

THE TOXICITY OF STREPTOLYSIN O FOR BEATING  
MAMMALIAN HEART CELLS IN TISSUE CULTURE\*

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Since rheumatic fever is known to be initiated by streptococcal infections, considerable attention has been focussed on streptolysin O because of its profound effects on the heart (1-3). Appreciable circumstantial evidence has accumulated which is compatible with the concept that it may play an etiological role in this illness (4-6).

For example, the rapidly lethal effect of intravenous streptolysin O for rabbits and mice is preceded by a dramatic disruption of heart function, as evidenced by electrocardiography (6-8). In vitro, Kellner et al. (2) have demonstrated the direct toxicity of extremely small doses of this toxin for isolated perfused mammalian hearts. Recently, Reitz et al. (9) have analyzed this in vitro toxicity for the guinea pig and rat heart in greater detail, and have demonstrated that two mechanisms are involved. The final cardiac arrest is due to an irreversible disturbance of the atrio-ventricular conduction system, while the atria are reversibly depressed via the release of acetylcholine. The exact nature of the former irreversible damage has not been clarified, but it has been shown that the contractility of ventricular muscle strips from intoxicated hearts was not impaired upon electrical stimulation. Of the numerous streptococcal extracellular products known to be released by the microorganisms in vivo during infection (10), evidence has suggested that streptolysin O may be the only one which is strikingly cardiotoxic. (2).

Streptolysin O has been shown to be cytolytic for a number of mammalian cell types in vitro in addition to erythrocytes; e.g., leukocytes, Ehrlich ascites tumor cells, macrophages, and platelets (11-14). It has been suggested that cell membrane disruption, and/or lysosome lysis may be important factors in cytotoxicity. Keiser et al. have also demonstrated some susceptibility of mitochondria to this toxin (15).

The special implications of the toxicity of streptolysin O for the heart prompted the present investigation of its effects on isolated pulsating mammalian heart cells in tissue culture, (16) in the hopes of shedding light on its cardiotoxicity at the cellular level.

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### *Materials and Methods*

The lyophilized purified streptolysin-O fractions were prepared from Group A and Group C-streptococcal culture supernates by a sequence of ammonium sulfate precipitation, electrophoresis and hydroxylapatite-column chromatography, as reported in previous publications (17, 18). The same preparations were used in earlier studies on the cardiotoxicity of this substance *in vivo* (6, 19). These fractions were contaminated with a maximum of 1.5% DPNase<sup>1</sup> on the basis of the purest fractions of that enzyme presently available. The lyophilized preparations contained about 160,000 and 40,000 hemolytic units (HU)/mg dry weight, respectively, and were approximately 95% in the reversibly oxidized state. Hemolytic assays were carried out as reported previously (17).

For the cytotoxicity tests, the streptolysin O was dissolved in complete growth medium, and activated by mixing with cysteine-HCl in the same medium, so that the final concentration of each was 1 mg/ml, at pH 7.2. After incubation at 30°C for 5 min, the reduced streptolysin was placed in an ice bath, and used within 3 hr. Dilutions of this in complete growth medium were made as needed, warmed to 37°C, and used immediately.

Antibody to group-C streptolysin O was prepared by Prager and Feigen (20) in rabbits, with the lyophilized preparation used in our experiments. Cholesterol suspensions were prepared by the method of Cohen et al. (21).

Ventricular or atrial cells from the hearts of 1-4-day old neonatal Sprague Dawley rats were grown in "Rose" chambers by the method of Mark and Strasser (22). An average of  $3 \times 10^6$  cells was inoculated per chamber, in total volume of 1.5 ml. Generally between 60 and 90% of these cells stretched out, and they were usually used for experiments on the second or third day of incubation. By this time, most of the beating cells had grown into a loose network which contracted synchronously at median rates of approximately 130 beats per min, ranging between 120 and 145 per min. In these cultures, virtually all of the myocardial cells were pulsating.

Beating rabbit myocardial cells were prepared in essentially the same way, from fetal rabbit hearts after 22 or 23 days of gestation. In cultures derived from both rat and rabbit hearts, endothelial cells were always present at 2 days, representing from 15% to 25% in rat heart cultures, and up to one half in the rabbit cultures.

Rat kidney cells were grown from trypsin dissociated 1-7-day kidney fragments, under conditions of culture similar to the heart cells. Growth resulted in a mixture of epithelial and fibroblast cells after 2 days.

Test solutions were added to the cells by injection through the silicone rubber gasket of the culture chamber. For visualization of the cultures, the microscope stage was maintained at 35°C by a Sage air curtain heater (Sage Instruments, Inc., White Plains, N. Y.). Most of the tests were visually observed by phase optics. In some instances, cultures were fixed with osmium tetroxide and stained by Heidenhain's hematoxylin, using picric acid differentiation.

For electron microscopy, in order to facilitate subsequent removal of embedded cultures from the coverslips, the latter were sometimes coated with a film of carbon by exposure to atomic arc-produced carbon for periods ranging from 8 to 15 sec (Mikros Carbon Evaporator, Varian, Palo Alto, Calif.). The ease of removal of cells from the coverslips, which was achieved by increasing the exposure of carbon, had to be balanced against the slightly inhibitory effect of the thicker carbon films on the culture growth. 10 sec was selected as producing an optimal film thickness. The coated coverslips were then sterilized in an oven at 175°C for 2 hr. Less satisfactory alternative methods for solving this difficulty involved coating the fixed cells

<sup>1</sup> According to recent terminology, this may be referred to as nicotinamide adenine dinucleotidase (NAD-ase).

with thin parlodion films, or growing the cells directly on glass coverslips which were later removed after embedding by alternate cooling with dry ice and warming.

Fixation was initially carried out in the culture chambers by rapid introduction of fixative through the silicone rubber gasket with a syringe, and consequent drainage of culture medium from an effluent syringe needle. The fixation was carried out at 37°C in 2.5% glutaraldehyde, maintained at pH 7.4 with 0.05 M cacodylate-buffer containing 0.17 M sucrose. After fixation for 1–2 hr, the cover slip culture was removed from the chamber, and placed in fresh fixative at 4°C overnight. The material was washed in several changes of buffer containing 0.34 M sucrose, then placed in buffered 1% osmium tetroxide for 1–2 hr, and dehydrated in an ethanol series. The fixed cultures on the coverslips were embedded in Araldite (Ciba, Cambridge, England), essentially after the method of Lavail (23) involving immersion of a Beem capsule filled with Araldite over the cells. After the removal of the coverslip, sections were cut on a Porter-Blum MT-1 ultramicrotome, double stained using saturated uranyl acetate in 50% ethanol and lead citrate (24), and finally examined in a Philips EM-100 electron microscope. Cultures which had been exposed to streptolysin O for 1–2 min were fixed and prepared for electronmicroscopy in an exactly similar manner.

