

## MOTION PICTURE STUDIES ON DEGRANULATION OF HORSE EOSINOPHILS DURING PHAGOCYTOSIS\*

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PLATES 20 TO 25

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It was shown in the preceding report (1) that eosinophil granules are lysosome-like structures, similar in many regards to cytoplasmic granules of rabbit polymorphonuclear leucocytes. Lysis of granules in intact polymorphonuclear leucocytes during phagocytosis has recently been demonstrated (2). The present study was designed to determine whether or not eosinophil granules also disrupt during phagocytosis.

### Methods

Eosinophils were separated from mixed horse leucocytes by sedimentation through 36 per cent albumin as described in the accompanying paper (1). They were then washed 3 times in physiological saline and finally suspended in saline at approximately  $10^8$  per ml. The suspension was held at room temperature in a stoppered lusteroid tube.

*Serum* was obtained from fresh clotted horse blood. Frequently the serum was adsorbed with glass powder prior to use. 10 ml serum was mixed with 1 gm of 325 mesh borosilicate glass powder over the course of 1 hour at room temperature, followed by centrifugation at 100 g for 10 minutes to remove the glass. On some occasions phenol red indicator was added to the serum to permit adjustment of the pH to approximately 7 with  $\text{CO}_2$ .

*Immune serum* was obtained from a horse which had been injected weekly  $\times 5$  intravenously with 5 ml volumes of human red cells or of yeast cell walls (zymosan). Human red cells were washed thrice in saline and a 5 per cent suspension was then prepared. Zymosan (Standard Brands, Inc., New York City) was boiled in physiological saline, washed on the centrifuge three times, and finally suspended at 10 mg per ml in saline. Horse serum containing precipitating antibodies against human albumin was obtained from Sylvania Chemical Company, Orange, New Jersey. Heat inactivation of serum was for 30 minutes at  $56^\circ\text{C}$ .

The *particulates* to be engulfed included: (a) human red cells from the same person whose red cells were employed to immunize the horse, (b) zymosan, and (c) an antigen-antibody precipitate prepared by mixing 1.5 mg of crystalline human albumin with 1 ml of horse anti-human albumin serum, followed by centrifugation and three washings in saline.

*Thin preparations* on glass slides were made as described previously (2). Slides and coverslips were coated with formvar by dipping them into a 0.2 per cent solution in ethylene dichloride.

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ride of formvar 15/95E (Shawinigan Resins Corp., Springfield, Massachusetts), and allowing them to air dry.

*Cinemicrophotography* under oil-immersion phase contrast and enlargements from the movie film were done employing equipment and procedures recorded in detail elsewhere (2).

#### RESULTS

*General Features of Eosinophil Behavior in the Coverslip Preparations.*—Speirs reported that mouse eosinophils showed little or no locomotion on glass surfaces, but moved about rapidly if the glass were coated with thiolated or formalinized gelatin (3). Our experience was similar; on glass slides the horse eosinophils rounded up and failed to migrate. Two procedures were found to favor eosinophil locomotion: (a) adsorption of the serum used with borosilicate glass powder, and (b) coating of the slides and coverslips with formvar. Either one of these procedures resulted in moderately good eosinophil locomotion in the slide preparations; when both glass adsorption of serum and formvar coating of glass were used, as was the case for the studies reported here, the eosinophils moved about and engulfed suitable objects with much the same rate and facility as observed previously for human or rabbit polymorphonuclear leucocytes (2). The appearance of living horse eosinophils under phase contrast illumination is shown in Fig. 1.

The behavior of horse eosinophils in these slide preparations constituted good evidence that they had not been significantly damaged as a result of isolation by sedimentation through concentrated albumin solution.

Locomotion in eosinophils had many features in common with neutrophils. The advancing edge usually consisted of a broad pseudopod with a ruffled border. Locomotion was accomplished without signs of general cytoplasmic streaming; granules retained their relative position over long periods of time. The trailing portion of the migrating eosinophil often showed a rounded, tail-like structure.

*Phagocytosis of Zymosan by Horse Eosinophils.*—Zymosan (yeast cell walls) was avidly engulfed by horse eosinophils in the presence of immune (horse antizymosan) serum. Phagocytosis of zymosan under these conditions was accompanied by granule rupture, as is seen in Fig. 2. For clear illustration of this phenomenon, a cell was selected in which fairly extensive degranulation occurred; on the average, however, only 1 to 2 eosinophil granules disrupted for each zymosan body ingested.

