MOTION PICTURE STUDY OF THE TOXIC ACTION OF STREPTOLYSINS ON LEUCOCYTES*

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PLATES 10 TO 16

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The preceding communication presents evidence that streptolysins bring about release of hydrolytic enzymes from lysosomes isolated from various tissues (1). This finding, considered along with previous reports establishing the lysosomal nature of leucocytic granules (2) and the leucocidal action of streptolysins (3), led to speculation that these bacterial products might disrupt granules in intact white cells, releasing autolytic enzymes to digest other leucocyte structures and result eventually in cell death. Such a sequence of events, if supported by experimental observations, would be in keeping with de Duve's (4) concise and colorful designation of lysosomes as "suicide bags."

This communication presents cinemicrophotographic studies done to elucidate detailed morphologic aspects of streptolysin toxicity on rabbit polymorphonuclear leucocytes and macrophages.

Methods

The streptolysin O preparation was the same as that designated preparation A in the preceding paper (1). The streptolysin S used was a lyophilized product described previously (5). Streptolysins were dissolved in phosphate-buffered saline and centrifuged at high speed to remove any particles which might otherwise confuse observations on degranulation. Streptolysin O was activated by addition of 3.5 mM cysteine.

Rabbit granulocytes were obtained from peritoneal exudates as described previously (2). Rabbit alveolar macrophages, collected by a modification of the technique of Myrvik *et al.* (6), were kindly supplied by Dr. Zanvil Cohn. Leucocytes were washed twice in saline by centrifuging at 250 *g* for 5 minutes at room temperature, and were then suspended in Hanks' solution containing 10 per cent rabbit serum at a cell concentration of approximately 20,000 per mm^3.

Small drops of the white cell suspension and of appropriate dilutions of streptolysin solution (or, for control, Hanks' with or without cysteine but no streptolysin) were mixed and made

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into thin preparations between glass slides and coverslips (7). The action of streptolysin O on white cells was very rapid; in order to visualize and photograph the toxic events from their inception, it was necessary to dilute the streptolysin to a concentration approximately twice that at which its toxic action was no longer seen. The sequence of toxic events was slowed further by making observations at room temperature rather than at 38°C.

Cinemicrophotography and enlargements of sequences from the motion picture film were made as described in detail elsewhere (7).

RESULTS

The appearance of normal rabbit polymorphonuclear leucocytes is shown in Fig. I. Under the conditions employed, these cells did not change detectably in the thin preparations during a 1 hour period of observation.

Addition to the rabbit granulocyte suspensions of streptolysin O at low concentration (500 to 1000 hemolytic units per ml) resulted in a striking sequence of changes in white cell morphology. The initial sign of streptolysin O damage was rapid and extensive degranulation. The phase dense cytoplasmic granules, often gathered in the center of the leucocyte, appeared to explode, and were replaced by tiny clear zones, which either rapidly faded, or in some instances coalesced into small, poorly demarkated light areas. Granules lysed in all parts of the cell and seemed to discharge directly into the cytoplasm rather than into vacuoles, in contrast to the degranulation process reported during phagocytosis (7).

Approximately 1 minute after granule disruption, long filamentous processes appeared at the surface of the leucocyte membrane. Next the affected cell rounded up. Liquefaction of the cytoplasm commonly was noted at this stage, as evidenced by Brownian movement of all the remaining granules. Finally the nuclear lobes began to swell and frequently fused. The end stage of streptolysin O toxicity on the granulocyte was commonly a round cell with clear cytoplasm and a single ovoid eccentric nuclear structure. The cell membrane, although obviously abnormal, appeared to be intact.

The sequence of events described and depicted in Figs. II to IV was seen with regularity in numerous rabbit polymorphonuclear leucocytes damaged by streptolysin O. At low concentrations of streptolysin O many cells in a given preparation were affected, whereas others in the same vicinity did not change (see Fig. II, for instance). This apparent variation in leucocyte susceptibility to streptolysin toxicity was also noted in a previous study (3), and is unexplained.

Streptolysin S also damaged rabbit white cells, and the initial toxic event observed was granule lysis. Fig. V shows a polymorphonuclear leucocyte affected by streptolysin S (700 hemolytic units per ml) and Fig. VI illustrates rupture of an individual granule in this cell. Under the conditions used, degranulation on exposure to streptolysin S was accompanied by formation of cytoplasmic vacuoles which slowly disappeared. Filamentous change in the cell membrane, and nuclear fusion were sometimes seen, but were in general less striking than that seen with streptolysin O.

