

CINEMICROPHOTOGRAPHIC OBSERVATIONS ON GRANULE LYSIS  
IN POLYMORPHONUCLEAR LEUCOCYTES DURING  
PHAGOCYTOSIS\*

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PLATES 108 TO 114

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In a previous study, techniques were described for isolating in a reasonable state of purity the cytoplasmic granules of rabbit polymorphonuclear leucocytes (1). Several hydrolytic enzymes and the bactericidal agent phagocytin were found to be localized in these structures. Exposure *in vitro* of leucocyte granules to a pH below 5.0, or to certain surface-active substances resulted in lysis with liberation of their enzymes in a soluble form. Cytoplasmic granules in polymorphonuclear leucocytes thus resemble closely the structures called lysosomes by deDuve and coworkers (2).

Disappearance of cytoplasmic granules following ingestion of bacteria was first observed by Robineaux and Frederic in phase contrast motion pictures of guinea pig leucocytes (3). Degranulation and release of granule-associated enzymes into soluble cell fractions have also been demonstrated during phagocytosis of microorganisms by rabbit polymorphonuclear leucocytes (4, 5). Two possible mechanisms were proposed for granule rupture associated with particle ingestion: (a) acid lysis, with the fall in intracellular pH resulting from the increased lactic acid production known to occur during phagocytosis (6), and (b) fusion of the granule membrane with the invaginated cell membrane surrounding the engulfed material, in which event granule contents would be discharged directly into the phagocytic vacuole. This communication presents the findings on direct microscopic observation and motion picture recording of granule rupture accompanying phagocytosis of microorganisms by human, rabbit, and chicken polymorphonuclear leucocytes.

*Methods*

*Leucocytes* were prepared from heparinized human blood by the dextran sedimentation technique (7), from rabbit peritoneal exudates induced by glycogen (8), and from buffy coats of heparinized chicken blood. White cells were suspended in homologous plasma or serum.

*Microorganisms* to be engulfed were *Bacillus megaterium* and zymosan (yeast cell walls),

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selected because of their large size and susceptibility to phagocytosis. *B. megaterium* was cultured overnight at 38°C in penassay broth, collected and washed in saline on the centrifuge, and finally suspended in saline. Zymosan (Standard Brands, Inc., New York) was boiled in saline for 10 minutes, followed by repeated washing and suspension in saline. Concentrations of leucocytes and of organisms to be ingested were adjusted to yield satisfactory densities on the slide preparations.

*Thin preparations* of the mixture of phagocytes and organisms were essential for adequate visualization of intracellular events. Standard microscope slides and 20 × 40 mm coverslips were washed in non-ionic detergent and rinsed thoroughly. At the time of use these were polished with a lint-free cloth and all dust was removed with the aid of a camel's hair brush. Approximately 0.02 ml drops of the cell and particle suspensions were mixed on one end of the slide, after which 0.003 ml of the mixture was transferred to the coverslip by means of a platinum loop. On inversion of the coverslip a rapid and uniform spread of the suspension indicated adequate cleanliness. The coverslips were sealed with petroleum jelly and placed in a microscope stage incubator at 38°C (human cells) or 40°C (rabbit and chicken cells). On microscopic examination three zones were apparent in these slide preparations: (a) a large central area too thick to permit adequate definition of intracellular structures, (b) a peripheral very thin zone in which cells were ruptured or so confined as to interfere with locomotion, and (c) a narrow strip between the above two, showing well spread cells exhibiting normal movements.

*Cinemicrophotography* under phase contrast was accomplished using a Zeiss ultraphot II with neofluar phase 100 × oil immersion objective, a monocular tube with a Zeiss kpl 10 × ocular, and an arriflex 16 mm motor-driven camera without lenses. Motion pictures were recorded under tungsten illumination on kodak tri X negative film at a speed of 10 frames per second.

*Illustrations* shown in this communication were enlarged in the ultraphot from single frames of the motion pictures. An adapter to accommodate 16 mm film was constructed to replace the microscope stage. Enlargements were made employing a 1 × objective with no condenser. Kodabromide F-4 paper was placed in the 4 × 5 inch film cassettes. Final magnification could not be calculated with precision, but ranged from 1000 to 2000 (Figs. 1 to 8).

## RESULTS

Under the conditions employed polymorphonuclear leucocytes spread out and began to move almost immediately. Direction of locomotion was random, usually in a zigzag pattern, until the granulocyte came within 10 to 20 microns of a zymosan particle or *Bacillus megaterium*, at which time chemotaxis became evident with directed straight line movement towards the microorganism. Phagocytosis was accomplished readily. On contact the leucocyte and particle appeared to become firmly adherent; the cell cytoplasm then flowed around the particles until the process of ingestion was completed by fusion of cell membranes. At all stages cell membrane was seen to be in close apposition to the material being ingested; no visible extracellular fluid was taken in along with the particle, and no vacuoles were seen surrounding the engulfed material until the degranulation reaction began (see below). Completion of phagocytosis usually required 30 seconds to 2 minutes, depending on rate of locomotion of the cell and size of the objects to be ingested.

During ingestion of zymosan or of *B. megaterium* by human neutrophilic leucocytes, cytoplasmic granules seemed to become adherent to the surface of

the engulfed particle. Disappearance of granules was readily noted during the over-all process, but the small dimensions of human leucocytic granules rendered it impossible to observe directly individual granule lysis or to establish with certainty the site of granule rupture. Clear zones formed slowly about both types of particles and frequently it appeared that granule lysis preceded and was associated with vacuole formation.