Degranulation in the horse eosinophil resembled closely in intimate morphologic aspects that previously observed in rabbit or chicken polymorphonuclear leucocytes. Granule rupture was seen early in the course of engulfment, often before the process had been completed. Those granules which lysed were always situated adjacent to the particle being ingested, or alongside the clear zone remaining from previous granule disruption. Following rupture of the horse

eosinophil granules, a considerable amount of phase-dense amorphous material was often seen lying in the clear zones. In Fig. 2 such an amorphous deposit is visible alongside the zymosan body in the 10 and 15 second prints.

Eosinophils also ingested zymosan in normal horse serum or in heat-inactivated immune serum, and phagocytosis was sometimes accompanied by degranulation. The rapidity of phagocytosis and the degree of degranulation were distinctly reduced in normal or heated immune serum as compared to that seen in the presence of fresh zymosan immune serum.

*Phagocytosis of Human Erythrocytes by Horse Eosinophils.*—The horse eosinophils did not engulf human red cells suspended in normal horse serum. When immune (horse anti-human red cell) serum was employed, erythrophagocytosis by the eosinophils was marked. The sensitized red cells were ingested avidly so long as hemolysis had not yet occurred. In order to delay the hemolytic reaction and give a better opportunity to photograph phagocytosis, human red cells were held at 0°C for 1 hour in the horse immune serum, and slide preparations made by mixing this cell suspension with eosinophils were examined at room temperature rather than at 38°C. Approximately 3 to 4 minutes of microscopic observation was possible before extracellular hemolysis occurred. Eosinophils appeared to move about and engulf as rapidly at room temperature as they did at 38°C in this particular experimental situation.

The horse eosinophils seemed to be attracted to the sensitized human erythrocytes. They attached firmly to the red cells and rapidly began to ingest them. Almost without exception the eosinophil was unable to engulf the entire erythrocyte. Eosinophil cytoplasm flowed over approximately half the red cell and seemingly could go no further. The eosinophil then appeared to constrict about the erythrocyte, which soon assumed a dumb-bell shape. In many instances this constrictive reaction proceeded to bisection of the red cell. The general picture suggested that a human red cell was too large an object for the eosinophil to engulf; the eosinophil accordingly surrounded the red cell to the maximum of its capacity, and proceeded to "bite" it in half.

Degranulation of horse eosinophils during ingestion of sensitized human red cells was striking. The detailed morphological aspects of granule lysis under these circumstances are presented in Figs. 3 and 4. Rupture of an individual eosinophil granule was less abrupt than that previously reported for rabbit and chicken polymorphonuclear leucocytes. The first change was often swelling and decrease in phase density of a granule; over the course of the following 1 to 2 seconds lysis gradually occurred, usually resulting in a clear zone and amorphous phase-dense deposits alongside the erythrocyte.

Not shown in the illustrations was the fact that lysis of the erythrocyte often occurred at approximately the same time as granule rupture. Whether the red cell lysis was due to attack by released granule enzymes, or due to the effects of activated complement in the immune system could not be ascertained.

Human red cells suspended in heat inactivated immune (horse anti-human red cell) serum did not undergo hemolysis, thus allowing more prolonged observation. Under these circumstances erythrocytes were slowly engulfed by horse eosinophils but granule lysis was not seen. This system proved particularly suitable for observations on the "pinching off" phenomenon. As is illustrated in Fig. 5, the eosinophil surrounded approximately half the erythrocyte and then began to constrict the red cell at its middle, eventually cutting it in half. The half of the erythrocyte remaining outside did not lyse; it retained a perfectly normal general appearance, often floating off in the form of a small but otherwise undistinguished red cell. Apparently the constrictive process permitted the erythrocyte membrane to reform or close by fusion without loss of the intracellular hemoglobin.

Also illustrated in Fig. 5 is the absence of eosinophil degranulation on engulfment of a human erythrocyte in heat-inactivated immune serum. This cell and many others like it were observed for periods up to 30 minutes. No granule rupture occurred, and the ingested half of a red cell did not change in appearance.

*Interaction between Eosinophils and Antigen-Antibody Precipitates.*—Washed precipitates from human albumin and horse anti-human albumin were suspended in horse serum and mixed with horse eosinophils. These precipitates were strongly chemotactic for eosinophils. Similar precipitates suspended in heat-inactivated horse serum appeared to be less attractive. Small masses of precipitate were ingested completely, with accompanying degranulation. When encountering large clumps of precipitate the eosinophil cytoplasm flowed over part of the clump. Complete engulfment was not possible, and furthermore the eosinophils seemed incapable of pinching off a piece of the precipitate, in contrast to their behavior with sensitized red cells. Rather they usually assumed a crescent shape in intimate association with the precipitate. Granule lysis then followed, those granules lying adjacent to the precipitate usually being the first to burst (see Fig. 6). Over the course of an hour, such cells showed progressive and often complete degranulation with residual deposition of large amounts of the dense amorphous material.