In order to determine whether serotonin might be released from the beating heart cells after exposure to streptolysin O, the cultures were grown on a somewhat larger scale. Polystyrene "Cooper" dishes (Falcon Plastics, Los Angeles, Calif.) containing 6 ml of medium were inoculated with  $2 \times 10^6$  cells. After growing for 3 days, activated streptolysin O was added at a final concentration of 13,000 HU/ml. The culture fluid was then withdrawn as rapidly as possible, centrifuged at 4°C, and the supernate stored frozen at –20°C. Parallel cultures to which equivalent amounts of cysteine had been added, or untreated cultures, furnished the control samples. Bioassays of these specimens for serotonin were performed on two independent systems, the guinea pig ileum and the rat uterus.

The antiserotonin drug, 1-methyl-D-lysergic acid-butanolamide bimalate (UML-491) was obtained as a gift through the courtesy of Sandoz, Inc., Hanover, N. J. Serotonin-creatinine complex was purchased from Calbiochem, Los Angeles, Calif.

## RESULTS

Doses of activated streptolysin O at a final concentration of 8000 HU/ml caused cultures of synchronously pulsating networks of myocardial cells to cease beating in less than 20 sec, then to fibrillate vigorously. Numerous spherical cytoplasmic blebs, which extruded from the membranes of each cell, rapidly appeared within 1 min (as shown in Fig. 1) and cytoplasmic organelles became intensely granular. After 5 min, the double-walled nuclear envelope appeared swollen, and the contents of the nuclei were disorganized. At this dose level, all beating cells were killed. The remnants of the dead myocardial cells remained relatively intact over a period of 18 hr. The minimum toxic concentration at which about 10%–20% of the myocardial cells were rapidly killed was approximately 100 HU/ml, and a rather sharp endpoint was observed in the titrations of cytotoxic activity. In about one half the cultures exposed to 50% of the minimum toxic level, a temporary arrest in beating of the cells was observed, followed by a brief period of fibrillation, with subsequent recovery within 1 or 2 hr. In controls to which an equivalent volume of medium with cysteine was added, there was no alteration in beating rate or amplitude of contraction. Streptolysin-O preparations from Group A and Group C

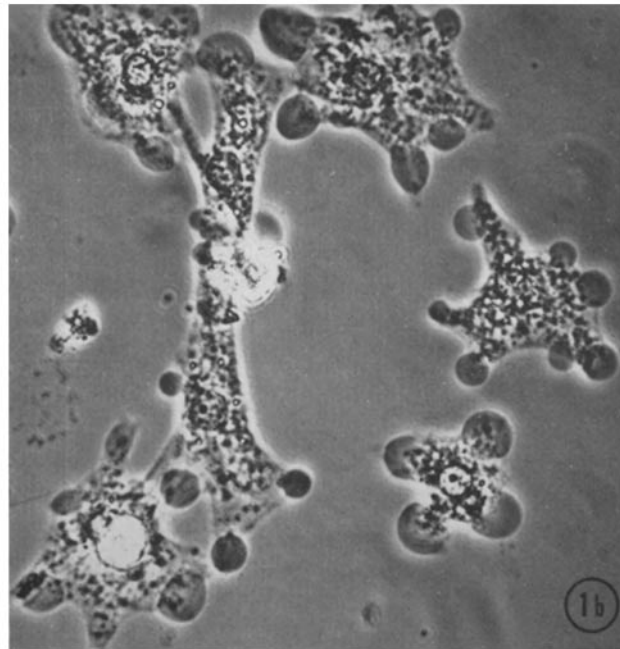


FIG. 1. Culture of rat heart ventricle cells after 2 days of growth; unstained, phase contrast visualization.  $\times 600$ . (a), Before treatment: the cells on the left are myocardial cells, are clearly striated, and were pulsating vigorously. Those on the right were not beating and were considered to be "endothelial" cells. (b), The same culture 3 min after exposure to 8000 HU/ml of reduced group-C streptolysin O. Note the multiple plasma membrane blebs and the cytoplasmic granulation of the killed myocardial cells.

streptococci revealed identical toxic effects on rat heart cells, and the minimal toxic levels were roughly equivalent in terms of hemolytic units/ml.

At lower toxic concentrations of streptolysin O (80–120 HU/ml), the sequence of cytotoxic effects was somewhat slower, requiring up to 10 min for completion. In all instances, however, beating ceased prior to the appearance of cell membrane blebs. At these minimal toxic levels, not all of the pulsating



FIG. 2. Culture of rat heart ventricular cells 30 min after addition of group-C streptolysin O at a final concentration of 100 HU/ml. Note the intact surviving myocardial cells (arrows), which were still beating, although surrounded by killed cells. The difference in morphology of the surviving cells shown represent the extremes normally seen in the untreated cultures. Unstained; phase contrast.  $\times 600$ .

myocardial cells were affected. Up to 80–90% of them continued to beat and appeared undisturbed, suggesting an “all-or-none” type of lethality. Killed cells immediately adjacent to beating, apparently healthy ones are shown in Fig. 2.

A limited number of similar tests on pulsating rabbit myocardial cells in tissue culture indicated that they were equally susceptible to the toxicity of streptolysin O.

On several occasions, myocardial cells were seen by phase contrast in which several dense concentrations of cytoplasm would form just inside the cell

membrane, after exposure to streptolysin O. These would bulge outwards, but the membrane would not be broken. After several minutes, such "incipient blebs" would disappear into the interior of the cell, which would then resume its normal appearance and beating function.

The myocardial cell cultures which had been fixed and stained with hematoxylin 2-5 min after intoxication by streptolysin O, revealed extruding blebs which appeared to be bounded by a membrane, as well as many swollen dense intracellular vacuoles outlined by a dark boundary, when viewed by light microscopy (Fig. 3). The myofibrils themselves looked relatively intact, although the vacuolation and displacement of cytoplasm into blebs, masked many of them. They did not appear to be as well organized as they were in the controls, where they were the chief visible intracellular structure in many cells, extending uniformly across their width. 2 min after streptolysin exposure, the nucleus and its surrounding double-membrane looked similar to those in untreated controls by light microscopy; but by 5 min, the nuclear disorganization was quite obvious, with dense aggregations of presumably chromatinic material throughout the nucleoplasm, and at the inner boundaries of the envelope.

When viewed by the electron microscope, normal myocardial cells prepared from 2-day neonatal rats, after 3 days in tissue culture, appeared fairly uniform with regard to the maturity of their intracellular organization, although less mature cells were also common. In addition to well defined sarcomeres, characteristic mitochondria and ribosomes were evident in the sarcoplasm (Figs. 4 and 5). Prominent intercalated discs occurred in some sections (Fig. 5).

After exposure to 8000 HU/ml of streptolysin O for between 1 and 2 min, followed by immediate fixation, the cells showed dramatic changes. The blebs that were so apparent under phase contrast, could be resolved in the electron microscope as protrusions of the plasmalemma, into which there was comparatively sparse leakage of organelles from the cytoplasm (Fig. 6). The chief cytoplasmic components of the bleb were ribosomes; occasional mitochondria and fragments of endoplasmic reticulum were present at the base of the bleb.