Rabbit polymorphonuclear leucocyte granules also adhered to the surface of the engulfed microorganisms, and their size was sufficiently large to allow observations on individual granule disruption. Granule lysis characteristically began early in the course of engulfment; as soon as firm attachment between phagocyte and particle had been made and the enveloping process had begun, granules in contact with the invaginating cell membrane began to rupture. As ingestion proceeded additional granules which encountered the zymosan or *B. megaterium* lysed. Upon completion of engulfment most of the granules had disappeared except for those located in other parts of the cell. With passage of time and shift in intracellular position of the engulfed organism, additional granules contacting it frequently ruptured. In healthy cells granule lysis was never seen spontaneously (*i.e.* in the absence of phagocytosis), nor did granules rupture in cytoplasmic sites removed from the engulfed particle. Rabbit leucocytic degranulation consisted of rapid, almost explosive disappearance of the phase-dense granules, leaving in their place bright empty-appearing spaces which themselves soon vanished. The over-all effect was one of flashes of light replacing the dark granules. In many instances vacuoles were seen to form about the engulfed organisms as soon as granule lysis began; these vacuoles enlarged with continuing granule rupture, giving the impression that granule contents were discharged into the space between the microorganisms and the invaginated cell membrane surrounding it.

Studies on chicken polymorphonuclear leucocytes made possible observations on detailed morphologic aspects of granule lysis. Cytoplasmic granules in these cells are oblong bodies much larger than those of rabbit white cells. Those granules adjacent to the particle being ingested began to lyse early in the course of phagocytosis. Approximately 0.1 second prior to rupture they commonly rounded up. Rupture was usually completed in 0.1 to 0.2 second, leaving in place of the phase-dense granule a bright clear space with a tiny round phase-dense body in its center. Concurrent with bursting of the granule a rim of darkening was visible on the adjacent surface of the particle being ingested. Lysis of chicken leucocyte granules appeared to be a violent process, accompanied by recoil of adjacent granules. The instantaneous rupture was followed by rapid (usually several seconds in duration) disappearance of all residuals; first the tiny dark remnant in the clear zone faded, then the phase-dense area on the surface of the adjacent engulfed particle lightened, and the clear zone began to shrink towards the organism, finally to disappear completely. On

occasions granule rupture spread contiguously; *i.e.*, following initial lysis an adjacent granule or granules ruptured into the remaining clear zone. Spreading lysis of this type occasionally led to formation of a clear zone occupying up to one-fourth of the cell cytoplasm. As was the case with individual ruptured granules, these large clear zones gradually contracted towards the engulfed particle and eventually disappeared.

#### DISCUSSION

The observation of Robineaux and Frederic (3) that cytoplasmic granules disappeared in guinea pig polymorphonuclear leucocytes which had engulfed microbes has been confirmed by Sbarra *et al.* (9). Our previous studies (4, 5) and the present work establish that degranulation also follows phagocytosis in human, rabbit, and chicken leucocytes.

Robineaux and Frederic suspected that granule disruption occurred principally in sites adjacent to the engulfed particles, but their techniques were not adequate to provide a clear picture of detailed aspects of granule lysis. Two technical factors employed in the present work made it possible to visualize these detailed aspects: (*a*) Use of the chicken polymorphonuclear leucocyte was advantageous because of the large size of its cytoplasmic granules; and (*b*) motion pictures were taken at a "fast motion" speed of 10 frames per second, rather than employing a time lapse system. The degranulation reaction proceeds with such rapidity that much of the process would be missed under conditions of time lapse exposure.

Possible mechanisms proposed for degranulation during phagocytosis were: (*a*) fusion between granule membrane and the leucocyte membrane overlying the engulfed particle, with discharge of granule contents into the phagocytic vacuole, and (*b*) rupture of the granule membrane by acidity developed during the ingestion process, with discharge of granule contents into the cytoplasm. Results of the present investigation may now be discussed in relation to these two working hypotheses. Five points may be considered:

1. Lysis occurred only in those granules situated adjacent to the engulfed organism (or, more precisely, the invaginated cell membrane surrounding it), or in apposition to clear zones remaining from prior granule rupture. Obviously this finding fits well with the membrane fusion concept. If, on the other hand, degranulation were due to acidity or other physicochemical changes in the cytoplasm, then it becomes necessary to explain why these conditions develop only in the immediate vicinity of the engulfed material.

2. Visible vacuoles commonly began to form about the ingested organisms shortly after initial granule rupture, and these vacuoles grew larger as degranulation progressed. This observation also supports the notion of membrane fusion, with discharge of granule contents into the "extracellular" space surrounding the microorganism leading to the formation of a visible vacuole.

Conceivably, however, degranulation and vacuole formation could be correlated in time but not be directly related one to the other.

3. Rupture of an individual granule in the chicken polymorphonuclear leucocyte was rapid and violent, with recoil of adjacent structures and creation of a sharply demarcated clear zone somewhat larger than the original granule, thus suggesting that granule contents were under tension. At first thought these findings seem to favor rupture of granules free in the cytoplasm. However, if a rigid granule membrane were to fuse with a cell membrane having distensible properties, a similar picture might result. Firm attachment between leucocytic cell surface and the engulfed particle could impair immediate spread of granule contents in the phagocytic vacuole; such spread when it did occur could then account for contraction of the clear zone towards the ingested organism as observed.

