ELECTRONMICROGRAPHIC STUDIES OF PHAGOCYTIC CELLS

I. MORPHOLOGICAL CHANGES OF THE CYTOPLASM AND GRANULES OF RABBIT GRANULOCYTES ASSOCIATED WITH INGESTION OF ROUGH PNEUMOCOCCUS*†

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THE essential role played by phagocytic cells in destruction of matter foreign to the host and in defense against bacterial disease has been recognized for many years. A clear understanding of the morphological alterations that cells undergo during the process of ingestion of particulate material has been obtained even though the underlying mechanisms have not been firmly established. The intracellular transport and subsequent fate of engulfed matter has been less thoroughly studied. In general, destruction of phagocytosed material has been thought to depend upon enzymatic degradation. This problem was discussed by Florey (1962*a*) in a recent textbook.

The process whereby phagocytosed matter is brought into contact with cellular enzymes has been the object of several recent studies. Robineaux and Frederic (1955) noted that the granules of polymorphonuclear leucocytes disappeared as these cells ingested bacteria and other particulate matter. Hirsch and Cohn (1960) have subsequently made similar observations with leucocytes from several species including man. They also found that certain enzymes—phagocytin, cathepsin, lysozyme, acid and alkaline phosphatase, beta glucuronidase, nucleotidase, ribonuclease, and desoxyribonuclease—were predominantly localized within a granular fraction obtained by differential centrifugation of disrupted granulocytes (Cohn and Hirsch, 1960*a*). Furthermore, Cohn and Hirsch (1960*b*) demonstrated that the supernatant from the granular fraction contained higher concentrations of these enzymes if phagocytosis had occurred before disruption of the leucocytes. It was concluded, therefore, that phagocytosis induced the granules to release their contents of antimicrobial and hydrolytic enzymes into the cytoplasm of the cell. More recently, using phase contrast cinemicroscopy, Hirsch (1962) recorded events thought to represent actual discharge of granule contents into vacuoles containing either bacteria or zymosan particles.

In this report morphological evidence will be presented supporting the concept that as part of the phagocytic process the contents of polymorphonuclear leucocyte granules are released into intracellular vacuoles containing bacteria. Findings thought to represent steps in the transport of ingested material through the cytoplasm to vacuoles will also be presented, and the appearance of the granulocytes in control preparations will be discussed.

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MATERIALS AND METHODS

Preparation of exudate

Adult male rabbits of mixed New Zealand and Flemish stocks maintained on Purina rabbit chow and tap water were given intraperitoneal injections of 400 ml. of 0·1 per cent shell fish glycogen in 0·85 per cent NaCl. After 16–18 hr. the resulting exudate was removed aseptically, and sufficient heparin was added to give a final concentration of 4 units per ml. The exudate contained from 5–10 million cells per ml., more than 90 per cent of which were granulocytes. Altogether, 15 rabbits were used. All glassware for viable leucocyte-bacterium mixtures was siliconized and rendered pyrogen-free by dry heat.

Bacterial culture

A rough strain pneumococcus (R36NC) obtained from Dr. W. B. Wood, Jr. was stored at 4° in defibrinated rabbit blood. Subcultures, grown for 15–18 hr. in brain-heart infusion broth (Difco), were washed once and then resuspended in 0·85 per cent saline before use.

Experimental procedure

Five ml. aliquots of peritoneal exudate prepared as described above were placed in siliconized glass tubes with screw caps and mixed with the washed, rough pneumococcus. The bacterium-leucocyte ratio was approximately 20 to 1. Preparations from which bacteria were omitted were prepared simultaneously in many but not all experiments. The tubes were tightly capped and rotated mechanically at 7–9 r.p.m. in an incubator maintained at either 37° ± 1° or at 25° ± 1°. Following rotation for 5, 10, 15, 30, 60, 90 and 120 min., tubes were removed from the revolving racks and to the contents was added 5 ml. of 1 per cent osmium tetroxide buffered to pH 7·3 either with phosphate in a balanced salt solution or with chromate buffer (Dalton, 1955). A total of 40 such preparations were made. Thirty-three controls were similarly prepared. The tubes were placed in an ice bath and after 25 min. their contents were sedimented at 65 *g* for 5 min. The supernatant was removed by suction, 5–10 ml. of ice cold 10 per cent phosphate buffered formalin (pH 7·1) was added, and the pellet was gently resuspended and allowed to stand for 1 hr. at 4°. The cells were again sedimented and after the fixative was decanted, they were quickly warmed and suspended in 0·1 or 0·2 ml. 3 per cent agar that had been prewarmed to approximately 80°. The agar mixture was promptly chilled, cut into small cubes, and embedded for electron microscopy according to the methods of either Luft (1961) or Low and Clevenger (1962). In some instances, either agar or formaldehyde was omitted from the procedure, and the cell mixtures were concentrated by centrifugation between each change of solution. These variations from the basic procedure did not induce detectable morphological alterations in the cells. Sections cut with glass knives on a Porter-Blum microtome were floated onto distilled water and picked up on either bare or Formvar coated grids. Most sections were stained with 2 per cent aqueous uranyl acetate, but lead staining according to the methods of Millonig (1961) or Karnovsky (1961) was used occasionally. In addition, sections 2–4 μ thick were prepared for phase contrast microscopy.