One other difference was also noted between the toxicity of streptolysins O and S in these preparations. Streptolysin S first began to exert its damaging action 15 to 30 minutes after mixing with white cells, in contrast to the prompt action of streptolysin O.

Lysosomes have recently been isolated from rabbit alveolar macrophages (8). It was of interest to determine whether or not exposure to streptolysin O would also cause degranulation and other toxic changes in these cells. Fig. VII (top) shows an untreated alveolar macrophage as seen by phase contrast cinemicrophotography. Cytoplasmic granules were numerous indeed, and of several densities and sizes. In general, however, two classes of granules could be distinguished: (*a*) very dark, sharply circumscribed bodies which varied considerably in size, and (*b*) much less dark, poorly demarkated granules. Exposure of macrophages to streptolysin O at a concentration of 1000 hemolytic units per ml caused lysis of the latter type of granule (see Fig. VIII). Other cell changes which followed degranulation of macrophages exposed to streptolysin O included cytoplasmic liquefaction, flattening of the cell, and alterations in the membrane. The affected cell commonly lost from its membrane the normal microvillous projections, and assumed a coarse, scalloped outline (Fig. VII, lower print).

DISCUSSION

The morphologic observations here reported on toxicity of streptolysins for rabbit leucocytes fit well with the working hypothesis, *i.e.* since streptolysins release hydrolases from isolated liver lysosomes, they might also break lysosomes in intact leucocytes, and thus release autolytic enzymes with resulting general cell damage and death. The initial morphologic alteration of leucocytes exposed to streptolysin was, in fact, degranulation. The granule contents appeared to be discharged directly into the cytoplasm rather than into a vacuole. Subsequent changes, such as cytoplasmic liquefaction and nuclear fusion, are consistent with activation of autolytic enzymes. It must be emphasized however, that these observations only support, and by no means prove, the postulated intracellular sequence of toxic events. Streptolysins might damage directly multiple structures at different times, or on the other hand these toxins might cause a metabolic or morphologic insult, undetected in these studies, which in turn affects granules, cell membrane, nucleus, etc.

If inappropriate release of lysosomal enzymes from leucocyte granules does in fact lead to cell death, perhaps these substances are eventually liberated from the leucocyte into tissues and cause damage to other cells or structures in the area. In other words, leucocyte granule enzymes could conceivably play an important role in the inflammatory reaction. Definitive evidence in support of such a concept is not available at present.

The striking difference in fate of the rabbit polymorphonuclear leucocyte following degranulation associated with exposure to streptolysin and that ac-

companying phagocytosis deserves mention. Streptolysin toxic degranulation is rapidly followed by severe cell damage, whereas after the degranulation of phagocytosis (7) the leucocyte shows none of these changes and continues to move about quite normally. The lack of general cell injury in this latter instance might be explained by the postulated discharge during phagocytosis of granule contents into the phagocytic vacuole, in essence an extracellular locus, rather than into the cell sap.

The present studies confirm and extend the previous report (3) on streptolysin toxicity for leucocytes. Products of staphylococci, called leucocidin, also damage white cells, and the investigations of Gladstone and van Heyningen (9) and Woodin (10, 11) indicate that leucocyte granules are disrupted in the process. Perhaps the mechanism of leucocyte damage by these substances is similar to that reported for streptolysins here.

As reported in the preceding paper on release of hydrolases from isolated lysosomes, streptolysin O was approximately 10 times less active than streptolysin S in this regard, when calculated on the basis either of weight or of units of hemolytic activity. In contrast, streptolysin O was, if anything, more active than streptolysin S in producing degranulation and other damage to intact leucocytes. The minimal concentration of streptolysin O resulting in leucocyte degranulation was only slightly higher than that required for release of hydrolases from isolated lysosomes. Streptolysin S, on the other hand, degranulated intact cells only when added at a much higher level than that required for action on lysosome suspensions; streptolysin S effects on leucocytes were further characterized by a 15 to 30 minute delay. These findings suggest that streptolysin O rapidly penetrates the leucocyte membrane to act on its postulated target, the granule; whereas streptolysin S reaches the granule with difficulty, perhaps because of relative impermeability of the cell membrane to this substance.