4. Associated with lysis of chicken leucocytic granules was a rim of darkening on the adjacent surface of the particle being engulfed. One possible explanation of this phenomenon is that some granule contents combine with and alter the phase properties of the surface of the organism (if membrane fusion occurs) or the cell membrane surrounding the organism (if free lysis takes place). Alternatively, the rim of darkening might be an optical "artifact," the phase density in this case being merely a consequence of change in the adjacent medium; *i.e.*, replacement of adjacent cytoplasm by a clear zone.

5. A small, phase-dense, round body was often seen in the clear zone resulting from lysis of chicken white cell granules. The possibility that this body is contracted granule membrane comes to mind, a concept which does not fit with the membrane fusion hypothesis. The perfectly round nature and very rapid disappearance of this structure speak somewhat against its being granule membrane. Perhaps this dense body is contained within the granules, to be liberated and then dissolved following their rupture.

The membrane fusion hypothesis cannot in any event be established firmly by observations made with the light microscope. Further evidence may be obtained by electron microscopy; specimens for this purpose would need to be fixed early in the ingestion process and would probably require considerable searching, since lysis proceeds so rapidly (0.1 second or less) that the likelihood of catching a granule in the act of discharging would be small.

Why does lysis not occur in granules situated near the membrane of the normal cell which has not engulfed foreign material? In ameboid cells a clear zone, the hyaloplasm, lies immediately beneath the cell membrane and might well prevent contact between cytoplasmic organelles and the inner membrane surface; it is thus necessary to suppose that the hyaloplasm disappears from invaginated cell membranes overlying engulfed particles. In addition, change in charge or other physicochemical properties of cell membrane to which a microorganism has become attached might lead to development of an attractive force or a lytic system for granular membrane.

Markedly degranulated leucocytes survived for periods up to 1 hour as evidenced by continuing cell locomotion. Since the mature polymorphonuclear leucocyte has a short life span *in vitro* (less than 24 hours), no observations could be made on the possibility of regeneration of granules. These highly specialized cells are incapable of reproduction and deficient in endoplasmic reticulum; it seems on these grounds highly unlikely that regeneration of granules can occur.

Metchnikoff originally coined the name phagocyte (eating cell) to signify their similarity to free-living motile cells whose nutrition was dependent on ingestion of food particles from the environment. He also pointed out that phagocytes were richly endowed with a variety of ferments to enable them efficiently to perform their task (10). We may now add to these early concepts of Metchnikoff the fact that, in mammalian polymorphonuclear leucocytes, digestive enzymes and some antibacterial substances are sequestered within cytoplasmic granules (lysosomes), to be discharged at time of need directly into or about the "stomach" or digestive vacuole surrounding engulfed foreign matter. It remains to be determined whether or not similar mechanisms operate in other types of phagocytic cells such as macrophages and free-living amebae.

#### SUMMARY

Phagocytosis of yeast cell walls and of *Bacillus megaterium* by human, rabbit, and chicken polymorphonuclear leucocytes has been observed by phase contrast microscopy and recorded on motion picture film. In suitably thin preparations intracellular events could be visualized well.

Lysis of cytoplasmic granules began early in the course of the ingestion process, rupture occurring only in granules adjacent to the microorganism being engulfed. Formation of a visible vacuole about the ingested particle frequently followed degranulation.

Chicken polymorphonuclear leucocytes, with their large phase-dense granules, were particularly suitable subjects for observations on detailed morphologic aspects of granule lysis. Rupture took place rapidly (0.1 second or less); in place of the granule there appeared a clear zone, often with a small phase-dense round structure in its center. Also accompanying granule lysis was an increase in phase density of the adjacent surface of the microorganism. Over the course of the following few seconds the darkening on the organism faded, the dense small body disappeared from view, and the clear zone contracted towards the engulfed particle.

The observations are discussed in relation to the hypothesis that fusion takes place between the granule membrane and the invaginated cell membrane overlying the ingested particle, with discharge of granule contents directly into the phagocytic vacuole.