The initial magnification of the electronmicrographs ranged from 2,500–17,600 diameters. The original negatives were enlarged optically to attain final magnifications of 10–70 thousand diameters on the prints. Approximately 2000 micrographs were made in the course of this study.

EXPERIMENTAL FINDINGS

Observations on control preparations

Granulocytes.—Two distinct cell types were encountered in the control preparations. These were the polymorphonuclear granulocyte (PMNG) and large mononuclear cells. This paper is chiefly concerned with the former. Cellular viability (measured by trypan blue staining, 99 per cent of PMNG were viable at the beginning of experiments) was unchanged after rotation at 37° for 120 mins. In general, cells that were not mixed with bacteria appeared oval in outline with comparatively few, relatively simple, surface projections (Fig. 1). Pseudopod

formation was not striking in control samples or in those containing pneumococcus, in contrast to the findings of Goodman, Moore and Baker (1956) who apparently used a stationary preparation. Control cells usually remained separated although sometimes they aggregated into small loose collections. There was little or no tendency for dense clumps of agglutinated cells to form (Tullis, 1953).

As has been found to be characteristic of PMNG, the granulocytic cell found within peritoneal exudate of rabbits contained multilobed nuclei, rare mitochondria, poorly developed endoplasmic reticulum, a small but well developed Golgi membrane complex and numerous inclusions corresponding to the granules of light microscopy (Fig. 1). In well prepared specimens, the nuclei were finely granular, and in general the electron dense material was more densely packed along the nuclear membranes than in the centre of the nuclei.

The granules seen in PMNG by light microscopy, herein referred to as granules or leucocytic granules, were of several types as characterized by size and electron density. The outlines of the granules were generally circular, but many oval and dumb-bell shapes were recorded. All were enclosed by an electron dense membrane. The most prominent of these bodies contained coarsely granular dark-staining material (DG in Fig. 1). Occasionally the material was in the form of large grains congregated along the limiting membrane in clumps to form a rosette about a clear centre. Less striking but more numerous was a smaller variety of leucocytic granule (LG in Fig. 1). These small bodies were not nearly so dark as those just described, and they contained fine-grained material. These resembled the granules of the human neutrophil. Occasionally it could be seen that these smaller bodies contained structures indistinguishable from cristae mitochondriales so that one could not say with absolute certainty that they were not mitochondria. In such instances they differed from conventional mitochondria in that their internal membranes were poorly delineated. A third type of granule resembled a target with a dark centre and a light outer ring (TG in Fig. 1).

Vacuoles were found within some cells from control preparations, but they were not generally prominent, occupying only a small fraction of the cellular cross section. Vacuolization in the control preparations was never as marked as that seen after phagocytosis. In a rare cell, small vacuoles were so numerous they imparted a foamy appearance to the granulocyte. As these small vacuoles were the same size as the small light-staining granules, it is possible that they were derived from such granules. If this were true there was no clue as to the fate of the electron dense contents formerly contained in these granules. It is to be emphasized that this finding was very uncommon.

Occasionally vacuoles containing material with morphological characteristics similar to endogenous leucocytic pyrogen as described by Goodale, Fillmore and Hillman (1962) and by Gander, Fritz and Goodale (1962) were found in control preparations following extended rotation. This material was also found extracellularly in control and experimental preparations.

Otherwise the cells of the control preparations remained unaltered after 2 hr. of rotation at 37° and there was no evidence of change suggestive of cellular dissolution or death.