SUMMARY

The initial morphologic alteration in rabbit polymorphonuclear leucocytes exposed to streptolysin is rapid and extensive lysis of cytoplasmic granules. The granules appear to rupture directly into the cell sap. Within a few minutes following degranulation, the leucocyte rounds up, filamentous processes appear on the cell membrane, the cytoplasm liquefies, and finally the nuclear lobes swell and fuse.

Streptolysin O causes these changes in intact leucocytes when added in concentrations only slightly higher than those required for release of hydrolases from isolated liver lysosomes, and furthermore exerts its action on granulocytes promptly. On the other hand streptolysin S acts on white cells only after a 15 to 30 minute delay, and the levels necessary to disrupt granules in leucocytes are considerably higher than those which act on lysosome suspensions.

Exposure of rabbit alveolar macrophages to streptolysin O also results in lysis of granules, soon followed by alterations in the cytoplasm and membrane.

The observations are in accord with the hypothesis that streptolysins penetrate the leucocyte membrane and bring about lysis of granules. Autolytic enzymes released from the granules might then be responsible for the subsequent damage seen in various other cell structures.

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EXPLANATION OF PLATES

PLATE 10

FIG. I. Appearance of normal rabbit polymorphonuclear leucocytes as viewed by oil immersion microscopy under phase contrast. The nuclear lobes and numerous phase-dense cytoplasmic granules are well defined. The light zone about each cell is due to phase halo. Approximately × 1800.

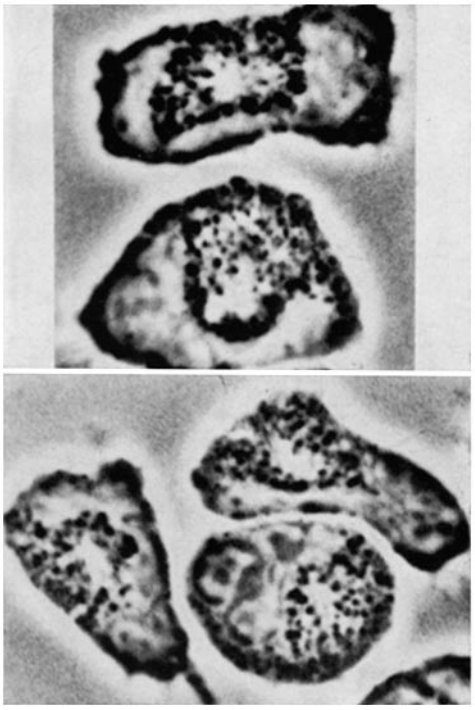

FIG. I

(Hirsch, *et al.*: Streptolysin and leucocytes)

PLATE 11

FIG. II. A sequence of changes at 30 second intervals in the cell on the right following exposure to streptolysin O. The cell on the left was not affected and serves as a standard for comparison. The initial event in the chain of streptolysin toxicity was explosive rupture of granules, shown here in scenes 1 through 5; note the loss of granules in the central portion of the cell. The granules seemed to rupture directly in the cytoplasm, leaving small clear zones in their place. No large vacuoles were formed. Shortly after degranulation, the cell surface began to show numerous long filamentous projections, first seen in these illustrations in the 3rd picture and most prominent in picture 6. The affected cell next rounded up, the cytoplasm liquefied as evidenced by Brownian motion of all remaining granules, and finally the nuclear lobes began to fuse, as is seen in 7 and 8. The leucocyte membrane, though altered, remained intact; no granules or other material was seen to escape. Approximately × 1500.