#### DISCUSSION

In confirmation of the earlier studies of Speirs (3), we found that eosinophils in serum did not function well when exposed to glass surfaces. The cytotoxic effect in this system could be reduced either by preabsorbing the serum with glass or by coating the glass surfaces with formvar. The serum component which inhibits eosinophil function was not identified, but plasma serum (4), *i.e.* serum obtained after clotting of cell and platelet-free plasma, was much less toxic to eosinophils on glass than was ordinary serum. The results suggest that some substance present in serum from clotted blood reacts with glass,

perhaps by simple adsorption, to produce an environment unfavorable for eosinophil locomotion.

Eosinophil degranulation accompanying phagocytosis resembled in its general aspects that previously observed for rabbit and chicken polymorphonuclear leucocytes (2). Granules which lysed were always those situated adjacent to the particle being engulfed or to the clear space left from prior granule disruption, suggesting the possibility that eosinophil granules discharge into the phagocytic vacuole rather than into the cell cytoplasm.

Sensitized erythrocytes were ingested in either fresh or in heat-inactivated serum, but degranulation occurred only in the presence of unheated serum. Recent studies have established that many hemolytic agents, such as acid and streptolysin, rupture lysosomes (5) and granules in rabbit polymorphonuclear leucocytes (6). Perhaps activated serum complement, another hemolytic agent, is the heat-labile factor required for disruption of eosinophil granules during engulfment of erythrocytes. It should be noted, however, that eosinophils did sometimes degranulate in heat-inactivated serum when taking in yeast cell walls or antigen-antibody precipitates. The mechanism for granule rupture might conceivably differ depending on the type of material being ingested.

The "pinching off" phenomenon, in which horse eosinophils stretched over sensitized human red cells to the limit of their capacity, and then constricted eventually to cut the red cell in half, is of interest from two points of view. First of all, in the "eating" parlance commonly applied to phagocytes, this observation demonstrates that these cells can, at least in some circumstances, bite off and swallow a portion of a food object too large to be ingested whole. Secondly, this bisection of a red cell did not lead to destruction of the half erythrocyte remaining outside the eosinophil; it remained as a small but otherwise normal looking red cell. This observation demonstrates a "self-sealing" property of the erythrocyte membrane in some situations.

The phagocytic capacity of eosinophils, the lysosomal nature of their granules, and the degranulation which accompanies ingestion suggest that one function of these cells is the engulfment and digestion of particulate matter in blood or tissues. One such possible role for eosinophils, namely phagocytosis of antigen-antibody precipitates, was demonstrated *in vitro* in the present work, and has also recently been shown to occur under certain conditions *in vivo* (7).

In addition to the proposed phagocytic function, it is possible that release of hydrolases, peroxidase, and basic proteins from eosinophils into the surrounding tissues might play a role in the inflammatory reaction.

From the over-all point of view, neutrophils and eosinophils seem to have many features in common. They are produced in bone marrow from undifferentiated stem cells, requiring approximately 4 days for maturation (8). Upon release from the marrow, both types of cells stay in the circulation for only a few hours (9-11), and pass into the tissues. Both are found in relatively large

numbers in skin, respiratory tract, and intestinal membranes (11), *i.e.* sites of contact between the animal and the outside world. Both cell types have an abundance of cytoplasmic granules, and we can now state that these granules contain a variety of hydrolytic enzymes. Under appropriate conditions eosinophils and neutrophils exhibit active locomotion and respond to chemotaxis. Both are capable of phagocytosis, and both degranulate during ingestion of particulate matter.

In addition to well known differences in their appearance and staining properties, neutrophils and eosinophils also differ in some other regards. For example, on administration of adrenal cortical steroids, neutrophil levels in the blood rise but eosinophils disappear from the circulation. Neutrophils function well on glass surfaces, whereas eosinophils do not. Although incompletely studied, there may well also be significant differences between these cells in their response to chemotaxis, or in the types of foreign particles they are capable of engulfing and digesting. In our experiments, for instance, specific immune serum was required for vigorous phagocytic attack on red cells or zymosan by eosinophils, and these cells showed but little inclination to take in bacteria under any circumstances.

The granules of eosinophils and neutrophils, though both of lysosomal nature, differ in their content of certain enzymes and proteins. The presence in eosinophil granules of a crystalline structure, of an acidophilic basic protein, and of peroxidase in large amounts are, at the moment, the main distinguishing features. These features are not, however, enlightening in terms of possible specific functions. Additional studies on eosinophils and their granules are thus required before we can assign a specific role to this cell in mammalian physiology.