The myofibrils of well differentiated cells appeared somewhat disorganized, but were still readily discernible (Figs. 7 and 8). In myocardial cells, such as that illustrated in Fig. 8, which is presumably less well differentiated into myofibrils, but distinguishable from endothelial cells by the presence of filaments in thick bands, the effect on the endoplasmic reticulum was striking (see Fig. 8). The cisternae were considerably dilated, and the contents became condensed.

There was widespread vacuolation of the cytoplasm after streptolysin-O treatment (Figs. 7 and 8), accompanied by similar changes in the mitochondria, with some swelling of the cristae. Where the Golgi complex was seen, this too had become vacuolated. The nuclear envelope remained intact, and its apparent

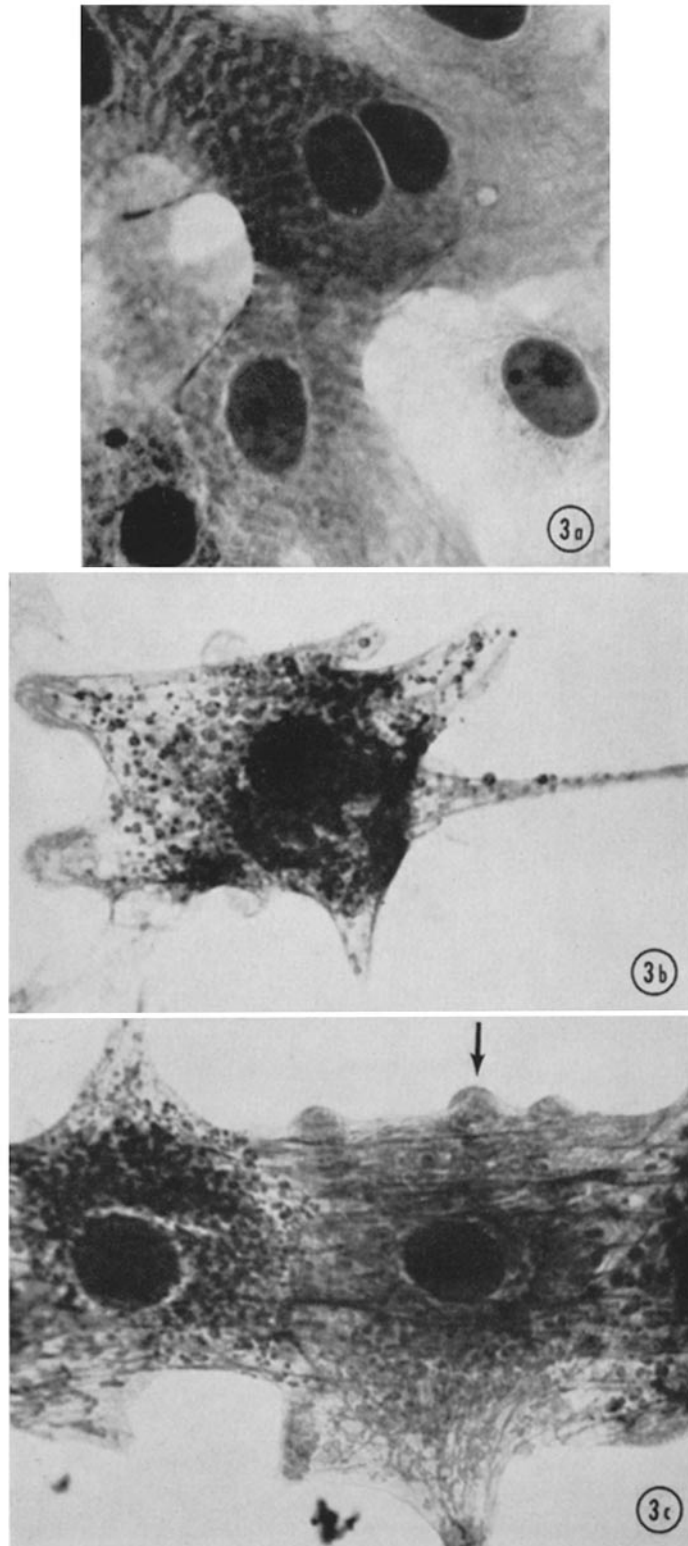
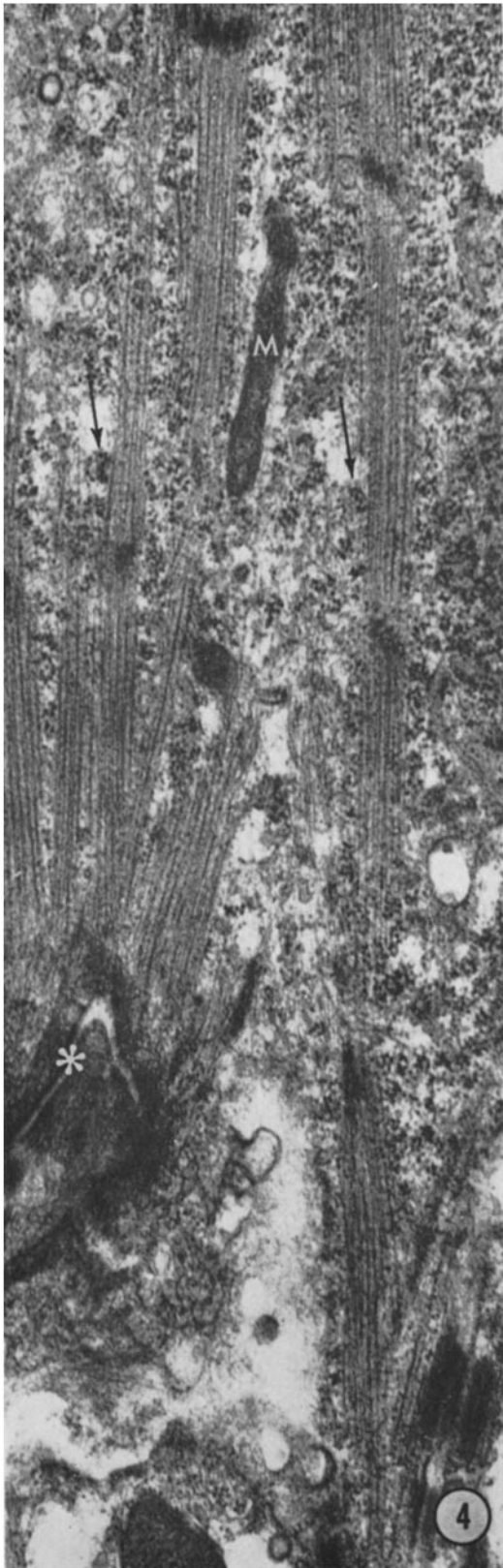


FIG. 3. Culture of rat heart ventricular cells fixed with osmium tetroxide and stained with hematoxylin. (a) Control, untreated.  $\times 1150$ . (b) Isolated myocardial cell after exposure to group-C streptolysin O (8000 HU/ml), 4 min prior to fixation.  $\times 1250$ . (c) Three cardiac cells damaged by group-C streptolysin O, under the same conditions as in (b). The central cell shows alterations which suggest early or reversible, incipient blebbing (arrow).  $\times 1150$ .





thickening observed by phase microscopy was seen to be due to the piling up of dense material, presumably chromatin, against the inner side of the nuclear envelope. The nucleoplasm appeared less dense than normal.