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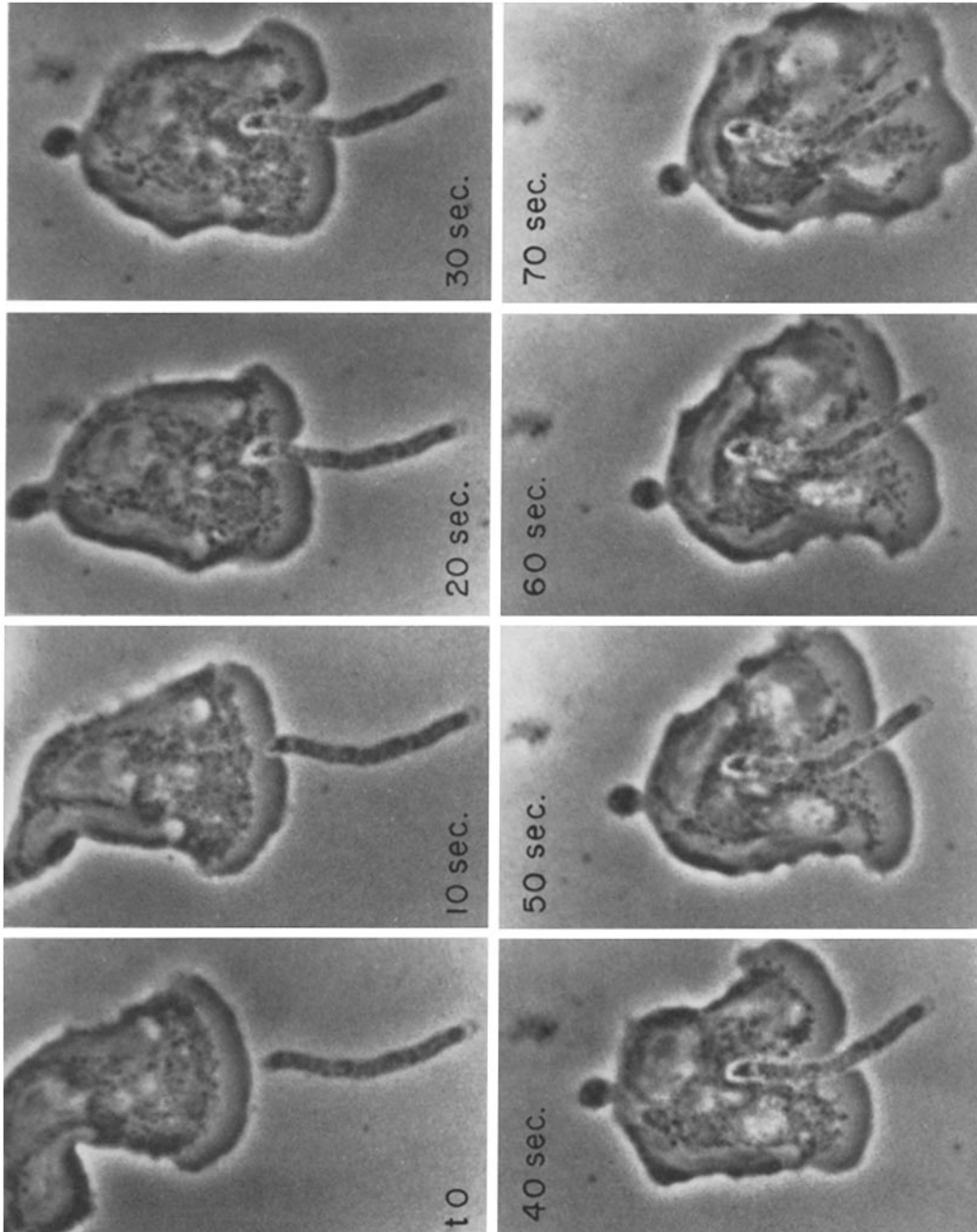
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## EXPLANATION OF PLATES

## PLATE 108

FIG. 1. Phagocytosis of *Bacillus megaterium* by a human polymorphonuclear leucocyte. Note the over- all reduction in content of cytoplasmic granulations by the time ingestion has been completed. Approximately X 2000.





(Hirsch: Granule lysis in leucocytes)

PLATE 109

FIG. 2. Comparison of a normal human polymorphonuclear leucocyte (left), and a granulocyte which has engulfed large numbers of *Bacillus megaterium* (right). Large vacuoles have formed about the bacteria; in the living cell the microorganisms floated freely in these clear zones. The cell at right also shows nearly complete disappearance of cytoplasmic granules and increased phase contrast definition of nuclear structures. This degranulated leucocyte exhibited normal locomotion and phagocytic function, proceeding to ingest in normal fashion the bacillus seen at the bottom of the picture. Approximately  $\times 2000$ .

FIG. 3. Phagocytosis of *Bacillus megaterium* by a rabbit polymorphonuclear leucocyte. Note the disappearance of cytoplasmic granules, and the formation of a visible vacuole about the microorganism following granule lysis. Approximately  $\times 1000$ .

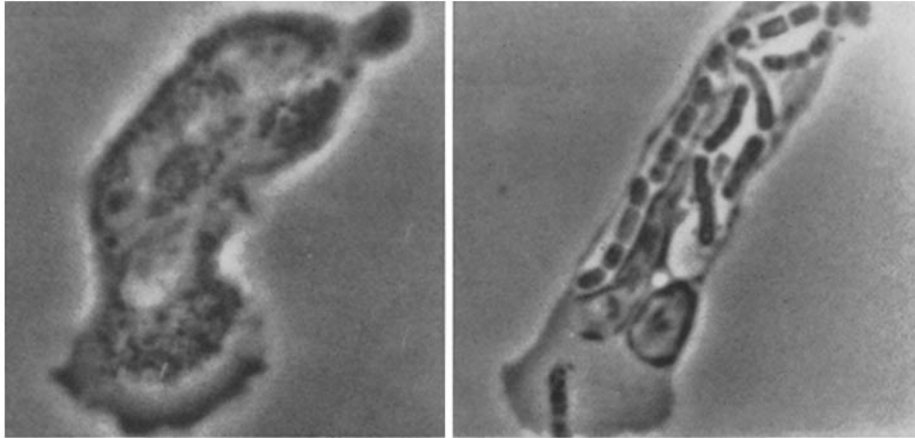


FIG. 2

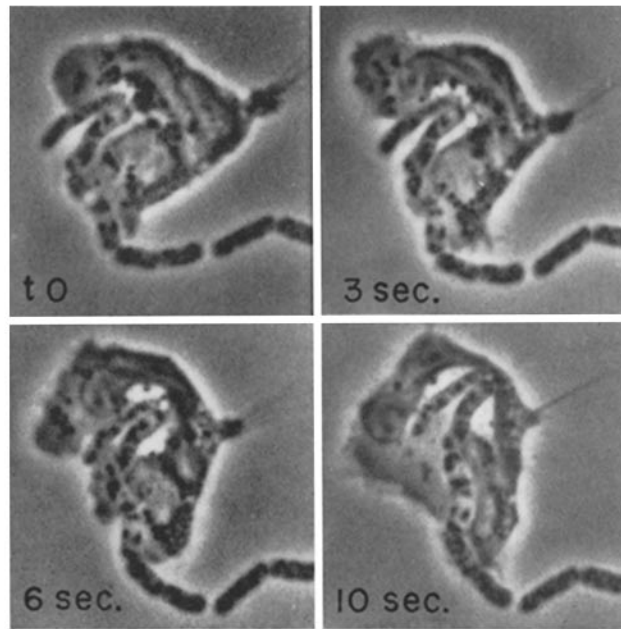


FIG. 3

(Hirsch: Granule lysis in leucocytes)