#### SUMMARY

Horse eosinophil function has been studied *in vitro* by means of phase contrast cinemicrophotography.

Locomotion of horse eosinophils was inhibited by serum factors reacting with glass surfaces. Under appropriate conditions which eliminated this inhibitory effect, eosinophils moved about and ingested some particles as rapidly as did neutrophils.

Eosinophils were attracted to and readily engulfed such diverse materials as yeast cell walls, foreign erythrocytes, and antigen-antibody precipitates. Specific antibody was required for phagocytosis of red cells, and greatly accelerated the uptake of yeast cell walls.

Horse eosinophil granules situated adjacent to material being engulfed disrupted with discharge of granule contents into or alongside the phagocytic vacuole. Granule disruption resulted in a clear zone and deposition of amorphous, phase-dense material.

A heat-labile serum factor was required for degranulation of eosinophils ingesting foreign red cells, but not for degranulation during engulfment of yeast cell walls or antigen-antibody precipitates.

Horse eosinophils were incapable under these conditions of engulfing an entire human red cell. The eosinophil commonly put out a large pseudopod to surround about half the red cell, and then appeared to constrict this pseudopod distally to cut the erythrocyte in half.

It is concluded that eosinophils are phagocytic cells, resembling neutrophils in many of their properties. Any specific functions of eosinophils, distinguishing them from other phagocytes, remain to be discovered.

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## EXPLANATION OF PLATES

## PLATE 20

FIG. 1. The appearance under phase contrast of normal horse eosinophils moving about on formvar-coated glass slides. Locomotion is in the direction of the clear pseudopod with a ruffled edge. In the upper illustration *N* points to one of the nuclear lobes, and *G* indicates some large cytoplasmic granules. Approximately  $\times 2000$ .



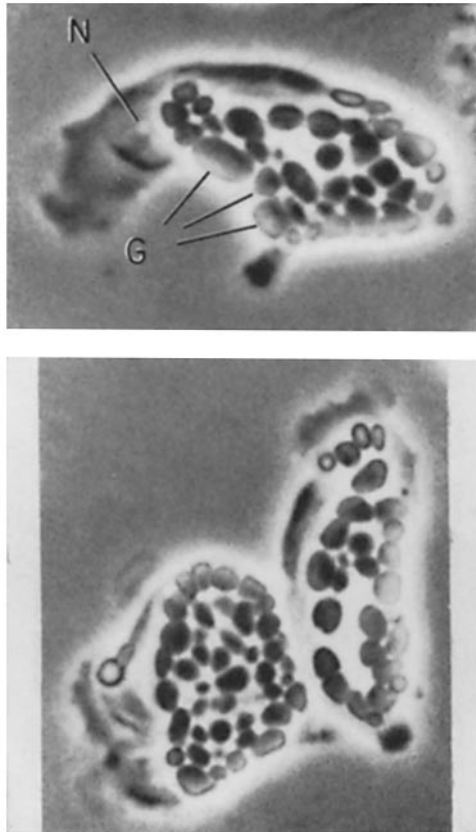


FIG. 1

(Archer and Hirsch: Degranulation of eosinophils)

PLATE 21

FIG. 2. A sequence of enlargements from the motion picture film, illustrating rupture of cytoplasmic granules in an eosinophil during ingestion of a yeast cell wall (zymosan) particle. Granules (*G*) situated adjacent to the zymosan (*Z*) disrupt with creation of a clear zone and deposition of phase-dense amorphous material alongside the zymosan. Approximately  $\times 2000$ .