Since the cultures employed contained an appreciable proportion of non-beating cells which have been identified as endothelial cells (22), it was possible to directly compare their susceptibility to streptolysin O with that of the pulsating myocardial cells. Although no clearcut quantitative differences in their sensitivity to this toxin could be seen, the endothelial cells were affected more slowly, and in a different way. At doses which caused widespread rapid death of myocardial cells with multiple blebbing within 2-3 min, the endothelial cells were still relatively intact after 10 min (Fig. 9). In addition, blebs in the latter cells were rarely seen, and they either disintegrated gradually over a period of 20 min, or became detached from the glass. At higher streptolysin-O concentrations, virtually no endothelial cell remnants could be seen after 18 hr, in contrast to the condensed residues of the myocardial cells.

Cell membrane blebs also appeared very rapidly when rat kidney cells were exposed to streptolysin O. Cytoplasmic granulation appeared within 1 to 2 min, followed by changes in the nuclear membranes which were similar to those seen in myocardial cells. However, usually only single large cytoplasmic blebs were observed in the damaged kidney cells, instead of the numerous ones visible in affected myocardial cells (see Fig. 10). The sensitivity of renal cells to this toxin appeared to be equivalent to that of the heart cells. No differences in susceptibility were noted in renal cells which were morphologically epithelial or fibroblast in nature.

Because of the presence of small amounts of contaminants in the streptolysin-O preparations, control tests were performed to rule out their role in the observed cell destruction. Good correlation was found between cytotoxicity and the loss of hemolytic properties under conditions known to inhibit the latter. Thus, pretreatment of the streptolysin O with equivalent amounts of cholesterol on an equal weight basis, completely inhibited the toxicity to the heart cells. When the proportion of cholesterol to streptolysin O was reduced (1:3), the myocardial cells were not killed, but they temporarily stopped

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FIG. 4. Part of a myocardial cell prepared for electron microscopy from a control culture 3 days old. The section is cut longitudinally with respect to the myofibrils. Mitochondria (M) and ribosomes (arrows) occur between the fibrils. An intercalated disc, cut obliquely, is included (asterisk). Glutaraldehyde and osmium tetroxide fixation; staining with uranyl acetate and lead citrate.  $\times 30,000$ .

FIG. 5. A section similar to that shown in Fig. 4 including a transverse section of an intercalated disc (asterisk) and aligned myofibrils in which the sarcomeres are defined by dense Z bands (Z). The large mitochondria (M), a characteristic feature of myocardial cells, are prominent in the sarcoplasm. Fixed and stained as in Fig. 4; arrow points to ribosomes.  $\times 17,000$ .

beating and then fibrillated, after which they recovered and contracted at normal rates and vigor within 1 hr.

Reversibly oxidized streptolysin O (about 95% oxidized), was much less acutely toxic for pulsating heart and kidney cells, the minimum toxic level

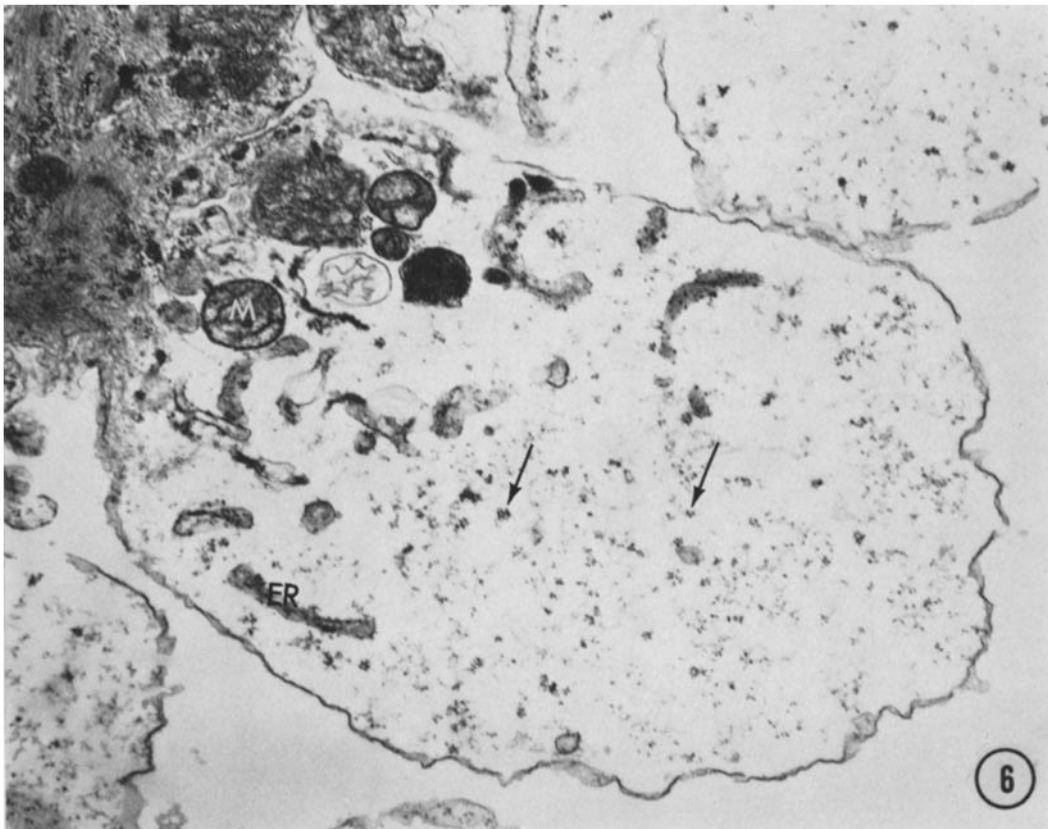


FIG. 6. Electronmicrograph of one of the blebs which occurred shortly after exposure of myocardial cells (1 min, 15 sec) to streptolysin O. The striking feature is that the components of the cell did not flow rapidly into the bleb. There is only slight leakage of cytoplasmic fragments such as mitochondria (M), cisternae of the rough surfaced endoplasmic reticulum (ER), and scattered ribosomes (arrows). Myofibrils (f) can be seen in the main body of the cell at upper left.  $\times 21,000$ .

being about 100 active HU/ml (2000 potential HU/ml). At these concentrations, the same cytolytic changes in the cells proceeded as rapidly as with the reduced preparations. The small residue of reduced active streptolysin (about 5%), correlated rather well with the acute cytotoxicity observed. At lower

concentrations of oxidized streptolysin-O, cytotoxic effects on pulsating cardiac cells were observed, but these usually required several hours for completion, although they were qualitatively similar. Because of this latter observed delay in the toxic process with the oxidized preparation, experiments were performed to determine whether its reduction may have been brought about in the medium by the presence of living cells. However, no reducing substances capable of activating streptolysin O could be detected in fluids harvested from 2- and 3-day old heart or kidney cell cultures. Furthermore, the residual oxidized streptolysin O detectable in the medium 2 hr after addition to the cultures, had not been significantly depleted. It is possible that the oxidized form is gradually converted to the active form by the presence of actively metabolizing tissue, and that these small activated quantities are attached and exert their effects on the cells immediately after reduction. They would, therefore, not be detectable by our methods.

