurophil population. Another suggestion from this sequential degranulation was the appearance of lactoferrin (the marker enzyme for the specific granules) in large amounts in the phagocytosis supernatants, suggesting

that degranulation of the specific granules was predominantly outward from the cell. This was in contrast to the azurophil granules, in which little or none of the myeloperoxidase (the marker enzyme for primary granules) was found outside the cell while significant amounts were found within the phagolysosomes.

Segal et al. (1980), on the other hand, did not find sequential degranulation in his phagocytosis experiments. He found that both the specific and azurophil granule populations degranulate at the same time. He mentions that the acid hydrolases appear in the phagolysosome at a much later time than myeloperoxidase and lactoferrin, thus indicating that these different enzymes are not likely to be located within the same granule population.

The work reported here partially supports both the work of Spitznagel et al. (1974) and Segal et al. (1980). Like in Segal's et al. (1980) experiments, sequential degranulation was not observed. Both myeloperoxidase and lactoferrin activity was initially detectable in isolated phagolysosomes at the same time, i.e., 3 minutes. The glycosidases in human neutrophils, which were characterized by Rest et al. (1978) as having a very complex distribution, were found to appear in isolated phagolysosomes much later than myeloperoxidase and lactoferrin. This again supported

Segal's work and suggested the possibility of a third or tertiary granule population.

Measurement of lactoferrin, as mentioned, proved to be quite difficult. Its content within the phagolysosome was zero to 4.5% of the whole cell homogenate. However, when examining the phagocytosis supernatants for lactoferrin content, it was found to contain between 40% to 80% of the whole cell homogenate. This observation supported Leffel's et al. (1974) work, in which they found degranulation of the specific granules being mainly outward from the cell.

Explanations for these differences and similarities are: (1) differences in the design of the experiments; (2) differences in particles to be phagocytized; and (3) differences in opsonization procedures.

There is no question as to the importance of phagolysosome formation and degranulation for the maintenance of an infection-free state. Perturbation in either phagolysosome formation or degranulation leads to life-threatening infection. More work is needed to clarify the differences seen in the kinetics of degranulation between investigations using dissimilar experimental designs. Extracellular degranulation and its function in the destruction of microorganisms also warrants further discussion.

CHAPTER 5

CONCLUSIONS

1. Attachment of opsonized lipopolysaccharide oil emulsions begins immediately.
2. Phagolysosome formation began at around 10 seconds with the appearance of a measurable increase in oil content of the phagolysosomal fractions.
3. Myeloperoxidase and lactoferrin activity in the phagolysosomal fraction was detectable at 3 minutes and continued to increase over time beyond 15 minutes.
4. Only N-acetyl-β-D-glucosaminidase and β-D-glucuronidase of the five human glycosidases assayed showed a consistent increase with time in the phagolysosomal fraction. Their appearance in phagolysosomal fractions at 10 minutes was much later than that of myeloperoxidase or lactoferrin.

REFERENCES

Ackerman, G. A. 1971. The human neutrophilic myelocyte. A correlated phase and electron microscopic study. Z. Zellforsch., 121:153-170.

Arnold, R. R., M. F. Ide and J. R. McGhee. 1977. A bactericidal effect from human lactoferrin. Science, 197:263-265.

Bainton, D. F. 1972. The origin, content and fate of polymorphonuclear leukocyte granules. In: Phagocytic Mechanisms in Health and Disease. R. C. Williams and H. H. Fudenberg, editors. Intercontinental Medical Book, New York.

Bainton, D. F. 1973. Sequential degranulation of the two types of polymorphonuclear leukocyte granules during phagocytosis of microorganisms. J. Cell Biol., 58: 249-264.

Bainton, D. F. and M. G. Farquhar. 1966. Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. J. Cell Biol., 28:277-301.

Bainton, D. F. and M. G. Farquhar. 1968. Differences in enzyme content and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. J. Cell Biol., 39:299-317.

Bainton, D. F., J. R. Ullyst and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. J. Exp. Med., 134: 907-934.

Bergmeyer, H., E. Bernt and B. Hess. 1963. Lactic dehydrogenase. In: Methods of Enzymatic Analysis. H. V. Bergmeyer, editor. Academic Press Inc., New York.