Mononuclear cells.—Mononuclear cells were easily distinguished from granulocytes by their slightly larger size and by the presence of a large single-lobed nucleus. The mitochondria of monocytes were larger and more prominent than those noted in granulocytes, and the endoplasmic reticulum was more clearly

developed. Granules were not commonly encountered in monocytes although an occasional dense body was present. The fibrillar component recently described in monocytes of several species by de Petris, Karlsbad and Pernis (1962) was not seen. Unlike granulocytes, preparation of monocytes for electron microscopy presented no particular difficulties.

Structure of the pneumococcus

The phagocytosed pneumococcus was readily recognized by a dense outer membrane enclosing granular cytoplasm containing filamentous material (Figs. 2, 3, 4 and 5). The bacteria found inside the cells were frequently coated with an electron dense shell. When the pneumococcus was found extracellularly, no capsule or shell could be seen. It was not the purpose of this study to delineate the structure of the bacterium further than required for accurate identification.

Alterations in the morphology of granulocytes associated with phagocytosis

The intracellular changes that took place after phagocytosis were progressive and could not be separated into distinct stages even though representative samples were studied at short intervals during protracted rotation. Consequently, no attempt was made to characterize the findings at each time studied. The most rewarding specimens were prepared after either 30 or 60 min. rotation at room temperature. Under these conditions the morphological changes associated with phagocytosis were advanced, yet cellular dissolution was not sufficient to make the cells unfit for study. Furthermore, since the rate of phagocytosis was known to be temperature dependent, and since the intracellular changes to be described apparently took place more slowly at 25° than at 37°, it was possible to obtain more examples of the early stages of vacuolization and discharge of granules in those experiments performed at the lower temperature. Those findings in the cytoplasmic contents thought to represent steps in the transport of ingested material through the cytoplasm to vacuoles and the deposition of granule contents in the vacuoles will be described.

EXPLANATION OF PLATES

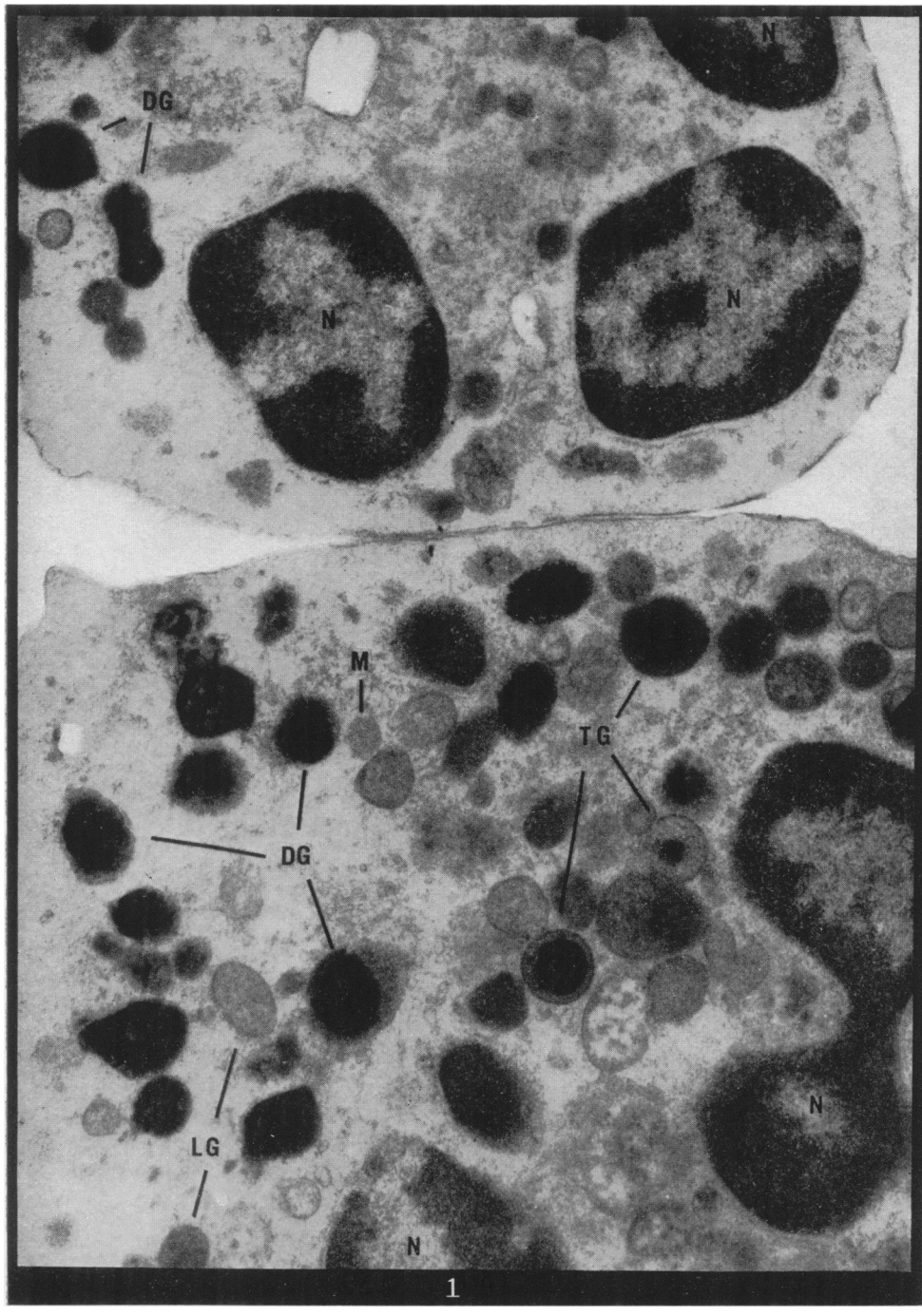
FIG. 1.—Polymorphonuclear granulocytes from control preparation. The large dark-staining (DG), small granular light-staining (LG) and target-like granules (TG) are illustrated. Note the relatively smooth cell membrane, multilobed nuclei (N) and the small mitochondria (M). Uranyl acetate stain. ×19,500.