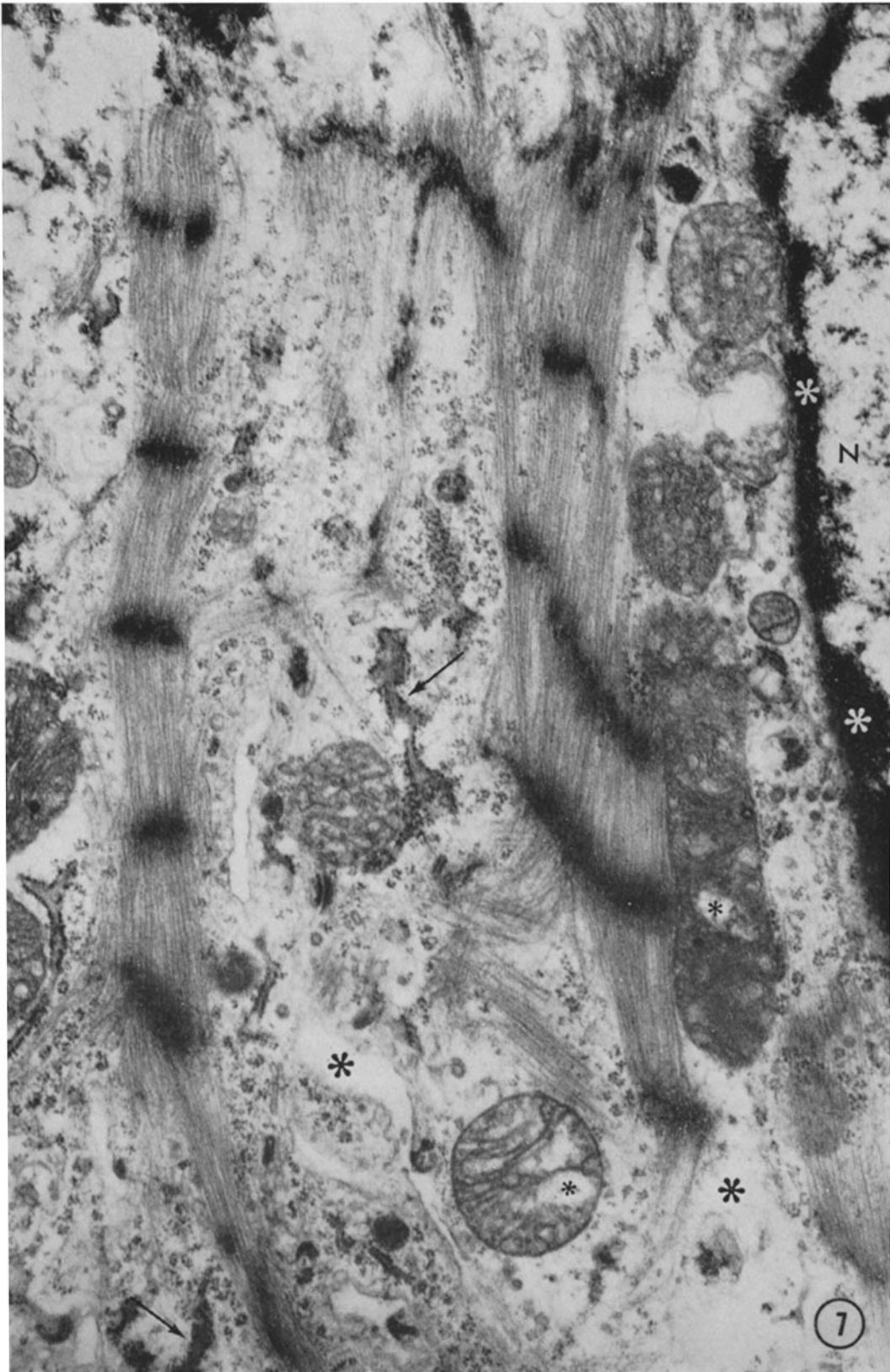
The reduced streptolysin O, after heating to 56°C for 30 sec was rendered innocuous for the beating heart or kidney cells. This treatment is known to destroy its hemolytic activity. Addition of rabbit antiserum to the reduced streptolysin, prior to its introduction to cell cultures, also completely prevented any cytotoxic effects. Control cysteine-HCl solutions in the growth medium, or inocula of growth medium itself did not cause significant alteration in any of the cell types studied. Analysis of the culture fluid in which beating heart cells had grown for 3 days failed to reveal the presence of an inhibitor analogous to that shown by Bernheimer et al. (1) with frog hearts, or by Rowen (25) in the plasma of mice receiving sublethal doses of streptolysin O.

Since several drugs with antiserotonin activity had been shown to protect mice against the acute lethal effects of streptolysin O, one of the most effective of these was studied in the pulsating heart cell system. UML-491, a lysergic acid derivative, was added to vigorously beating cultures at a final concentration of 40  $\mu\text{g}/\text{ml}$ , a level found in preliminary tests to be the maximum tolerated. After incubation for 15 min, one or five minimal toxic doses of activated streptolysin O were added. No protection against its toxicity was observed.

Serotonin assays of fluids obtained from heart cell cultures immediately after streptolysin-induced killing failed to reveal any evidence for release of this mediator. Moreover, addition of serotonin, in the form of serotonin-creatinine complex, to vigorously growing heart cells at final concentrations of 0.1 or 0.2 mg/ml failed to reveal any significant toxic effects.

#### DISCUSSION

These results demonstrate the extremely rapid susceptibility of functioning myocardial cells to the lethal effects of streptolysin-O *in vitro*. The virtually immediate cessation of beating, followed rapidly by multiple cell membrane bleb formation, support previous indications from other systems that this



toxin acts through disruption of the cell membrane integrity. Although the cardiac endothelial cells were susceptible, they were apparently affected more slowly, and cell membrane blisters were not conspicuous. The kidney cells were also affected rapidly, and there was a tendency for only single large blebs to be formed as they died.