Berry, L. J. and T. D. Spies. 1949. Phagocytosis. Medicine (Baltimore), 28:239-300.

Bretz, U. and M. Baggiolini. 1974. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. J. Cell Biol., 63:251-269.

Caner, J. E. Z. 1965. Colchicine inhibition of chemotaxis. Arthr. and Rheum., 8:757-760.

Capone, R. J., E. L. Weinreb and G. B. Chapman. 1964. Electron microscope studies on normal human myeloid elements. Blood, 23:300-320.

Chipman, D. M. and M. Sharon. 1969. Mechanism of lysozyme action. Lysozyme is the first enzyme for which the relation between structure and function has become clear. Science, 165:454-465.

DeChatelet, L. R. 1975. Oxidative bactericidal mechanisms of polymorphonuclear leukocytes. J. Infect. Dis., 131:295-303.

Densen, P. and G. L. Mandell. 1978. Gonococcal interactions with polymorphonuclear neutrophils. Importance of the phagosome for bactericidal activity. J. Clin. Invest., 62:1161-1171.

DePetris, S., G. Karlsbad and B. Pernis. 1962. Filamentous structures in the cytoplasm of normal mononuclear phagocytes. J. Ultrastruct. Res., 7:39-55.

Ehlenberger, A. G. and V. Nussenzweig. 1977. The role of membrane receptors for C3b and C3d in phagocytosis. J. Exp. Med., 145:357-371.

Elsbach, P. 1980. Degranulation of microorganisms by phagocytic cells. Rev. Infec. Dis., 2:106-128.

Fruhman, G. J. 1960. Inhibition of neutrophil mobilization by colchicine. Proc. Soc. Exp. Biol. Med. (N.Y.), 104:284-286.

Gallin, J. I., R. A. Clark and M. M. Frank. 1975. Kinetic analysis of the generation of the chemotactic factor in human serum via activation of the classical and alternate complement pathways. Clin. Immunol. Immunopath., 3:334-336.

Goldfinger, S. E., R. R. Howell and J. E. Seegmiller. 1965. Suppression of metabolic accompaniments of phagocytosis by colchicine. Arthr. and Rheum., 8:1112-1122.

Griffin, F. M., Jr., J. A. Griffin, J. E. Leider and S. C. Silverstein. 1975. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. J. Exp. Med., 142:1263-1282.

Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. II. Mechanisms of release during phagocytosis and adherence to non-phatocytosable surfaces. J. Immonol. 107:1547-1557.

Henson, P. M. 1976. Membrane receptors on neutrophils. Immunol. Commun., 5:757-774.

Hirsch, J. G. 1960. Antimicrobial factors in tissues and phagocytic cells. Bact. Rev., 24:133-140.

Holmes, B., A. R. Page and R. A. Good. 1967. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. J. Clin. Invest., 46:1422-1432.

Ishizaka, K., H. Tomioka and T. Ishizaka. 1970. Mechanisms of passive sensitization. I. Presence of IgE and IgG molecules on human leukocytes. J. Immunol., 105:1459-1467.

Jacques, Y. V. and D. F. Bainton. 1978. Changes in pH within within the phagocytic vacuoles of human neutrophils and monocytes. Lab Invest., 39:179-185.

Kane, S. P. and T. J. Peters. 1975. Analytical subcellular fractionation of human granulocytes with reference to the localization of vitamin B_{12}-binding proteins. Clin. Sci. Mol. Med., 49:171-182.

Kaplan, A. P., A. B. Kay and K. F. Austen. 1972. A pre-albumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. J. Exp. Med., 135:81-97.

Kay, A. B. and A. P. Kaplan. 1975. Chemotaxis and haemo-statis. Brit. J. Haematol., 13:417-422.

Kay, A. B., D. S. Pepper and M. R. Ewart. 1973. Generation of chemotactic activity for leukocytes by the action of thrombin on human fibrinogen. Nature (New Biology), 243:56-57.

Kay, A. B., D. S. Pepper and R. McKenzie. 1974. The identification of fibrino-peptide B as a chemotactic agent derived from human fibrinogen. Brit. J. Haematol., 27:669-677.