FIG. 2.—A vacuole (V) with protrusions believed derived from leucocytic granules (G). A pneumococcus (P) is within the vacuole. The small bodies (M) within the vacuole may represent portions of mitochondria. Lead stain. ×24,500.

FIG. 3.—Similar to Fig. 2 but at lower magnification. A pneumococcus and material similar to the contents of granules of the dense type (DG) are within protrusions from a vacuole. Other labels as before. Uranyl acetate stain. ×15,000.

FIG. 4.—Two dark bodies (DG) are closely applied to pneumococci within a vacuole. See Fig. 5. Uranyl acetate stain. ×53,000.

FIG. 5.—Vacuole in a PMNG containing dark material in round clumps (DG) and ill-formed masses (R) thought to represent the contents of leucocytic granules after discharge into the vacuole, before complete dispersal. Note the close relation between the pneumococci and the dark material. Beads (B) of electron dense material line the wall of the vacuole as was frequently seen. Uranyl acetate stain. ×30,500.



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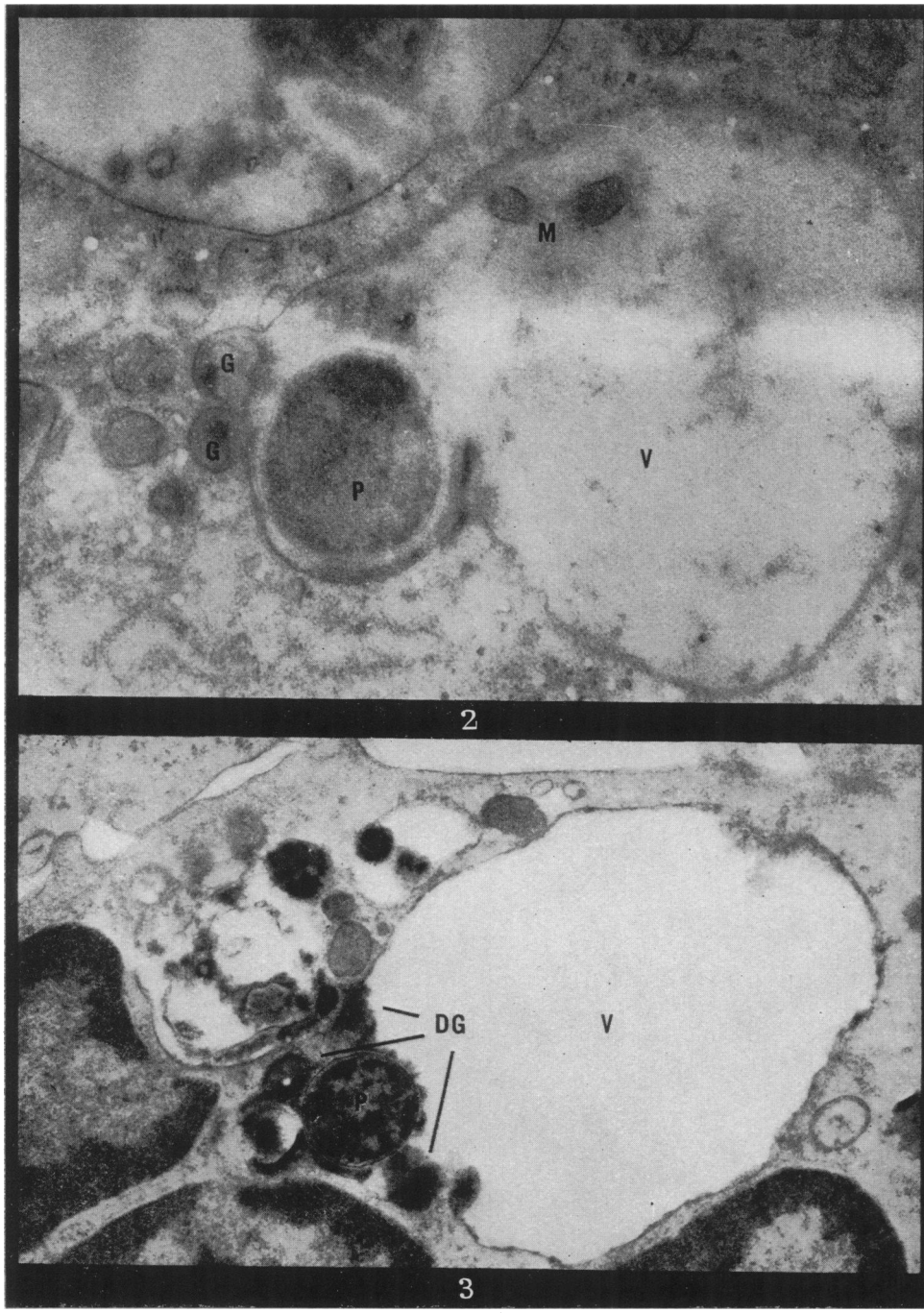

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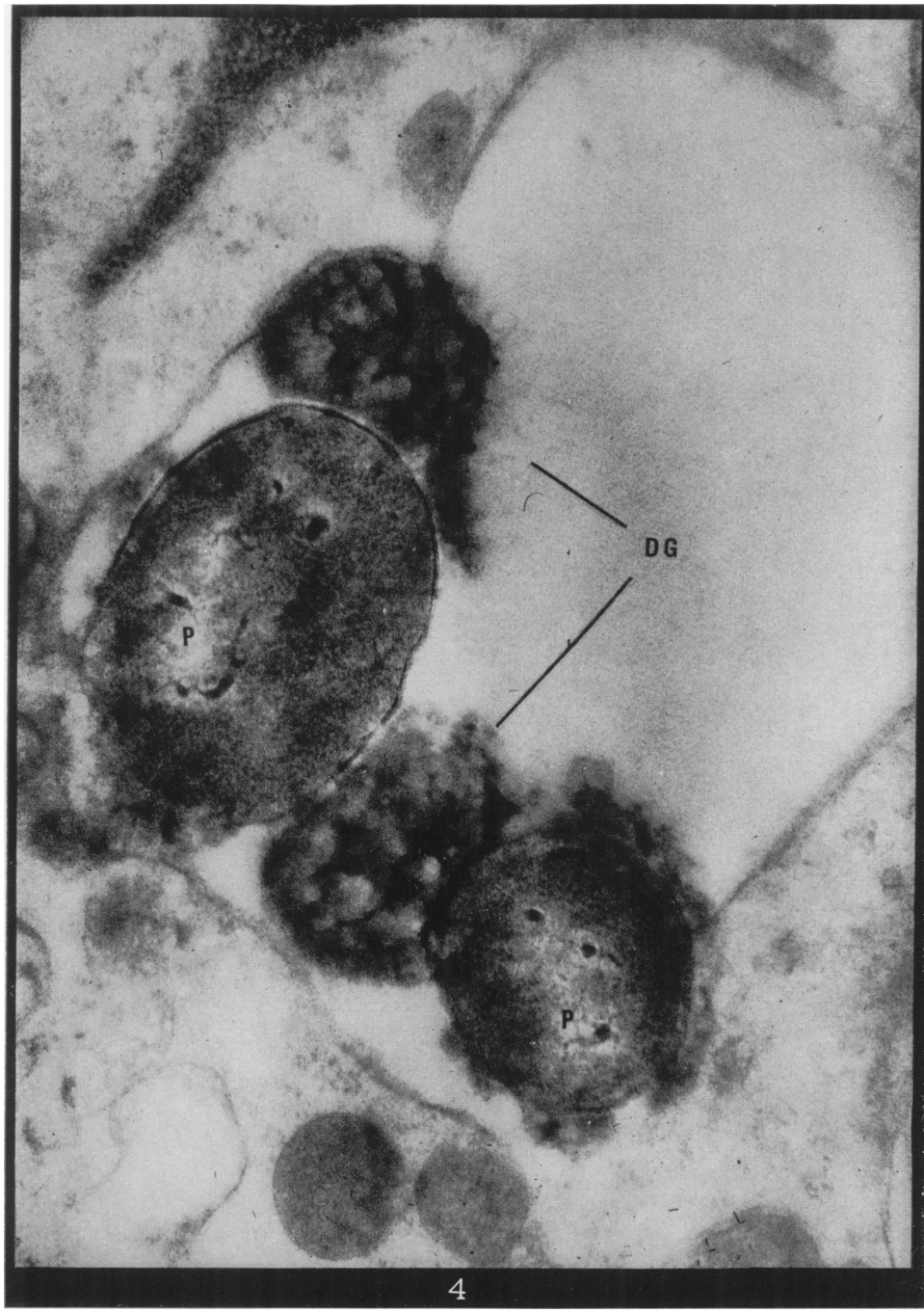