PLATE 110

FIG. 4. Phagocytosis of zymosan bodies by a chicken polymorphonuclear leucocyte. The phase contrast appearance of the zymosan particles (*Z*) is seen in the *t* 0 picture; they are composed of a central phase-dense nucleoid structure and a surrounding layer of cell wall which appears lighter.

The sequence of prints demonstrates over-all aspects of the phagocytic process. Note the close apposition of leucocytic membrane to the zymosan. Clear zones seen in the cytoplasm in 50 and 60 second prints are the result of granule lysis, demonstrated more clearly in following illustrations. Note the reduction in content of granules in the leading half of the leucocyte; *i.e.*, that part of the cell containing the zymosan particles. Approximately  $\times 1000$ .

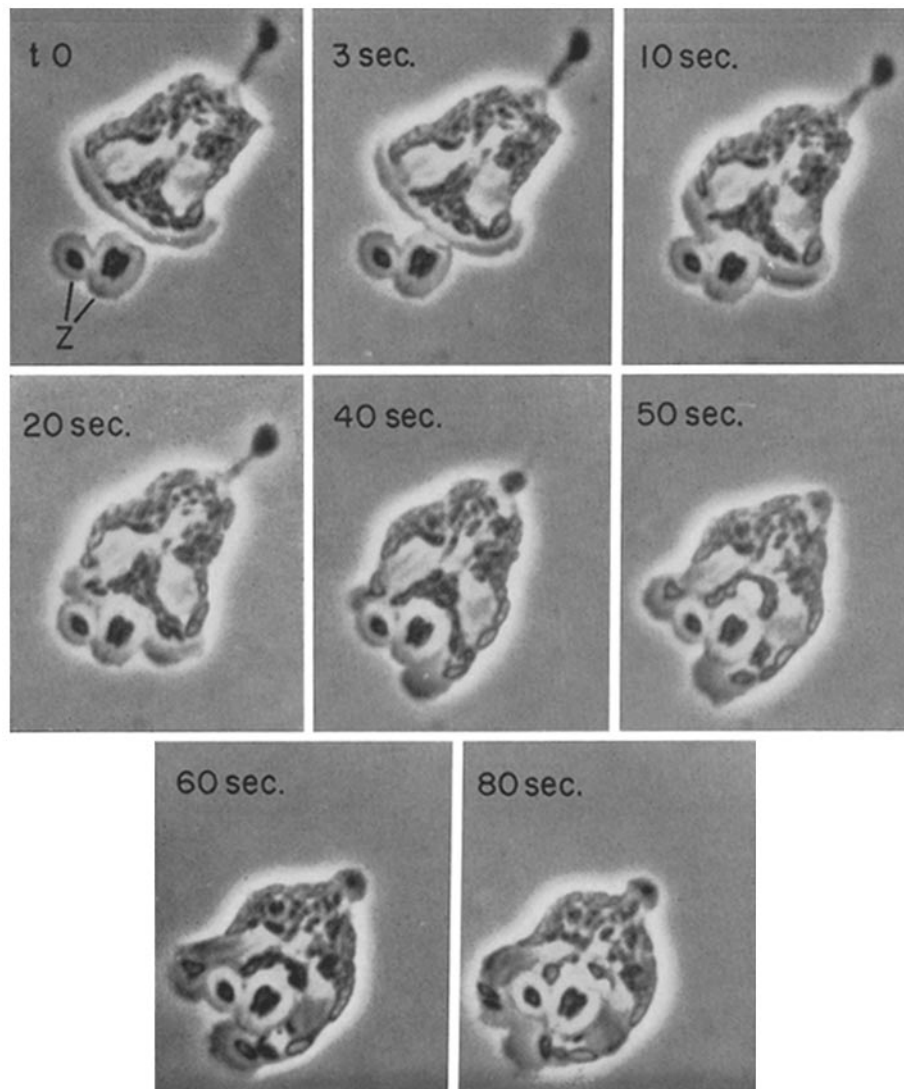


FIG. 4

(Hirsch: Granule lysis in leucocytes)

PLATE 111

FIG. 5. Sequence of enlargements at short time intervals to illustrate detailed aspects of chicken leucocyte granule rupture during phagocytosis of zymosan. Both the phase-dense oval bodies (*Z*) and the surrounding large transparent zones are parts of the zymosan. Phagocytosis of the two zymosan particles is at this stage not yet completed. Two cytoplasmic granules (*G*) which lie adjacent to one of the zymosan bodies are seen to round up (0.1 second), then disappear with formation of a clear zone in the cytoplasm (0.2 second and 0.3 second). Note at this stage also the two small phase-dense bodies within the clear zone, and the appearance of a phase-dense layer on the surface of the zymosan adjacent to the clear zone. During the following 4 seconds the small dense bodies disappear, the phase-dense appearance of the adjacent surface of the zymosan fades, and the clear zone gradually contracts towards the engulfed particles. Approximately  $\times 2000$ .