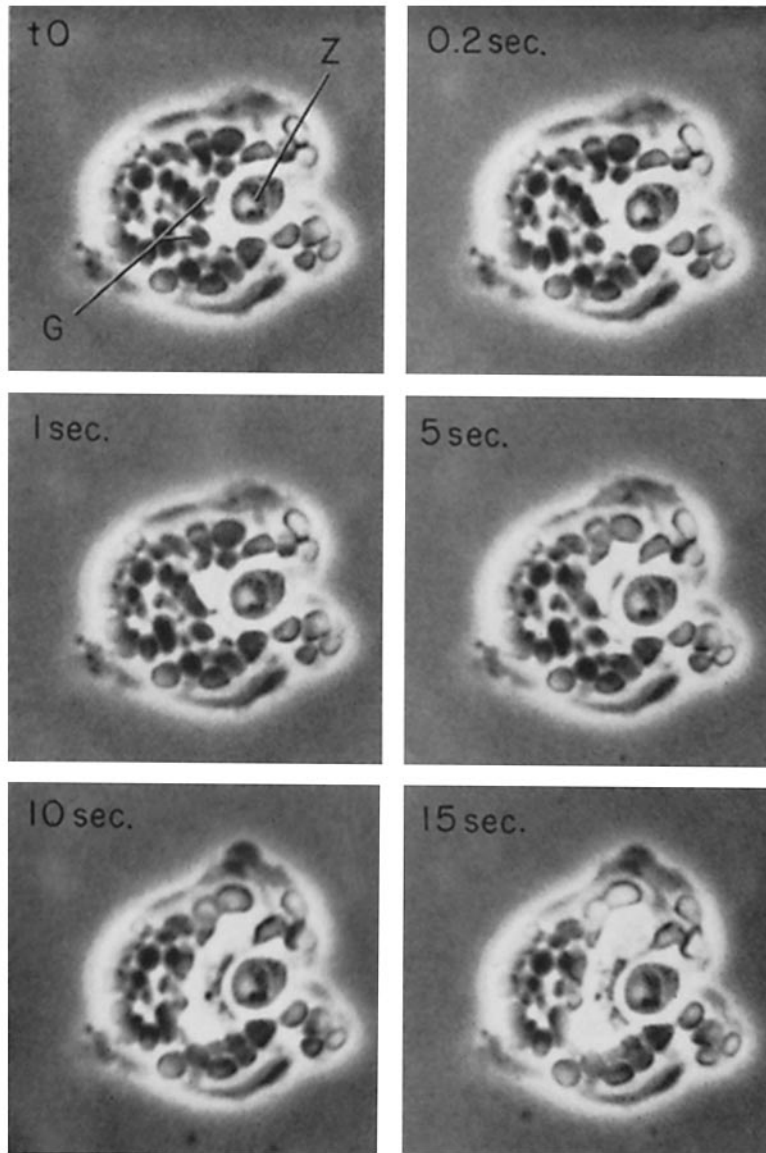


FIG. 2

(Archer and Hirsch: Degranulation of eosinophils)

PLATE 22

FIG. 3. Lysis of horse eosinophil granules in the course of phagocytosis of a human red cell (*E*) in the presence of specific immune (horse anti-human erythrocyte) serum. The eosinophil cytoplasm flows about the red cell but appears to be incapable of engulfing the entire erythrocyte; a pinching off of the red cell follows, approximately half being engulfed. In the circled area is seen disruption of several granules situated adjacent to the half of the red cell being ingested. Approximately  $\times 2000$ .