Keller, H. U. and E. Sorkin. 1967. Studies on chemotaxis. V. On the chemotactic effect of bacteria. Int. Arch. Allergy, 31:505-517.

Keller, H. U. 1972. Chemotaxis and its significance for leukocyte accumulation. Agents Actions, 2:161-169.

Keller, H. U., M. W. Hess and H. Cottier. 1975. Physiology of chemotaxis and random motility. Sem. in Hemat., 12:47-57.

Kitao, T., K. Hattori and M. Takeshita. 1974. Electrophoretic analysis of polymorphonuclear cell membrane. Clin. Chem. Acta, 56:211-213.

Klainer, A. S. and C. J. Betsch. 1973. Scanning electron microscopy of the attachment of human polymorphonuclear leukocytes to Staphylococcus aureus. J. Infect. Dis., 127:686-688.

Klebanoff, S. J. 1975a. Antimicrobial systems of the polymorphonuclear leukocyte. In: The Phagocytic Cell in Host Resistance. Bellanti and Dayton, editors. Raven Press, New York.

Klebanoff, S. J. 1975b. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin. Hemat., 12:117-142.

Klebanoff, S. J. and R. A. Clark. 1978. The Neutrophil: Function and Clinical Disorders. North Holland Publishing Co., New York.

Klebanoff, S. J. and C. B. Hamon. 1972. Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. J. Reticuloendothel. Soc., 12:170-196.

Lachman, P. J., A. B. Kay and R. A. Thompson. 1970. The chemotactic activity for neutrophil and eosinophil leukocytes of the trimolecular complex of the fifth, sixth, and seventh components of human complement C567 prepared in free solution by the reactive lysis procedure. Immunology 19:895-899.

Leffel, M. S. and J. K. Spitznagel. 1972. Association of lactoferrin with lysozyme in granules of human polymorphonuclear leukocytes. Infect. Immun., 6: 761-765.

Leffel, M. S. and J. K. Spitznagel. 1974. Intracellular and extracellular degranulation of human polymorphonuclear azurophil and specific granules induced by immune complexes. Infect. Immun., 10:1241-1249.

Leffel, M. S. and J. K. Spitznagel. 1975. Fate of human lactoferrin and myeloperoxidase in phagocytizing human neutrophils: Effects of immunoglobulin G subclasses and immune complexes coated on latex beads. Infect. Immun., 12:813-820.

Lehrer, R. I. 1972. Functional aspects of a second mechanism of candidocidal activity by human neutrophils. J. Clin. Invest., 51:2566-2572.

Lehrer, R. I. and M. J. Cline. 1969. Interaction of Candida albicans with human leukocytes and serum. J. Bact., 98:996-1004.

Lehrer, R. J., J. Hanifin and M. J. Cline. 1969. Defective bactericidal activity in myeloperoxidase-deficient human neutrophils. Nature, 223:78-79.

Logue, G. L., W. F. Rosse and J. P. Adams. 1973. Complement-dependent immune adherence measured with human granulocytes: Changes in the antigenic nature of red cell bound C3 produced by incubation in human serum. Clin. Immunol. Immunopath., 1:398-407.

Malwista, S. E. 1968. Vinblastine: Colchicine like effects on human blood leukocytes. Arthr. and Rheum., 11: 108-109.

Malwista, S. E. 1971. Vinblastine: Colchicine like effects on human blood leukocytes during phagocytosis. Blood, 37:519-529.

Malwista, S. E. and P. T. Bodel. 1967. The dissociation by colchicine and phagocytosis from increased oxygen consumption in human leukocytes. J. Clin. Invest., 46:786-796.

Mancini, G., A. O. Carbonara and J. F. Heremans. 1965. Immunochemical quantitation by single radial immunodiffusion. Immunochemistry, 2:235-254.

Mandell, G. R. 1974. Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. Infect. Immunity, 9:337-341.

Mantovani, B. 1975. Different roles of IgG and complement receptors in phagocytosis by polymorphonuclear leukocytes. J. Immunol., 115:15-17.