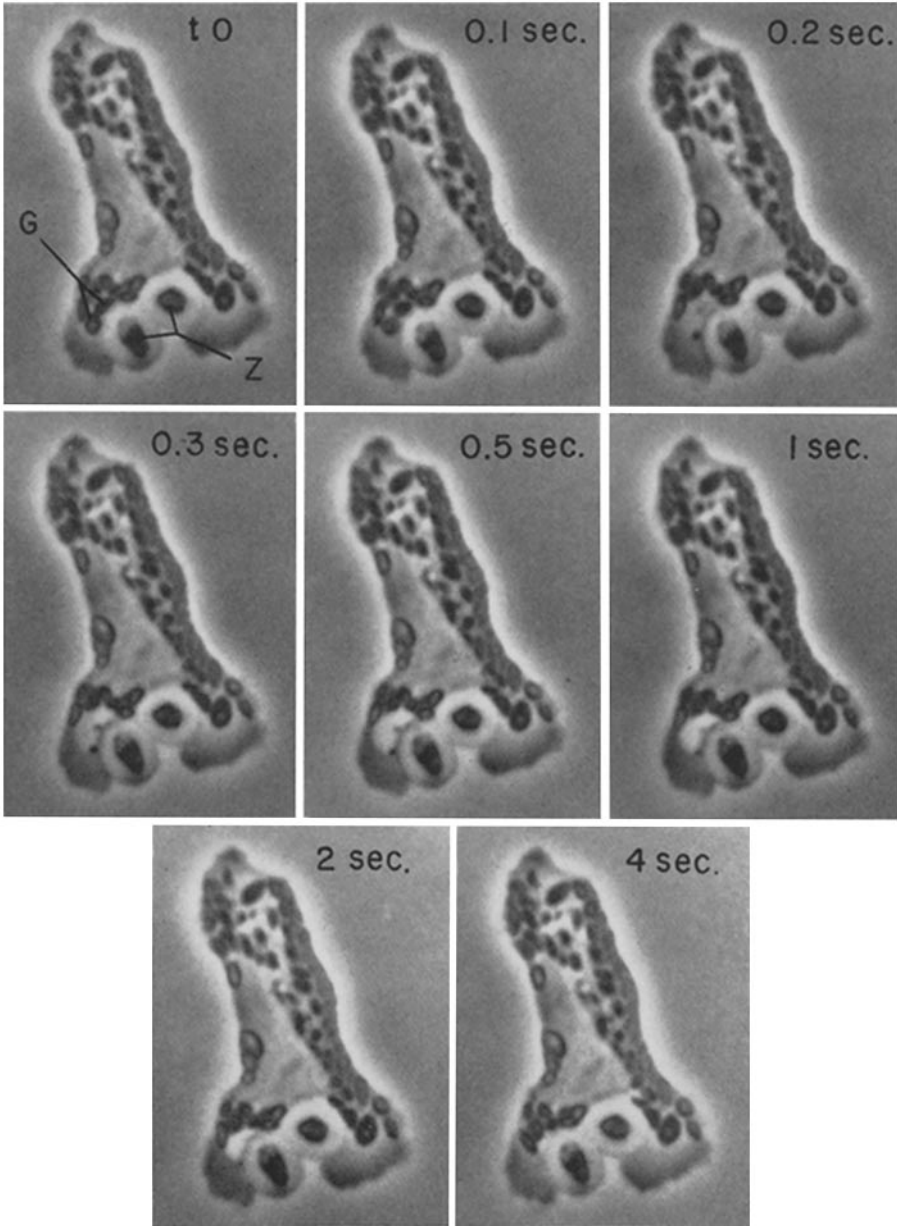


FIG. 5

(Hirsch: Granule lysis in leucocytes)

PLATE 112

FIG. 6. The same cell as in Fig. 5 at a slightly later stage of phagocytosis showing, in the circled areas, lysis of individual cytoplasmic granules. The rapidity and morphologic features of the lytic process are illustrated. Approximately  $\times 2000$ .