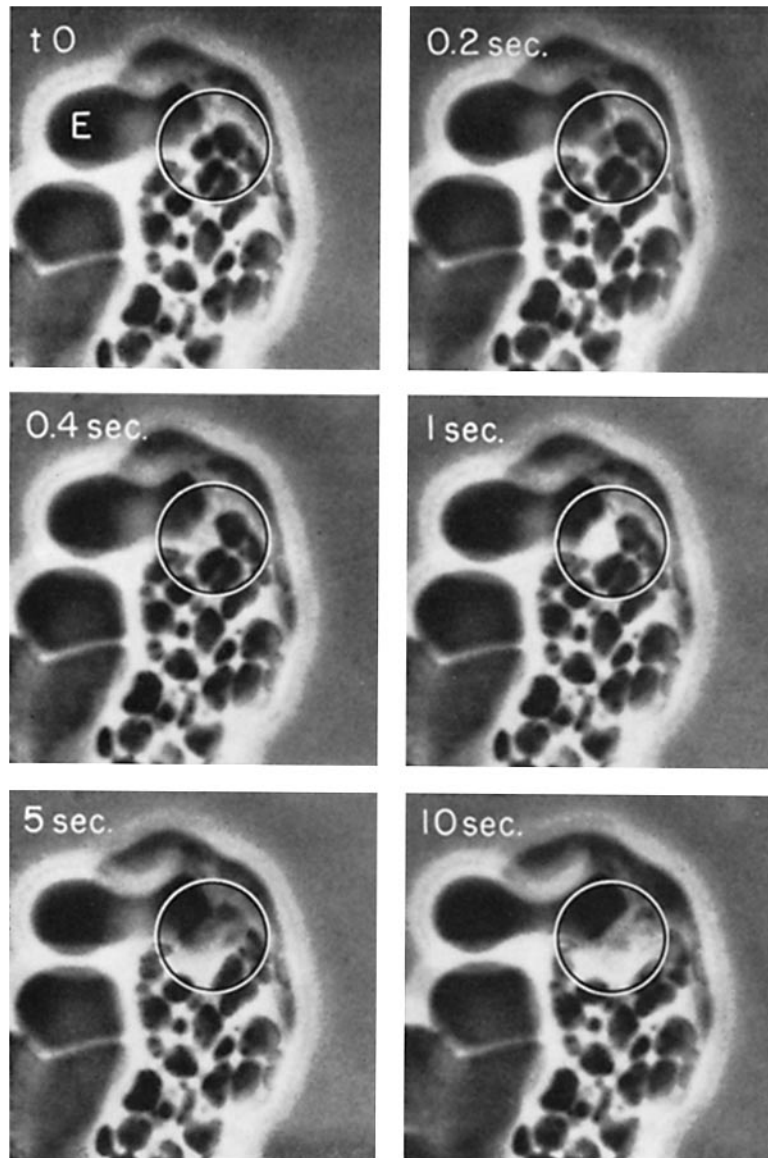


FIG. 3

(Archer and Hirsch: Degranulation of eosinophils)

PLATE 23

FIG. 4. Four sequences of enlargements illustrating varying morphologic features of granule lysis in a horse eosinophil engulfing a sensitized human red blood cell. The granule circled in the top sequence seems to rupture with simultaneous deposition of an irregularly shaped mass of phase-dense material at the tip of the red cell protruding into the eosinophil. The sequence on the next line shows lysis of another granule soon thereafter, with apparent fusion of its residue with that remaining from the initial granule lysis. A few seconds later this homogeneous mass of granule residue assumes a mottled appearance. The next sequence shows fusion of another granule with this residue; the granule becomes rounded and altered in phase density but retains for some time the appearance of a structural entity. Finally in the lowermost sequence is seen another type of granule rupture later in the same cell. The granule in the center of the circle is less dense and poorly demarkated at 0.1 second, and at 5 seconds has been replaced by a clear zone and a dense amorphous mass (bottom of the circle) adjacent to the edge of the phagocytic vacuole. The red cell has now lysed. Approximately  $\times 2000$ .