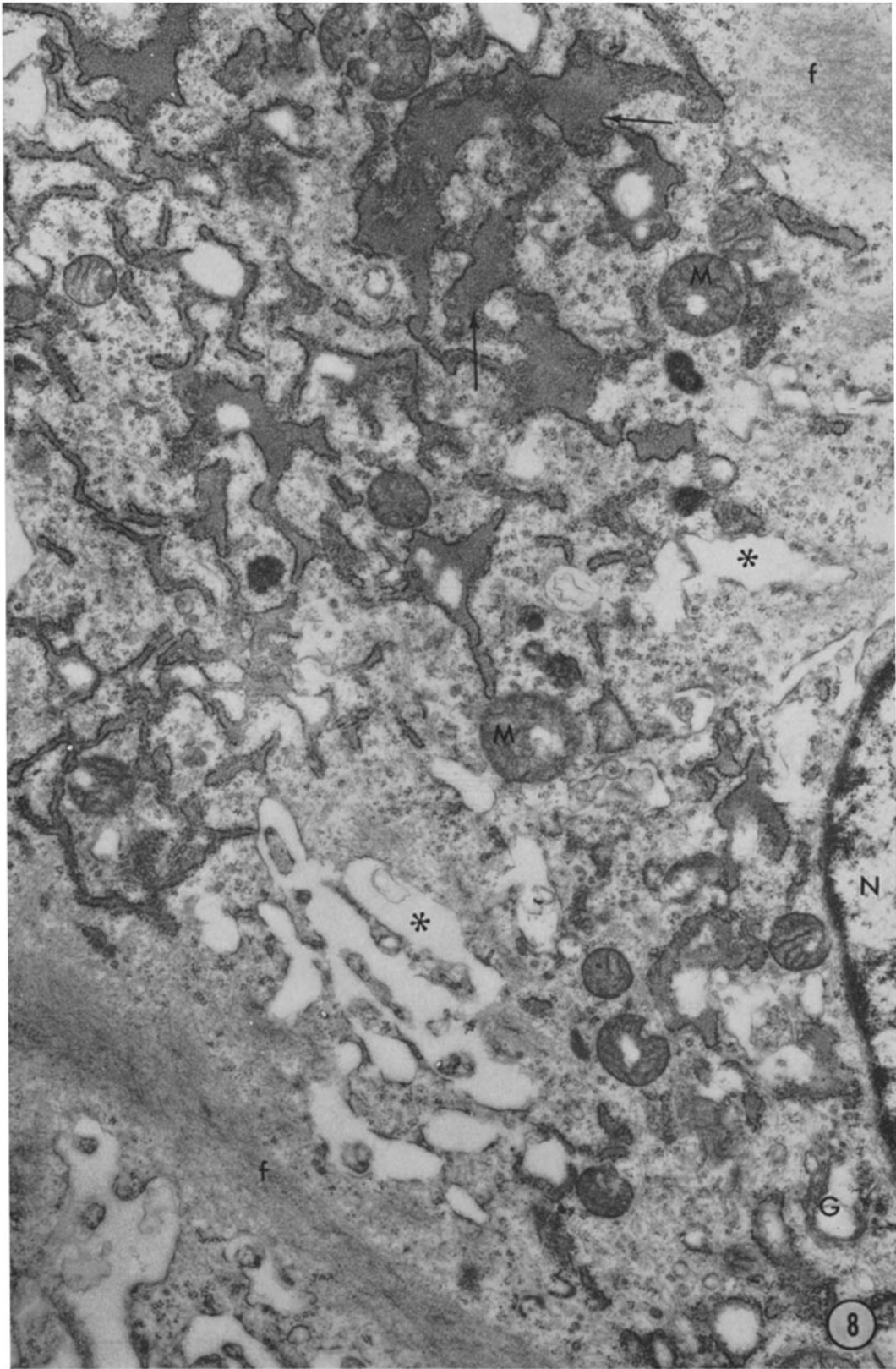
The findings at the electron microscope level indicate that the most dramatic and unusual early alteration of cardiac cells by streptolysin O intoxication was manifested in the endoplasmic reticulum. These structures became quite swollen, and their contents were considerably condensed. The rapidity of this change in the interior of the cell is of interest, since the cells were fixed  $1\frac{1}{2}$ -2 min after streptolysin exposure. The cell membrane also became rapidly altered, so that bulging occurred, with some emptying of the cell organelles into the bleb. No evidence of cell membrane rupture was seen. In addition, no evidence of lysosome lysis was evident in the cardiac cells killed by streptolysin O. Indeed, lysosomes were quite scarce in the normal cells. Those latter findings suggest that lysosome damage cannot be the primary point of attack of streptolysin O for heart cells, as has been indicated in the case of leukocytes (26, 27).

The cessation of beating was probably not due to the direct effects of streptolysin on the myofibers, since these were found by electron microscopy to be relatively intact after cell death. However, numerous vacuoles were noted in the cytoplasm, and these were also seen to some extent in the mitochondria and the Golgi apparatus. The changes in the nucleus were those commonly seen in cell death, but the nuclear membrane remained intact.

The relation of these findings to the studies of Reitz et al. (9) on the mechanism of streptolysin O cardiotoxicity for the isolated perfused mammalian heart, is worthy of comment. The irreversible phase of its toxicity for the heart *in vitro* was apparently damage to the conduction system, since ventricular myocardial strips from intoxicated hearts contracted normally upon electrical stimulation. Moreover, normal electrically-driven ventricular strips were not apparently affected by exposure to streptolysin O. The present findings demonstrate that streptolysin O can kill beating ventricular heart cells very rapidly and dramatically when they are directly exposed to this toxin. Penetration of the streptolysin O into the ventricular myocardial cells may not occur rapidly enough in the perfused heart system to kill an appreciable number of them. The conduction system may be more sensitive to the toxin, or it may simply

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FIG. 7. Electronmicrograph of a myocardial cell from a 3-day culture which had been exposed to streptolysin-O for 1 min, 15 sec. The nucleus (N) shows dark masses, presumably of chromatin, which are especially conspicuous against the nuclear envelope (white asterisks). Swollen endoplasmic reticulum (arrows) are seen, and evidence of cytoplasmic (large black asterisks) and mitochondrial (small black asterisks) vacuolation may be noted. The longitudinal myofibrils, with the dark Z bands, are relatively intact.  $\times 24,000$ .



be more available to it in the perfused heart. With adequate doses, beating atrial cells were also rapidly killed by streptolysin in the tissue culture system.

The relative sensitivities of cardiac endothelial, renal epithelial, and fibroblast cells to streptolysin O were of the same order of magnitude as that of

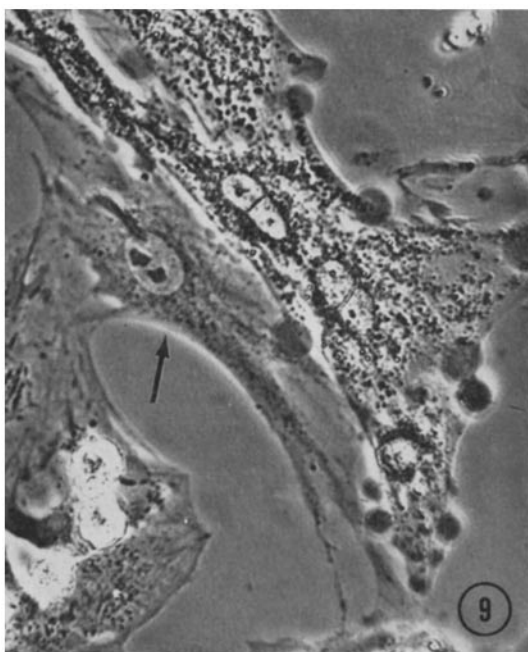


FIG. 9. Culture of rat heart ventricle cells 10 min after exposure to 400 HU/ml group-C streptolysin O. Killed myocardial cells with multiple blebs in close proximity to an endothelial cell (arrow), which at this stage appears unaffected. Phase,  $\times 600$ .