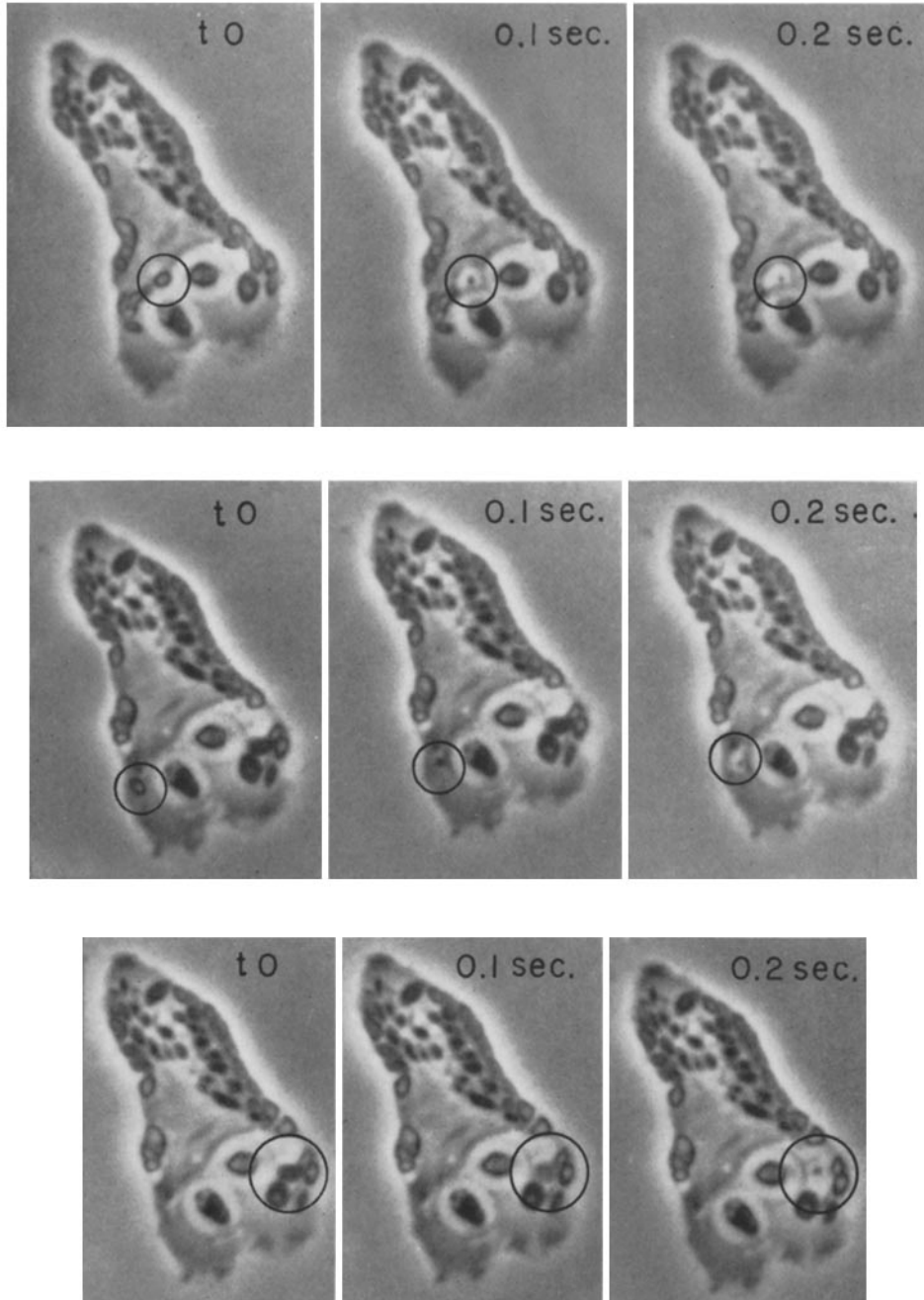


FIG. 6

(Hirsch: Granule lysis in leucocytes)

PLATE 113

FIG. 7. The same cell as that in Figs. 5 and 6, showing in the upper sequence a granule rupturing in apposition to a clear zone remaining from previous granule lysis, rather than in apposition to the engulfed particle. The lower sequence illustrates the lysis of a particularly large cytoplasmic granule (*G*) which results in formation of an unusually large dense body. This dense body gradually fades from view over the course of 5 seconds. Approximately  $\times 2000$ .