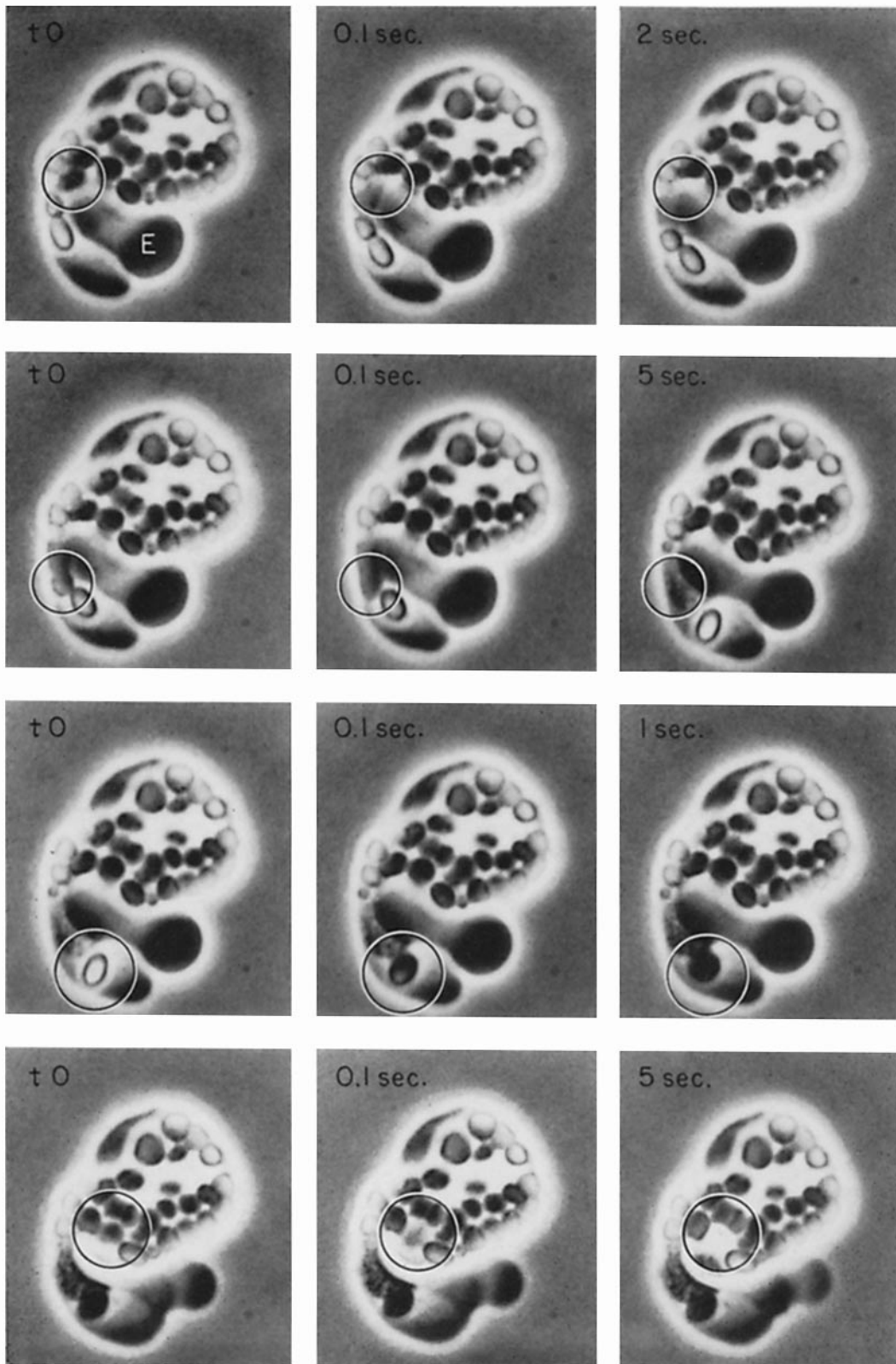


FIG. 4  
(Archer and Hirsch: Degranulation of eosinophils)

PLATE 24

FIG. 5. A sequence of pictures, at approximately 30 second intervals, of a horse eosinophil ingesting a human erythrocyte (*E*) in the presence of heat-inactivated immune (horse anti-human red cell) serum. Again the eosinophil seems unable to surround and engulf the entire red cell. When approximately one-half of the erythrocyte has been surrounded, a pinching-off process begins. The eosinophil apparently constricts the erythrocyte into the dumb-bell shape seen in illustration 1, and this process continues (2 and 3) until finally the red cell is cut in half. Most fascinating was the repeated observation that the extracellular half of the red cell remained quite intact and often floated away, appearing as normal erythrocyte except for its small size.

Also of interest and demonstrated in the picture sequence in Fig. 5 is the absence of granule rupture in eosinophils engulfing a portion of a sensitized human red blood cell in a medium containing heat-inactivated horse serum, in sharp contrast to the extensive degranulation seen accompanying similar erythrophagocytosis in fresh serum. Approximately  $\times 2000$ .



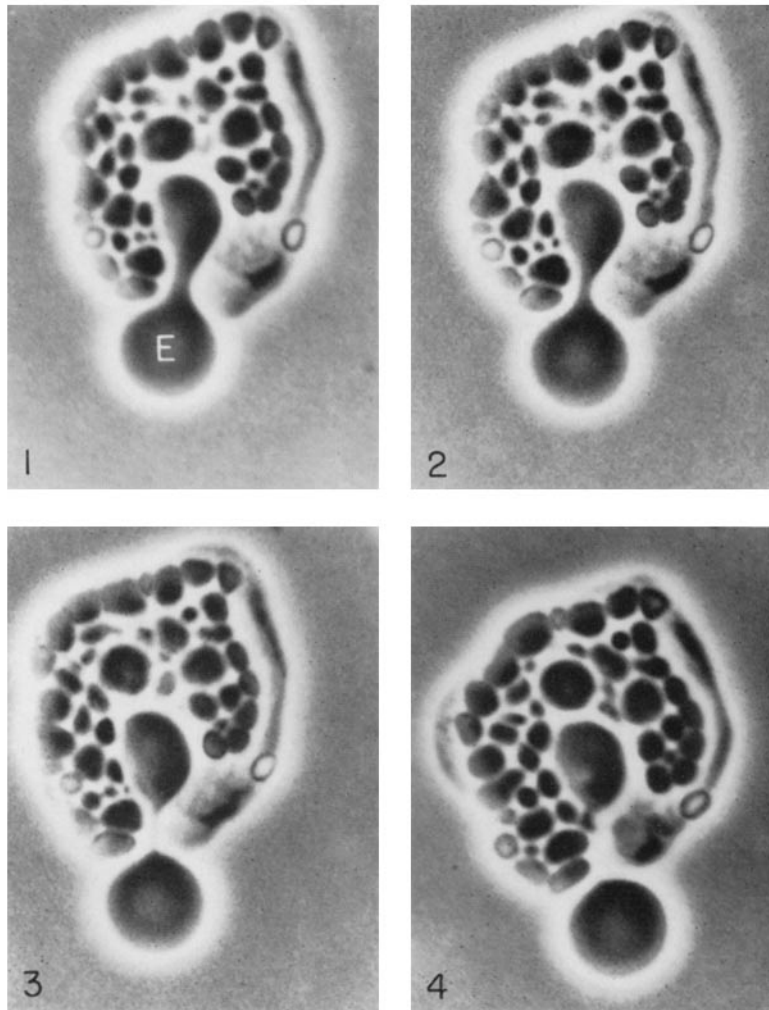


FIG. 5

(Archer and Hirsch: Degranulation of eosinophils)