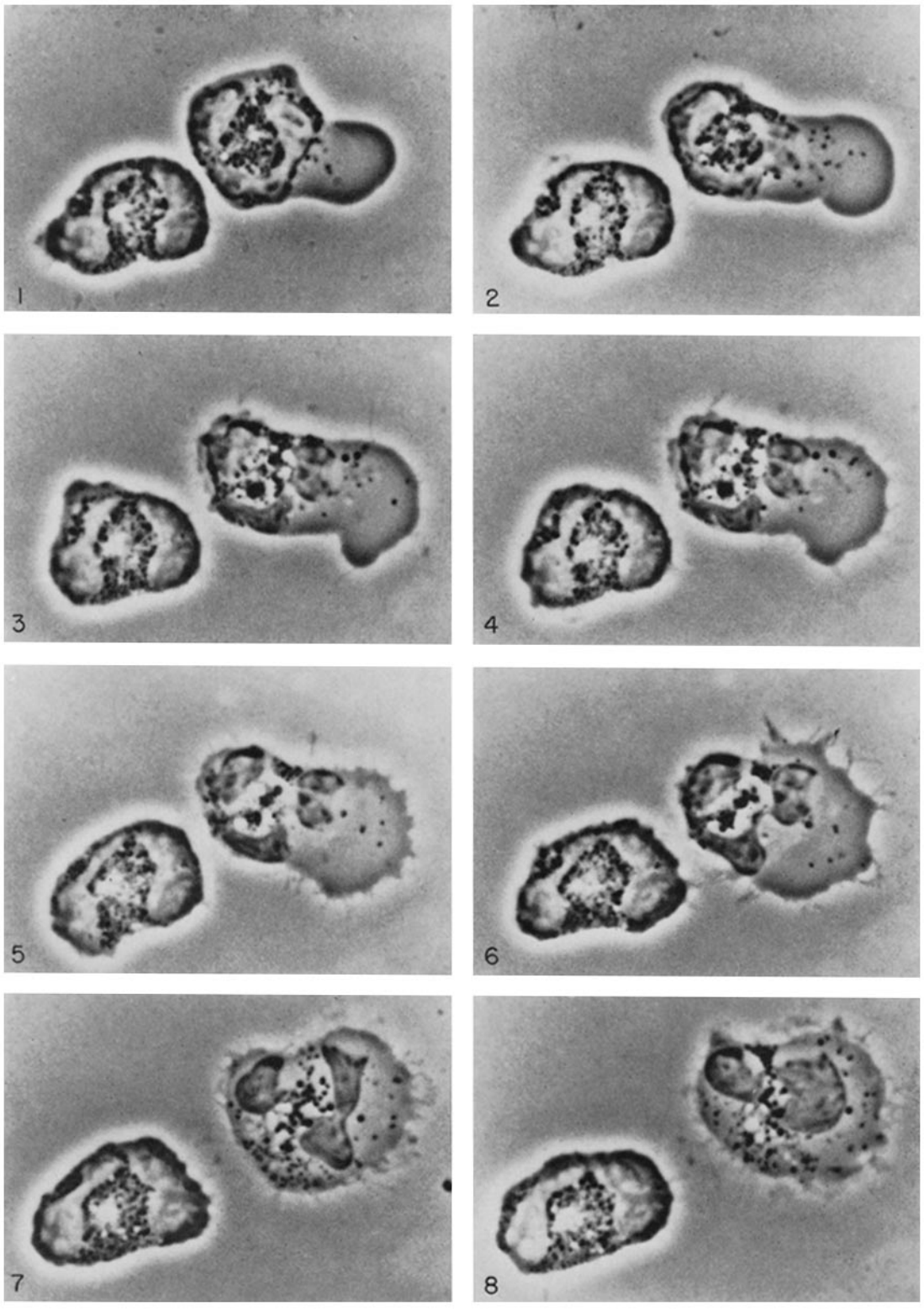

FIG. II

(Hirsch, *et al.*: Streptolysin and leucocytes)

PLATE 12

FIG. III. The upper two prints illustrate lysis of an individual granule in another rabbit polymorphonuclear leucocyte (same cell as that in Fig. IV) damaged by streptolysin O. The round phase-dense body in the center of the circle of the *t* 0 picture has disappeared 0.1 second later. Approximately × 2000.

The lower four prints show, at the time intervals indicated, nuclear fusion following streptolysin O damage (same cell as that in Fig. II). Morphologic aspects and rapidity of the fusion of the two nuclear lobes on the right are illustrated. Approximately × 2000.