Messner, D. J. and J. Jelinek. 1970. Receptors for human G. globulin on human neutrophils. J. Clin. Invest., 49:2165-2171.

Messner, R. P., T. Loxdal, P. G. Quie and R. C. Williams, Jr. 1968. Serum opsonin, bacterial and polymorphonuclear leukocyte interactions in subacute bacterial endocarditis. Antiglobulin factors and their interaction with specific opsonins. J. Clin. Invest., 47:1109-1120.

Metchnikoff, E. 1905. Immunity. In: Infective Diseases. Cambridge University Press, Cambridge.

Michaelis, T. W., N. R. Lorrimer, E. N. Metz and S. P. Balcerzak. 1971. Surface morphology of human leukocytes. Blood, 37:23-20.

Mudd, S., M. McCutcheon and B. Lucke. 1934. Phagocytosis. Physiol. Rev., 14:210-275.

Olsson, F., T. Olofsson and H. Odeberg. 1972. Myeloperoxidase-mediated iodination in granulocytes. Scand. J. Haematol., 9:483-491.

Olsson, I. and P. Venge. 1972. Cationic proteins of human granulocutes. I. Isolation of the cationic proteins from the granules of leukemic myeloid cells. Scand. J. Haematol., 9:204-214.

Penny, R., D. A. G. Galton, J. T. Scott and V. Eisen. 1966. Studies on neutrophil functions. I. Physiological and pharmacological aspects. Brit. J. Haematol., 12:623-632.

Querinjean, P., Jr., R. C. Chandan and K. M. Shahani. 1969. Isolation and characterization of human milk lysozyme. Arch. Biochem. Biophys., 103:59-65.

Quie, P. G., J. G. White, B. Holmes and R. A. Good. 1967. In vitro bactericidal capacity of human polymorphonuclear leukocytes: Diminished activity in chronic granulomatous disease in childhood. J. Clin. Invest., 46:668-679.

Rabinovitch. M. 1968. Phagocytosis: The engulfment stage. Semin. Hemat., 5:134-155.

Rest, R., M. H. Cooney and J. K. Spitznagel. 1978. Subcellular distribution of glycosidases in human polymorphonuclear leukocytes. Biochem. J., 174:53-59.

Scott, R. E. and R. G. Horn. 1970. Ultrastructural aspects of neutrophil granulocyte development in humans. Lab. Invest., 23:202-215.

Scribner, D. J. and D. Fahrney. 1976. Neutrophil receptors for IgG and complement: Their roles in attachment and ingestion phases of phagocytosis. J. Immunol., 116:892-897.

Segal, A. W., J. Dorling and C. Stephanie. 1980. Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. J. Cell Biology, 85:42-59.

Shiffman, E., H. J. Showell, B. A. Cocoran, P. A. Ward, E. Smith and E. L. Becker. 1975. The isolation and partial characterization of neutrophil chemotactic factors from E. coli. J. Immunol., 114:1831-1837.

Shin, H. S., M. R. Smith and W. B. Wood, Jr. 1969. Heat labile opsonins to pneumococcus. II. Involvement of C3 and C5. J. Exp. Med., 130:1229-1241.

Skarnes, R. C. and D. W. Watson. 1957. Antimicrobial factors of normal tissues and fluids. Bact. Rev., 21:273-294.

Spitznagel, J. K., F. G. Dalldorf, M. S. Leffel, J. D. Folds, I. R. H. Welsh, M. H. Cooney and L. E. Martin. 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab. Invest., 30:774-785.

Stecher, V. J. and E. Sorkin. 1972. The chemotactic activity of fibrin lysis products. Int. Arch. Allergy, 43:879-886.

Stecher, V. J., E. Sorkin and G. P. Ryan. 1971. Relation between blood coagulation and chemotaxis of leukocytes. Nature (New Biology), 233:95-96.

Stossel, T. P. 1973. Quantitative studies of phagocytosis: Kinetic effects of cations and heat labile opsonin. J. Cell. Biol., 58:346-356.

Stossel, T. P. 1974. Phagocytosis. New Engl. J. Med., 290: 717-723; 774-780; 833-839.

Stossel, T. P. 1975. Phagocytosis: Recognition and ingestion. Semin. Hemat., 12:83-116.