PLATE 25

FIG. 6. Sections *A* and *B* illustrate horse eosinophils engulfing antigen-antibody precipitates (the amorphous dark masses labeled *P*). The eosinophils seemed to be attracted to the precipitates, and were able to ingest small but not large masses of precipitate completely, again indicating a size limitation for particulates which can be taken in by these cells under these conditions. Granule lysis regularly accompanied ingestion of precipitate. Sections *C* and *D* illustrate degranulation in eosinophils attempting to engulf large clumps of antigen-antibody precipitates (*P*). The cells frequently assumed a crescent shape, and granule rupture was most commonly seen in portions of the cytoplasm adjacent to the precipitate. Approximately  $\times 2000$ .

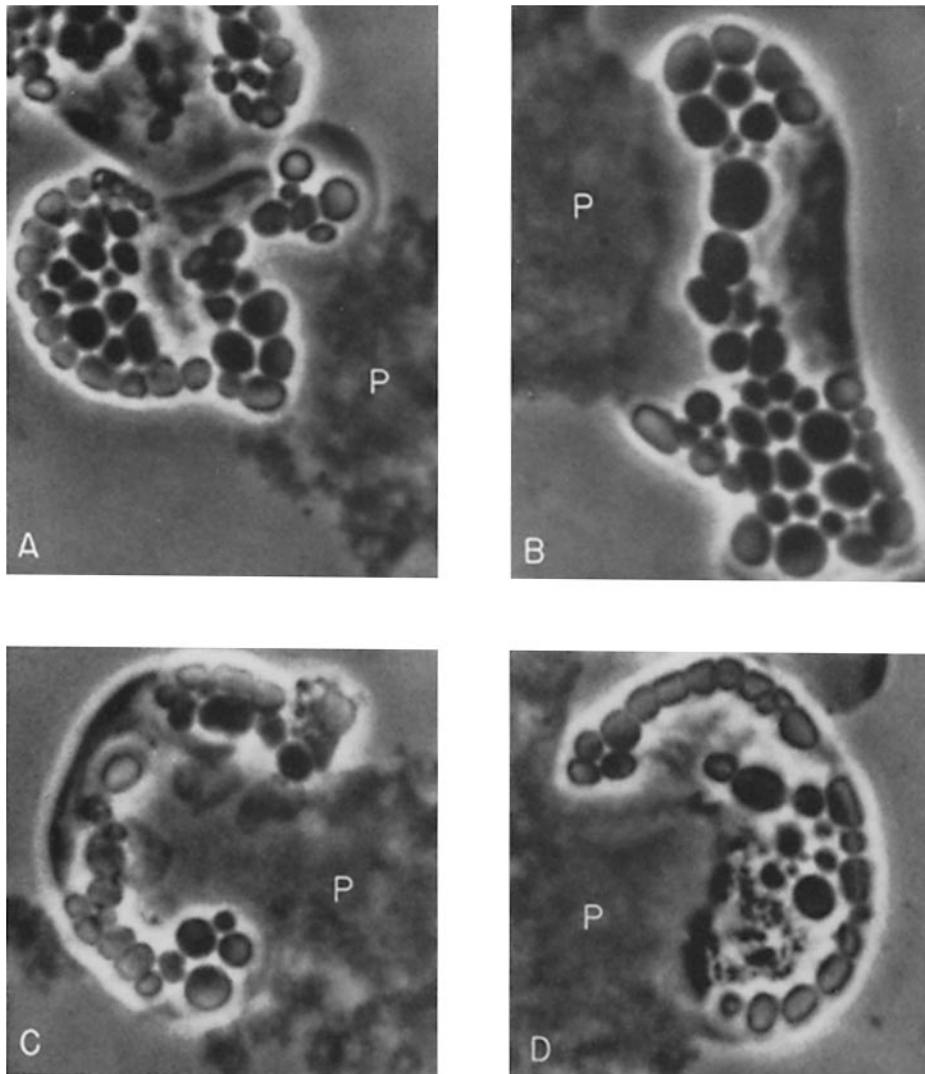


FIG. 6

(Archer and Hirsch: Degranulation of eosinophils)