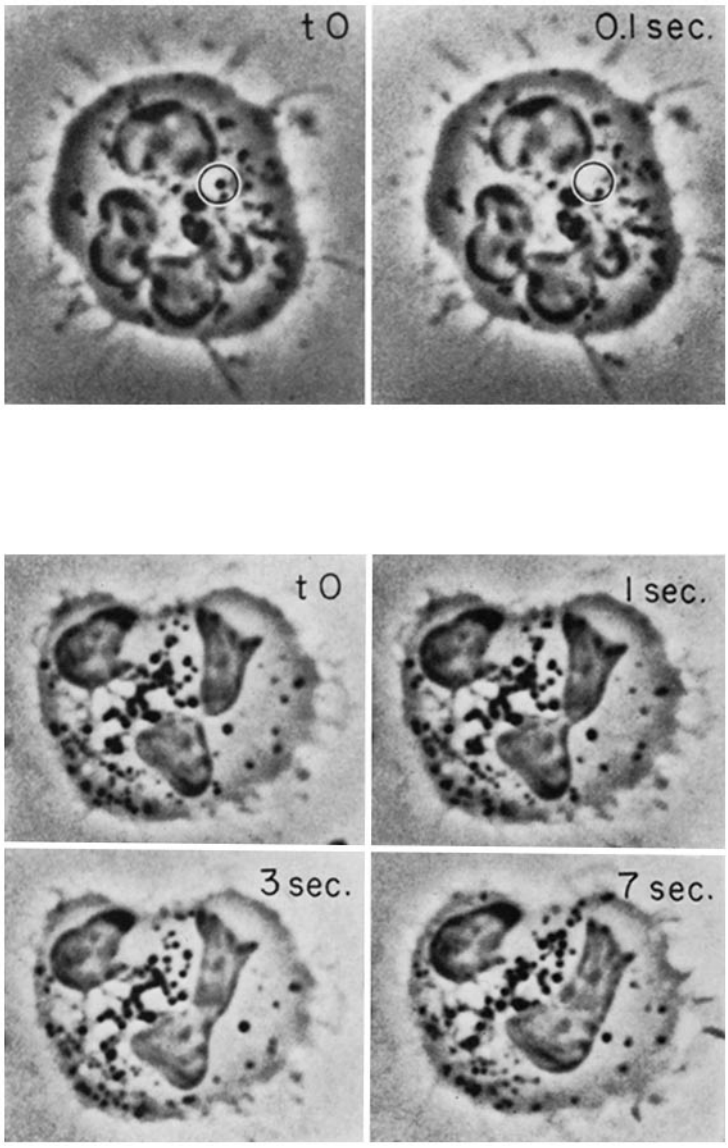

FIG. III

(Hirsch, *et al.*: Streptolysin and leucocytes)

PLATE 13

FIG. IV. Pictures of a leucocyte taken at approximately 1 minute intervals to show late stages of damage by streptolysin O. In print 1 the cell is partially degranulated and has developed hair-like processes on its membrane. The following sequences illustrate progressive, almost complete degranulation, formation of a cytoplasmic vacuole, and nuclear fusion progressing to its end stage, namely, formation of a single ovoid nuclear mass. The progressive decrease in phase contrast of nuclear structures is also evident. Approximately × 2000.