beating heart cells, but they appeared to be damaged somewhat more slowly than the latter. With any of the cell types, at lower toxic levels of streptolysin, an all-or-none killing was evidenced. It was common to see a normal intact beating cell in close proximity to one which had been lysed, showing the typical blebbed appearance. This tendency has been noted by other investigators with erythrocytes (28), leukocytes (11), macrophages (14), and lysosomes (27), as

FIG. 8. Electronmicrograph showing the striking and rapid effect of streptolysin O (1 min, 15 sec exposure) on the endoplasmic reticulum of a myocardial cell. The cisternae has become dilated and the contents condensed (arrows). The mitochondria (M) show signs of vacuolation, as do the Golgi apparatus (G) and extensive areas of the cytoplasm (asterisks). Fibrils (f) are visible. A portion of the nucleus (N) is seen at right.  $\times 19,000$ .

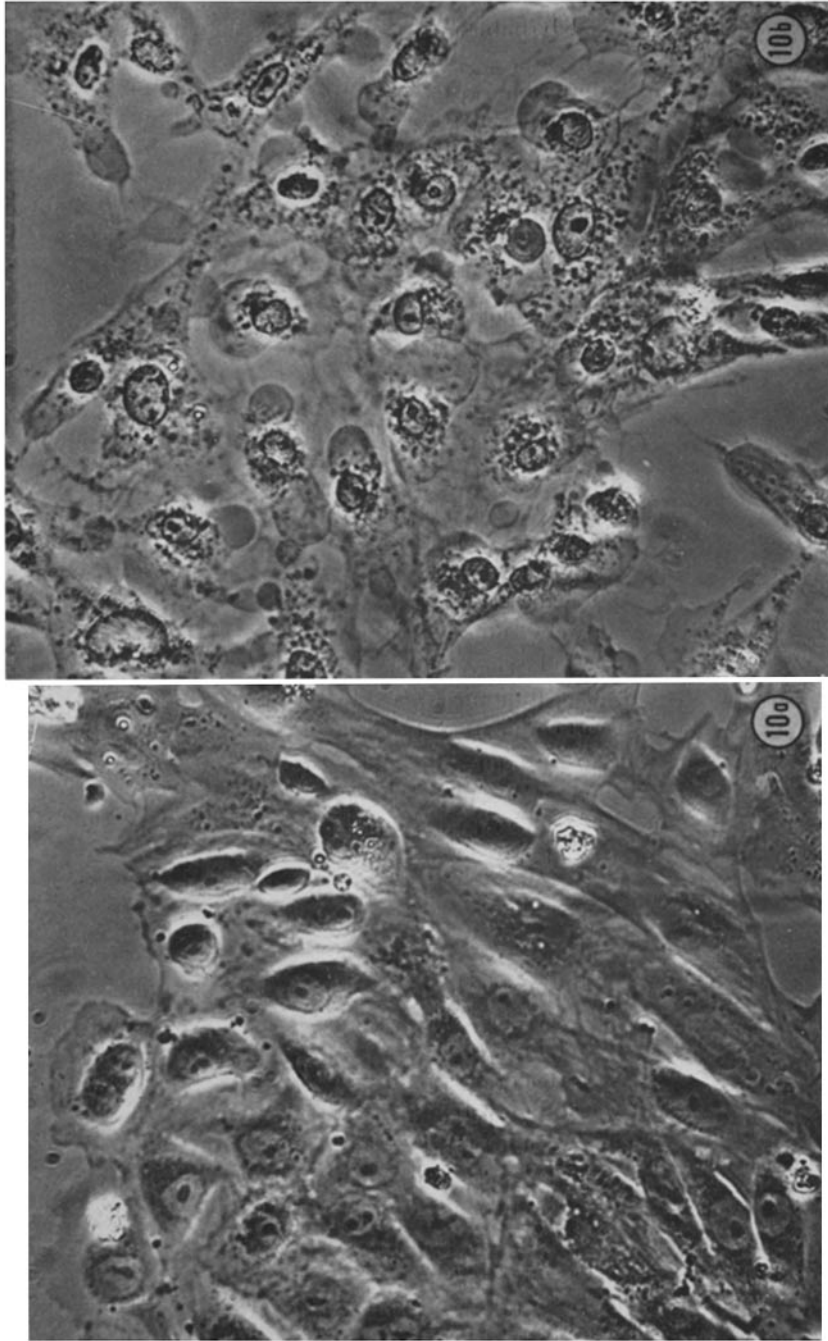


FIG. 10. Culture of rat kidney epithelium, unstained; phase contrast.  $\times 600$ . (a) Control, before treatment. (b) Same culture, 5 min after exposure to 8000 HU/ml of group-C streptolysin O. Fewer plasma-membrane blebs per cell are seen, compared with the changes induced in myocardial cells.



well as in the acute lethal effects seen upon intravenous injection into mice and other animals (6, 7, 29). The same type of all-or-none killing of superficial skeletal muscle cells was observed in guinea pigs after intradermal injection of this toxin (30).

At concentrations of streptolysin O which just failed to kill any beating myocardial cells, they stopped pulsating temporarily, fibrillated, and then recovered. This could indicate that there was sufficient streptolysin O to attach to the cell and cause depolarization of the membrane, but that there was an insufficient amount to effect rupture and cell lysis. Of some interest in this regard, was the earlier finding that sublethal doses of streptolysin O in vivo caused temporary and varied electrocardiographic changes in a high proportion of the animals (6).

The number of cytoplasmic blebs per cell was consistently higher in myocardial cells than in the kidney cells or endothelial cells. It is conceivable that this is a reflection of the number of "attack" sites on the cell membrane. If so, it could account for the apparent increased rapidity of effect of streptolysin O for cardiac cells.

The failure of a streptolysin protective antiserotonin agent to influence the cytotoxicity of streptolysin O for the beating heart cells, and the lack of effect of serotonin in rather high concentrations on these cells, indicates that the observed in vivo protection by this drug acts through a different mechanism. In addition, significant amounts of serotonin were not detected from isolated myocardial cells which had been killed by streptolysin O. Feigen has shown that serotonin is released in considerable quantities from the perfused mammalian heart when intoxicated by this streptococcal product.<sup>2</sup> He has also found that this antiserotonin drug does not protect against streptolysin O damage to the perfused whole heart, nor does serotonin itself have any significant effect on that system (9).