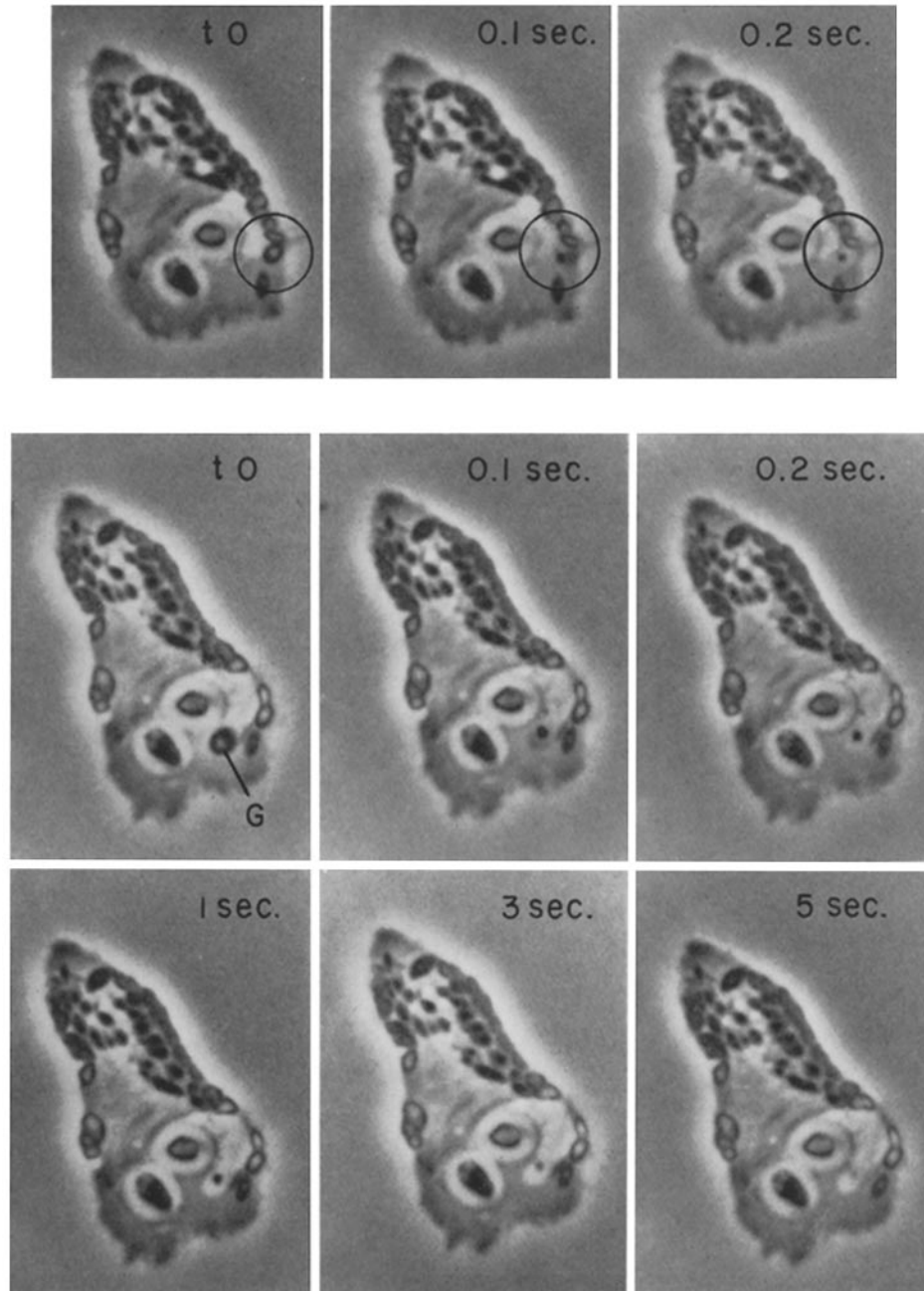


FIG. 7

(Hirsch: Granule lysis in leucocytes)

PLATE 114

FIG. 8. Lysis of chicken polymorphonuclear leucocyte granules (*G*) during ingestion of *Bacillus megaterium* (*M*). The upper sequence shows particularly well the change in phase contrast appearance of the bacterial surface following granule rupture. At 1 second a phase-dense area is seen on the bacterial surface adjacent to the clear zone resulting from lysis of several granules (*G*) seen in the *t* 0 print. At 3 seconds this dense area is less prominent, but now the entire surface of the engulfed bacillus appears darker. With continuing granule lysis the edges of the bacterium appear to darken further. Approximately  $\times 1000$ .

The lower sequence illustrates lysis of an individual large granule (*G*) in a chicken leucocyte engulfing *Bacillus megaterium*. The granule becomes somewhat rounded ( $\frac{1}{5}$  second) and disappears from view ( $\frac{2}{5}$  second). At this time no dense body or sharp clear zone is seen; rather there is seen on careful examination of the  $\frac{2}{5}$  second print a "ghost" of the granule, that is a fine membrane, oval in shape and appearing to be firmly attached to the surface of the bacterial cell, or to the invisible invaginated cell membrane overlying it. Approximately  $\times 1500$ .

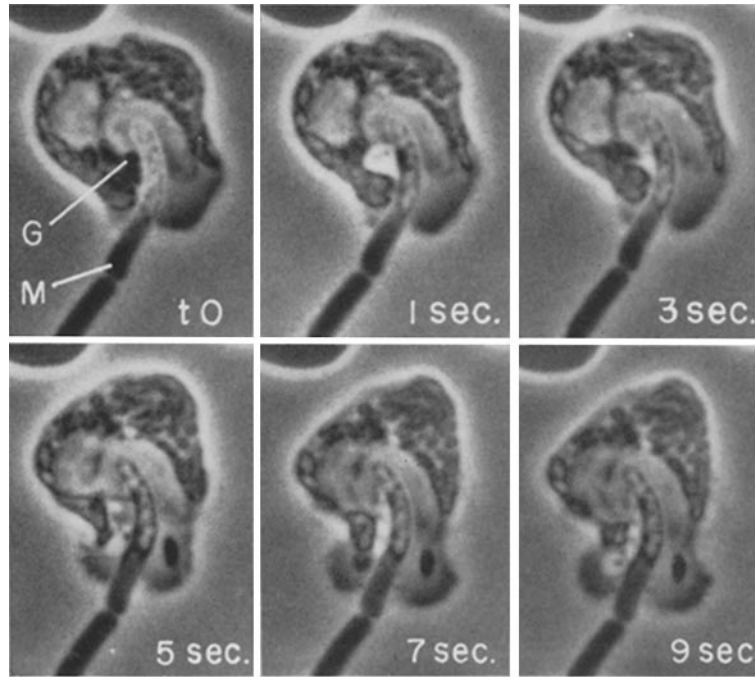


FIG. 8 (*Upper*)

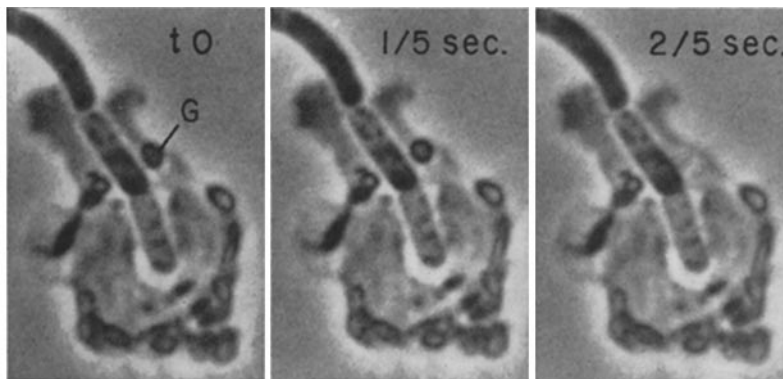


FIG. 8 (*Lower*)

(Hirsch: Granule lysis in leucocytes)