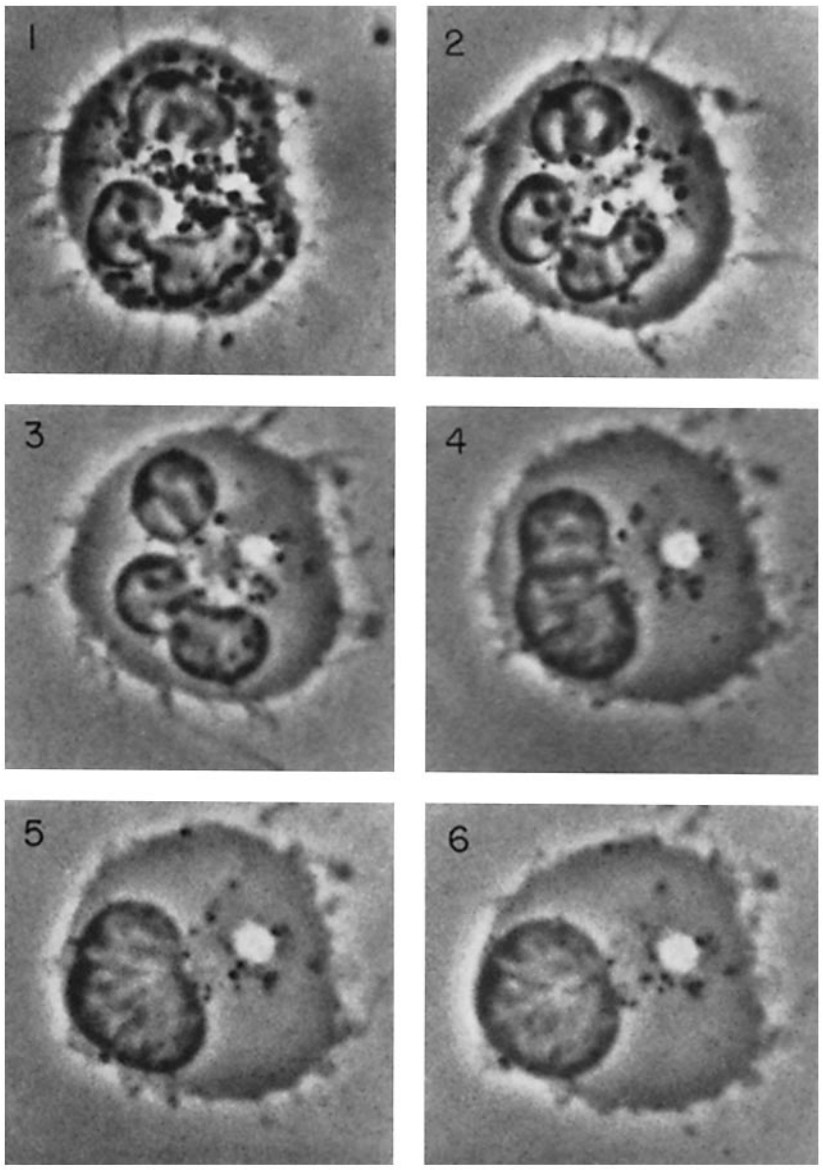

FIG. IV

(Hirsch, *et al.*: Streptolysin and leucocytes)

PLATE 14

FIG. V. Toxic effects of streptolysin S on a rabbit granulocyte. Streptolysin S exerted its toxicity only after a considerable delay, in contrast to the prompt action of streptolysin O. As is shown in these pictures, taken at 30 second intervals, degranulation occurred on exposure of leucocytes to streptolysin S; in this cell cytoplasmic vacuoles formed and granules often seemed to discharge into these clear zones. No filamentous processes were seen on the membrane, and nuclear changes were not striking. Approximately × 2000.