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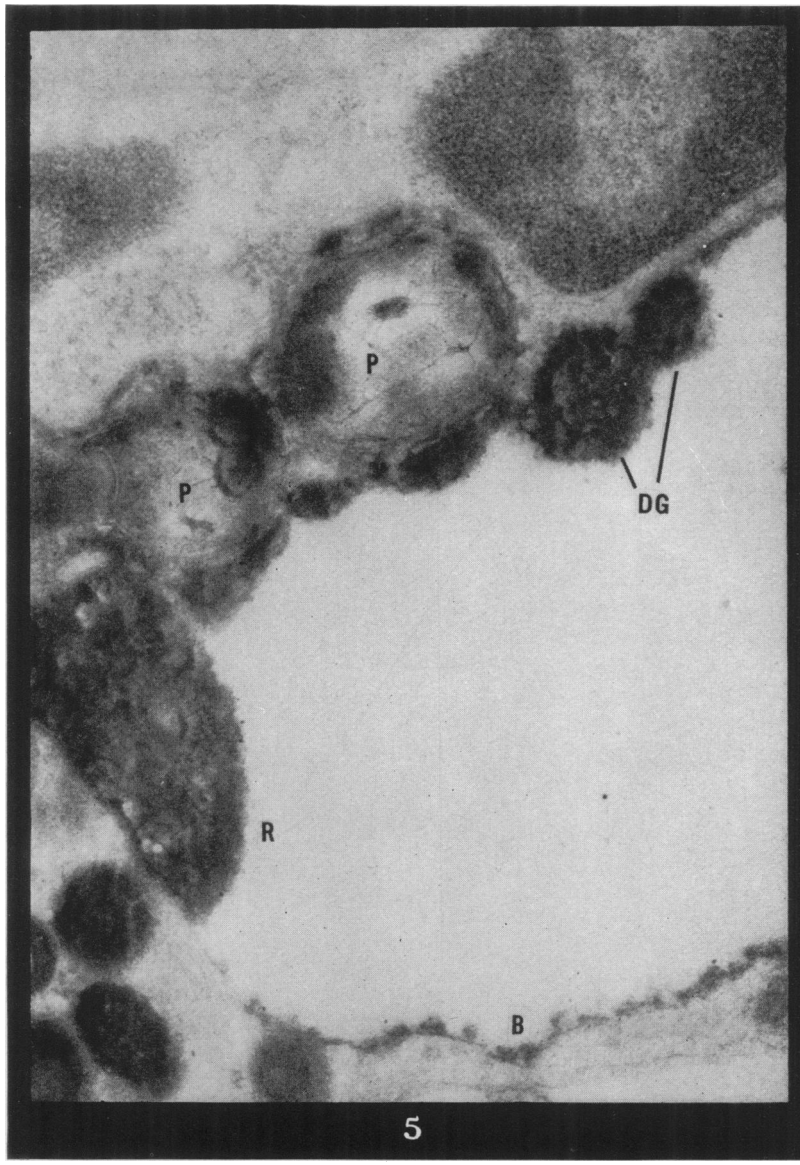