Stossel, T. P. 1976. The mechanism of phagocytosis. J. Reticuloendothel. Soc., 19:237-245.

Stossel, T. P., C. A. Alper and F. S. Rosen. 1973. Serum-dependent phagocytosis of paraffin oil emulsified with bacterial lipopolysaccharide. J. Expl. Med., 137:690-705.

Stossel, T. P., T. D. Pollard, R. J. Mason and M. Vaughan. 1971. Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. J. Clin. Invest., 50:1745-1757.

Suter, E. 1956. Interaction between phagocytes and pathogenic microorganisms. Bact. Rev., 20:94-132.

Tagesson, C. and O. Stendahl. 1973. Phagocytosis of Salmonella typhimurium 395 MR10 by rabbit polymorphonuclear leukocytes: Formation of phagolysosomes demonstrated by zonal separation. Acta. Path. Microbiol. Scand. (Section B), 81:481-46.

Tempel, T. R., R. Snyderman, H. V. Jordan and S. E. Mergenhagen. 1970. Factors from saliva and oral bacteriae, chemotactic for polymorphonuclear leukocytes: Their possible role in gingival inflammation. J. Peridont., 41:71-80.

Walters, M. N. I. and J. M. Papadimitriou. 1978. Phagocytosis: A Review. CRC Critical Reviews in Toxicology, 377-421.

Ward, P. A. 1967. A plasmin-split fragment of C'3 as a new chemotactic factor. J. Exp. Med., 126:189-206.

Ward, P. A., I. H. Lepaw and L. J. Newman. 1968. Bacterial factors chemotactic for polymorphonuclear leukocytes. Amer. J. Path., 52:725-736.

Ward, P. A. and N. J. Zvaifler. 1973. Quantitative phagocytosis by neutrophils. I. A new method with immune complexes. J. Immunol., 111:1771-1776.

Weksler, B. B. and C. E. Coupal. 1973. Platelet-dependent generation of chemotactic activity. J. Exp. Med., 137:1419-1430.

Welsh, I. R. H. and J. K. Spitznagel. 1971. Distribution of lysosomal enzymes, cationic proteins and bactericidal substances in subcellular fractions of human polymorphonuclear leukocytes. Infect. Immun., 4: 97-102.

West, B. C., A. S. Rosenthal, N. A. Gelb and H. R. Kimball. 1974. Separation and characterization of human neutrophil granules. Amer. J. Path., 77:41-62.

Winkelstein, J. A. 1973. Opsonins: Their function, identity and clinical significance. J. Pediat., 82:747-753.

Wood, W. B., Jr. 1960. Phagocytosis, with particular reference to encapsulated bacteria. Bact. Rev., 24:41-49.

Wood, W. B., Jr., M. R. Smith, W. D. Perry and J. W. Berry. 1951. Studies on the cellular immunology to acute bacteremia. I. Intravascular leukocyte reaction and surface phagocytosis. J. Exp. Med., 94:521-533.

Worthington Biochemical Corp. 1972. Worthington Enzyme Manual, pp. 43-45. Worthington Biochemicals Corp., Freehold, N.J.

Wright, C. S. and M. C. Dodd. 1955. Phagocytosis. Ann. N.Y. Acad. Sci., 59:945-950.

Yoshida, T., S. Cohen, P. E. Bigazzi, T. Kurazuji and A. Amsden. 1975. Inflammatory mediators in culture filtrates of E. coli: Macrophage migration inhibitory, neutrophil chemotactic, lymphocyte mitogenic and fibroblast cytotoxic activities. Am. J. Path., 81:389-400.

Zeya, H. I. and J. K. Spitznagel. 1968. Arginine rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. J. Exp. Med., 127:927-941.

Zeya, H. I. and J. K. Spitznagel. 1969. Cationic protein-bearing granules of polymorphonuclear leukocytes: Separation from enzyme-rich granules. Science, 163:1069-1971.

Zimond, S. H. and J. G. Hirsch. 1973. Leukocyte locomotion and chemotaxis. New methods for evaluation and demonstration of a cell-derived chemotactic factor. J. Exp. Med., 137:387-410.