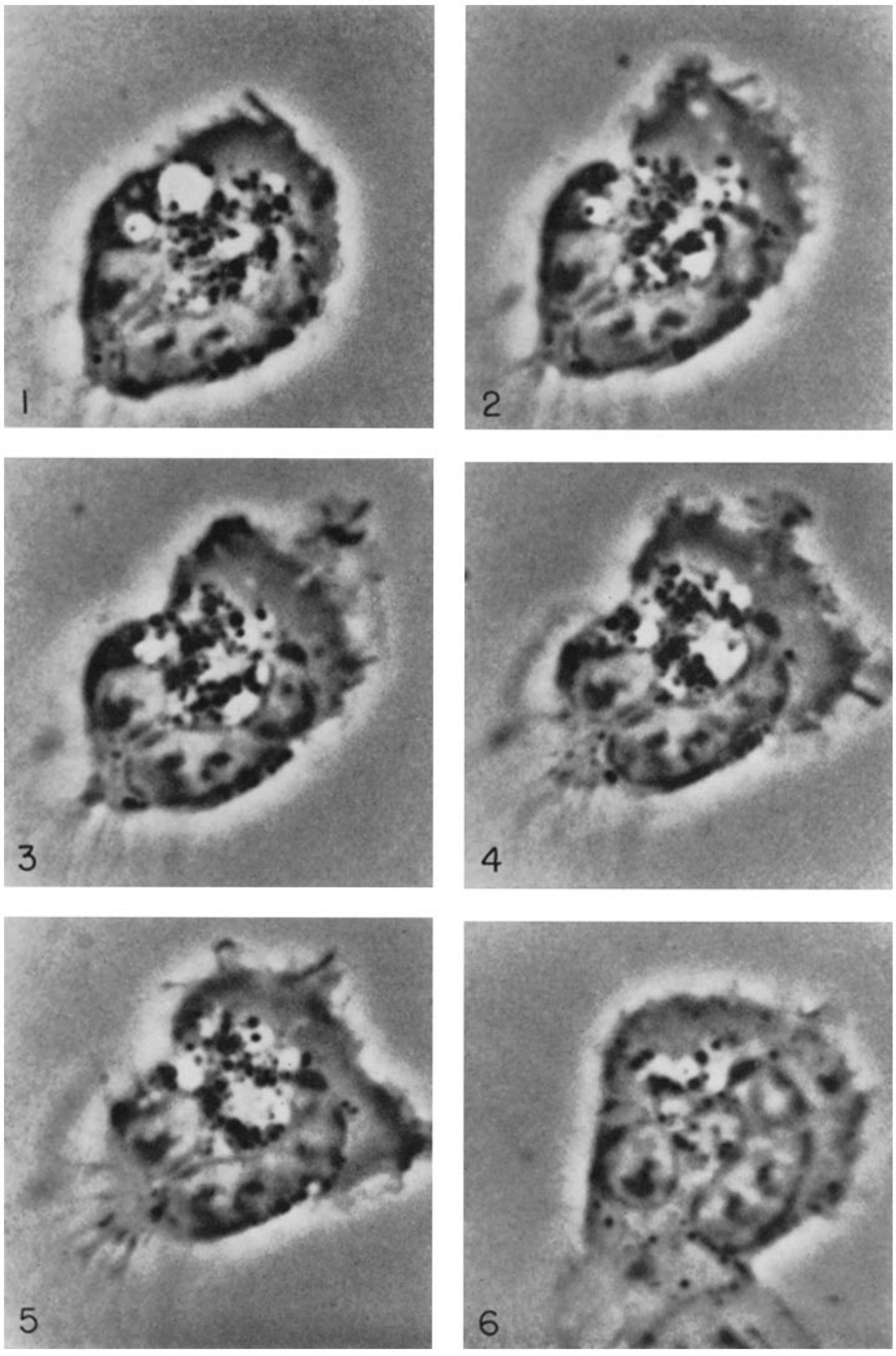

FIG. V

(Hirsch, *et al.*: Streptolysin and leucocytes)

PLATE 15

FIG. VI. Lysis of an individual granule in a leucocyte exposed to streptolysin S. In the t 0 print two dense granules are seen in the circled area; 0.1 second later, one of these granules has disappeared. Approximately × 2000.

FIG. VII. Upper print, a normal rabbit alveolar macrophage. Details of nuclear structure, variety of cytoplasmic granular elements, and microvillous membrane processes are seen. Lower print, another alveolar macrophage, damaged by streptolysin O. The cell has spread out and become irregular in shape. Membrane microvillae have disappeared. Very dark granular elements in the cytoplasm remain, but many of the less dark granules have vanished. Small clear zones have appeared in the perinuclear cytoplasm, giving it a moth-eaten or vacuolated appearance. Approximately × 2000.