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Phagocytosed pneumococci were found in the specimens prepared 5 min. after mixing. In specimens examined after such short periods of incubation, the intracellular bacteria were often found singly or in pairs, enclosed in vacuoles with dimensions only slightly larger than the pneumococcus. Frequently, the organisms were separated from the compartment wall by irregular collections of electron dense material. After 30 min. rotation, most cells contained ingested bacteria and large vacuoles. Although bacteria were still found in small vacuoles, the larger compartments usually contained one or more pneumococci. Frequently, there was direct communication between a small vacuole containing a pneumococcus and a larger compartment (Figs. 2 and 3).

In general the appearance of the leucocytic granules was altered after phagocytosis of pneumococci by their parent cell. In some instances, especially after brief periods of phagocytosis, no difference from control preparations could be detected, even though ingested bacteria were visible. At the other extreme was the very rare finding of cells containing only empty membrane-bound spaces, presumably the shells of granules after their contents were either discharged or altered so as to render them electron transparent as was discussed under controls. In other examples, rosettes of dense clumped matter, also similar to those found in some of the control preparations, lined the membranes of practically all visible leucocytic granules within the cells. Another finding, perhaps related to vacuole formation, was occasionally seen wherein the leucocytic granules were larger than those seen in control preparations. In this instance the electron dense contents were visible as widely separated chunks and imparted an appearance that might have resulted from distention of granules by the inflow of an electron transparent material.

When the pneumococcus was found in a protrusion from a large vacuole, collections of amorphous electron dense material usually surrounded the bacterium and coated the adjacent inner wall of the vacuole (Figs. 4 and 5). This amorphous material could not be distinguished morphologically from the contents of the large dark leucocytic granules. This matter also resembled closely the electron dense shell that enclosed the bacteria within the small vacuoles, but it was more abundant. As illustrated in Fig. 2, communication between vacuoles and membrane-bound bodies believed to be granules was found. Furthermore, dense round clumps of amorphous material with dimensions the same as those of the large dark granules were found near pneumococci within vacuoles (Figs. 3, 4 and 5). In many vacuoles, small dense beads, presumably dispersed granule material, lined the internal membrane (*B* in Fig. 5).

On a few plates mitochondria-like structures were found within vacuoles (Fig. 2). Subsequently this observation has been made with greater frequency in similar preparations of human leucocytes. The significance of this finding is obscure and it may represent an artifact.

In many vacuoles, small infoldings created irregularities in the membrane that was generally smooth. The significance of this finding remains speculative.

Dissolution and destruction of engulfed organisms was not studied. It was known that at least 95 per cent of an avirulent pneumococcus strain was killed shortly after ingestion by rabbit PMNG, and direct observation by phase contrast microscopy showed that intracellular bacteria disappeared gradually over several hours. This study provides no evidence pointing to what had happened to kill these vacuole-bound organisms since no consistent structural changes were noted.

DISCUSSION

These studies have documented with electronmicrographs the facility with which polymorphonuclear granulocytes ingest a particular type of bacterium. Of more fundamental importance, however, were the findings supporting the concept that the contents of the leucocytic granules were discharged into vacuoles containing bacteria. These observations not only confirmed but also extended the recent cinephotomicrograph findings of Hirsch (1962) who found that the granules of phagocytic leucocytes of several species seemed to disappear in the vicinity of vacuoles containing engulfed bacteria. Previously Cohn and Hirsch (1960*b*) had reported that the bactericidal and hydrolytic enzymes located within the granules of PMNG could be found within the cytoplasm of cells after phagocytosis had taken place. In keeping with this, in our electronmicrographs direct communication between the leucocytic granules and the interior of vacuoles containing ingested pneumococci was clearly apparent. Thus the hypothesis that granule contents are released intracellularly after phagocytosis was given morphological backing. The electron micrographic findings supporting the thesis were : the ingested bacteria, coated with material indistinguishable from from granule contents were found in small vacuoles, collections of material the same size, shape and appearance as the contents of large dark granules were found within the vacuoles, protrusions from walls of spaces containing pneumococci were not only the size of leucocytic granules but were filled with material identical in appearance with the contents of the granules.