#### SUMMARY

Pulsating mammalian myocardial cells were found to be highly susceptible in tissue culture to rapid destruction by streptolysin O. Cessation of beating occurred almost immediately, followed within minutes by multiple cell membrane bleb formation. Parallel with these changes, the cytoplasm became intensely granular and the nuclear membrane apparently thickened when viewed by phase microscopy. At the ultrastructural level, the cell membrane blebs were found to contain relatively small numbers of granular fragments. The endoplasmic reticulum of damaged heart cells was quite swollen, and its contents were considerably condensed. The myofibers were not strikingly altered, but cytoplasmic and mitochondria vacuoles were rather abundant.

<sup>2</sup> Feigen, G. 1968. Personal communication.

Cardiac endothelial, kidney epithelial, and fibroblast cells were also susceptible to lysis by this toxin, but the reactions occurred more slowly or bleb formation was less evident. An antiserotonin drug known to be protective against streptolysin-O in vivo (UML-491), did not protect against killing of cardiac cells at the tissue culture level. Serotonin could not be detected in the culture fluid after lysis of cardiac cells by streptolysin O.

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#### BIBLIOGRAPHY

1. Bernheimer, A. W., and G. L. Cantoni. 1945. The cardiotoxic action of preparations containing the oxygen-labile hemolysin of *Streptococcus pyogenes*. *J. Exp. Med.* **81**:295.
2. Kellner, A., A. W. Bernheimer, A. S. Carlson, and E. B. Freeman. 1956. Loss of myocardial contractility induced in the isolated mammalian heart by streptolysin O. *J. Exp. Med.* **104**:361.
3. Halbert, S. P., R. Bircher, and E. Dahle. 1961. Cardiac effects of streptolysin O in rabbits. *Nature (London)*. **189**:232.
4. Taranta, A. Factors influencing recurrent rheumatic fever. 1967. *Annu. Rev. Med.* **18**:159.
5. Stetson, C. A. 1954. The relation of antibody response to rheumatic fever. In *Streptococcal Infections*. M. McCarty, editor. Columbia University Press, New York. 208-218.
6. Halbert, S. P., R. Bircher, and E. Dahle. 1961. The analysis of streptococcal infections. V. Cardiotoxicity of streptolysin O for rabbits in vivo. *J. Exp. Med.* **113**:759.
7. Halbert, S. P., R. Bircher, and E. Dahle. 1963. Studies on the lethal toxic action of streptolysin O and the protection by certain antiserotonin drugs. *J. Lab. Clin. Med.* **61**:437.
8. Halpern, B. N., and S. Rahman. 1968. Studies on the cardiotoxicity of streptolysin O. *Brit. J. Pharmacol. Chemother.* **32**:441.
9. Reitz, B. A., D. J. Prager, and G. Feigen. 1968. An analysis of the toxic actions of purified streptolysin O on the isolated heart and separate cardiac tissues of the guinea pig. *J. Exp. Med.* **128**:1401.
10. Halbert, S. P. 1964. Analysis of human streptococcal infections by immunodiffusion studies of the antibody response. In *The Streptococcus, Rheumatic Fever and Glomerulonephritis*. J. Uhr, editor. The Williams and Wilkins Co., Baltimore, Md.
11. Bernheimer, A. W., and L. L. Schwartz. 1960. Leucocidal agents of hemolytic streptococci. *J. Pathol. Bacteriol.* **79**:37.
12. Bernheimer, A. W., and L. L. Schwartz. 1965. Effects of staphylococcal and other bacterial toxins on platelets in vitro. *J. Pathol. Bacteriol.* **89**:209.
13. Ginsburg, I., and N. Grossowicz. 1960. Effect of streptococcal hemolysins on Ehrlich ascites tumour cells. *J. Pathol. Bacteriol.* **80**:111.
14. Fauve, R. M., J. E. Alouf, A. Delauney, and M. Raynaud. 1966. Cytotoxic effects

- in vitro of highly purified streptolysin O on mouse macrophages cultured in a serum free medium. *J. Bacteriol.* **92**:1150.
15. Keiser, H., G. Weissman, and A. W. Bernheimer. 1964. Studies on lysosomes. IV. Solubilization of enzymes during mitochondrial swelling and disruption of lysosomes by streptolysin S and other hemolytic agents. *J. Cell Biol.* **22**:101.
  16. Harary, I., and B. Farley. 1960. In vitro studies of single isolated beating heart cells. *Science, (Washington)*. **131**:1674.
  17. Halbert, S. P. 1958. The use of precipitin analysis in agar for the study of human streptococcal infections. III. The purification of some of the antigens detected by these methods. *J. Exp. Med.* **108**:385.
  18. Halbert, S. P., and T. Auerbach. 1961. The use of precipitin analysis in agar for the study of human streptococcal infections. IV. Further observations on the purification of Group A extracellular antigens. *J. Exp. Med.* **113**:131.
  19. Halbert, S. P., E. Dahle, S. L. Keatinge, and R. Bircher. 1965. Studies on the role of potassium ions in the lethal toxicity of streptolysin O. *In* Recent Advances in Pharmacology of Toxins. H. W. Raudonat, editor. Pergamon Press Ltd., Oxford, England. 149.
  20. Prager, D., and G. Feigen. 1970. Response of the sensitized heart to oxidized and reduced streptolysin O. *Int. Arch. Allergy Appl. Immunol.* **38**:174.
  21. Cohen, B., H. Schwachman, and M. E. Perkins. 1937. Inactivation of pneumococcal hemolysin by certain sterols. *Proc. Soc. Exp. Biol. Med.* **35**:586.
  22. Mark, G. E., and F. F. Strasser. 1966. Pacemaker activity and mitosis in cultures of newborn rat heart ventricle cells. *Exp. Cell Res.* **44**:217.
  23. Lavail, M. M. 1968. A method of embedding selected areas of tissue cultures for electron microscopy. *Tex. Rep. Biol. Med.* **26**:215.
  24. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
  25. Rowen, R., and A. W. Bernheimer. 1956. The toxic action of preparations containing the oxygen-labile hemolysin of *Streptococcus pyogenes*. V. Mechanism of refractoriness to the lethal effect of the toxin. *J. Immunol.* **77**:72.
  26. Hirsch, J. G., A. W. Bernheimer, and G. Weissman. 1963. Motion picture study of the toxic action of streptolysins on leucocytes. *J. Exp. Med.* **118**:223.
  27. Zucker-Franklin, D., and J. G. Hirsch. 1965. Electron microscope study of degranulation of polymorphonuclear leucocytes following treatment with streptolysin O. *Amer. J. Pathol.* **47**:419.
  28. Alouf, J. E., and M. Raynaud. 1968. Some aspects of the mechanism of lysis of rabbit erythrocytes by streptolysin O. *In* Current Research on Group A Streptococcus. R. Caravano, editor. Excerpta Medica Foundation, New York. 194.
  29. Bernheimer, A. W. 1948. Properties of certain rapidly acting toxins as illustrated by streptolysins O and S. *Bacteriol. Rev.* **12**:195.
  30. Halbert, S. P. 1968. Pathogenic significance of streptococcal extracellular products. *In* Current Research on Group A Streptococcus. R. Caravano, editor. Excerpta Medica Foundation, New York. 173.