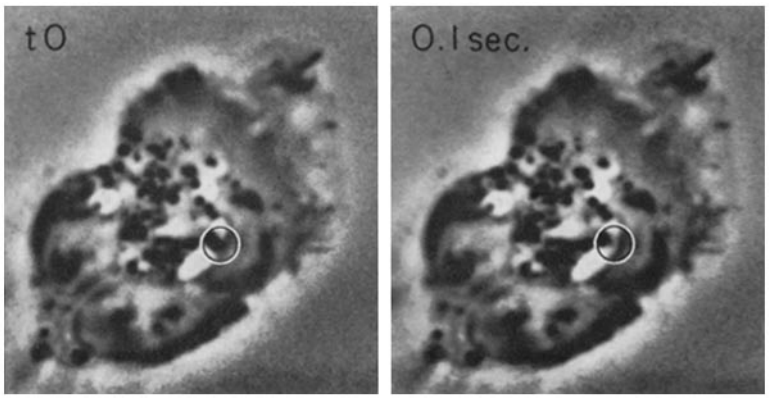

FIG. VI

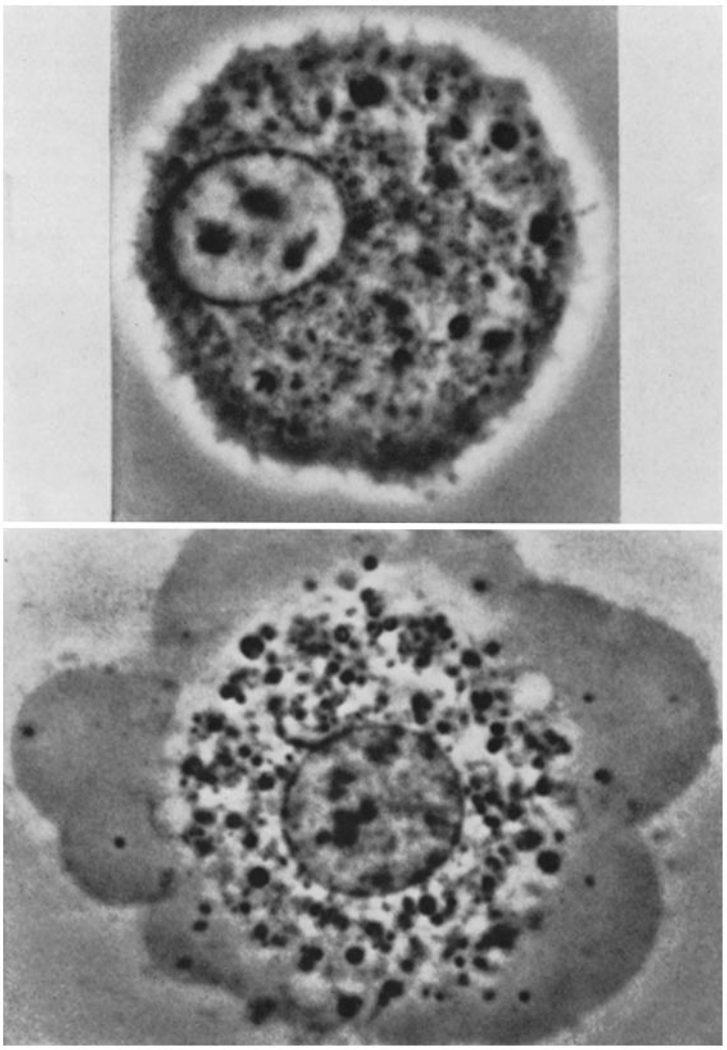

FIG. VII

(Hirsch, *et al.*: Streptolysin and leucocytes)

PLATE 16

FIG. VIII. Rupture of an individual granule in the cytoplasm of an alveolar macrophage exposed to streptolysin O. The degranulation is difficult to illustrate, since the number of granules is so large, and also because the granules which lyse are not the very dark, prominent ones, but rather the less dense bodies which are in many instances poorly demarkated from surrounding cytoplasm. In the circle on the *t* 0 print is one such poorly defined granule, which has lysed in the picture taken 0.1 second later. Although difficult to illustrate in prints, degranulation of macrophages exposed to streptolysin O was readily visible in the motion pictures.

Damage to this cell is also indicated by the smooth surface and vacuole formation in its cytoplasm. Approximately × 2000.

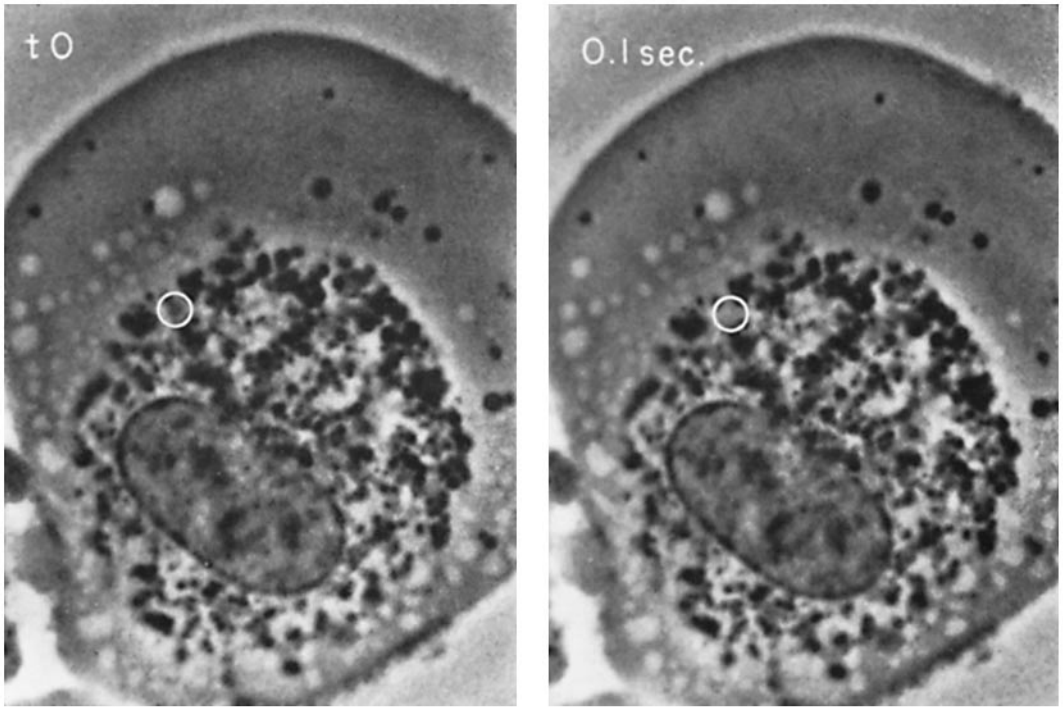

FIG. VIII

(Hirsch, *et al.*: Streptolysin and leucocytes)