The conditions under which phagocytosis occurred in this experiment were obviously highly artificial. Furthermore, the cells studied were stimulated to enter the peritoneal cavity by an irritating injection containing glycogen. These PMNG were not, therefore, identical with cells found in the circulating blood since they had responded previously to an irritant. No doubt of considerable physiological importance, the cells and bacteria were mixed in siliconized glass tubes. Nevertheless, the morphology of the PMNG did not differ materially from that of rabbit cells studied within blood vessels (Florey, 1962*b*) and they were efficient phagocytes.

Evidence supporting the validity of our observations was obtained from studies of experimental meningitis by Nelson, Blinzinger and Hager (1962). Although direct communication between granules and vacuoles of PMNG were not described from their electronmicrographs, several factors may have contributed to this. In the first place, the use of methacrylate embedding may have impaired the quality of the sections. Next, and possibly of even more importance, was the fact that in their experiment, organisms were injected into the subarachnoid space of healthy animals 2 hr. before the first specimens were taken. Unquestionably, there was a time lag before PMNG arrived in large numbers, but it is possible that phagocytosis had occurred an hour or more before the specimens were taken. In our *in vitro* studies, when the time between phagocytosis and fixation was prolonged, such extensive intracellular change had taken place that granule-vacuole communication was difficult to find. Even under experimental conditions designed to favour finding such an event, electronmicrograph documentation was comparatively rare. Thus, in keeping with Hirsch's cinephotomicrograph results, it seems most likely that discharge of granule contents should be found best shortly after phagocytosis.

Other aspects of the changes in the phagocyte were the formation of large vacuoles and the deposition of pneumococci within them. Five min. after mixing the phagocytes and bacteria, the organisms were commonly found either singly or in pairs within small cytoplasmic vacuoles but large vacuoles were rare. However, after 15 mins. or longer had elapsed, large vacuoles were common and as time passed the number of organisms within them increased substantially. This is in keeping with the observations of others who postulated that following ingestion, bacteria were confined to small vacuoles for transport through the cytoplasm of the PMNG for deposit into vacuoles that formed in response to phagocytosis (Florey, 1962*a*). There is the alternate possibility that the small compartments containing engulfed bacteria may become larger and eventually assume the appearance of large vacuoles, but the finding of bacteria in protrusions from large vacuoles apparently indicates that this is not always the case. Shortly after phagocytosis and presumably while *en route* from the cell membrane to the large cytoplasmic vacuoles, contact between the ingested bacteria and intragranular enzymes was established by discharge of granule contents into small pneumococcus-containing vacuoles. While these changes were taking place, the contents of other leucocytic granules were discharged into the large vacuoles. No clue was found pointing to the means by which enzyme-containing granules were transported to the vicinity of vacuoles. Neither the nature of the stimulus for vacuole formation nor the cause of vacuole-granule coalescence could be ascertained from our electronmicrographs. However, the combination of circumstances leading to formation of vacuoles, the discharge of digestive enzymes into vacuoles, and the agglutination of phagocytic cells into clumps had a deleterious effect upon both cellular integrity and viability.

Occasionally, extracellular material morphologically similar to granule contents was encountered. This material closely resembled the endogenous leucocytic pyrogen previously described by Goodale, Fillmore and Hillman (1962).

An unequivocal identification of leucocytic granule contents as either active enzyme or enzyme precursor was not achieved in these studies, but an approach to this problem using histochemical electronmicrograph techniques is planned.

SUMMARY

Cells from rabbit peritoneal exudate were mixed with a rough pneumococcus to permit phagocytosis. After rotation in siliconized glass tubes, samples of the mixtures were prepared for electron microscopy. Phagocytosis proceeded rapidly and pneumococci were found enclosed in small cytoplasmic vacuoles within 5 min. after mixing. The ingested organisms were usually coated with an electron dense material morphologically identical with the contents of the granules of leucocytes. Material believed to be granule material was found in the vacuoles. Direct contact between the ingested material and the contents of leucocytic granules was thus demonstrated. This work provides morphological evidence for the hypothesis that the contents of leucocytic granules are discharged into cytoplasmic vacuoles after phagocytosis of bacteria. This may represent part of a mechanism whereby cells attempt to destroy ingested material.

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