THE CYTOTOXIC PRINCIPLE OF THE

PHYTOFLAGELLATE *PRYMNESIUM PARVUM*

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ABSTRACT

The cytotoxic events leading to lysis induced in Ehrlich ascites tumor (E.A.) cells by *Prymnesium parvum* cell extracts were followed microscopically and measured quantitatively as changes in E.A. cell volume, uptake of trypan blue, and release of macromolecular constituents from the cells. Cell swelling was the most immediate response to *P. parvum* cytotoxin, while cell death and lysis were later events distinguished by a decline in cell volume, uptake of dye, and appearance of cellular macromolecules free in the incubation medium. The pH and temperature were shown to affect the outcome of the lytic sequence. At either low pH or temperature, cells swelled but did not lyse until the pH or temperature was raised. On the other hand, cells swollen at the higher pH or temperature could be protected from lysis by lowering either the pH or the temperature.

INTRODUCTION

Prymnesium parvum, a phytoflagellate of the Chrysomonadinae, produces toxic principles which cause death of many gill-breathing animals and have often been the cause of mass fish mortalities (Otterstrøm and Steemann-Nielsen, 1939; Reich and Aschner, 1947; Shilo and Aschner, 1953). Extensive studies have been carried out on the mode of action of the ichthyotoxin, and suitable assay systems for its quantitative determination have been developed (Yariv and Hestrin, 1961; Bergmann, Parnas, and Reich, 1963; Ulitzur and Shilo, 1964).

Cell extracts and culture supernatants of *P. parvum* exhibit a variety of biological activities which include hemolysis of mammalian erythrocytes (Yariv and Hestrin, 1961) and contraction of the isolated guinea pig ileum (Bergmann, Parnas, and Reich, 1964). In addition, various mammalian cells, including human amnion cells, Chang liver cells, and mouse peritoneal leukocytes, undergo morphological changes leading to lysis when brought in contact with *P. parvum* toxin

(Shilo and Rosenberger, 1960). Ehrlich ascites tumor cells are extremely sensitive to the action of the toxin (Dafni, 1964). Since these cells have been widely used in the study of the mechanism of action of various injurious agents, they seemed a suitable model system for studying the action of the *P. parvum* cytotoxin. The nature of the cytotoxic event induced by the toxin in this model system and the effect of environmental conditions are described in this paper.

MATERIALS AND METHODS

Preparation of P. parvum Cytotoxin

Axenic cultures of *Prymnesium parvum* Carter (from a strain isolated from brackish water fishponds in Israel (Reich and Kahn, 1954)) were grown on a modified medium of Droop (with the omission of glucose) and under conditions described previously (Shilo and Shilo, 1962). Cells were harvested after 21 days of growth, and the toxic principles extracted in ethanol (Shilo and Rosenberger, 1960). The single extract which served in all the experiments described

was prepared by extraction of 1 to 2 × 10⁸ *Prymnesium* cells per ml of ethanol. The dry weight of the toxic principles in each ml of ethanol was 4.5 mg. The extract was diluted in Krebs-Ringer phosphate buffer pH 7.4 just before addition to the incubation mixtures as described below.

Propagation, Collection and Maintenance of Ehrlich Ascites Tumor Cells

Ehrlich ascites tumor (E.A.) cells of the Landshütz strain (from the Department of Experimental Medicine, Hebrew University-Hadassah Medical School, Jerusalem) were grown intraperitoneally in albino mice of both sexes (12 to 15 g). Weekly i.p. passages were made using 0.2 ml of a freshly harvested peritoneal E.A. cell population. For the experiments, cells were harvested on the 6th day after inoculation. For collecting of the E.A. cells, mice were decapitated, drained of blood, and their peritoneal cavities rinsed repeatedly with physiological saline. The collected cells were washed twice (200 *g* for 3 min) with saline, and then resuspended and maintained in Krebs-Ringer phosphate buffer at different pH levels (as specified in the text). Cell suspensions containing 2.5 to 4 × 10⁶ cells/ml (optical density = 100 Klett units; Klett Summerson colorimeter, filter 42) served in all the experiments.

Incubation of E.A. Cells with P. parvum Cytotoxin

Incubation at 37° or 27° was carried out in 20-ml beakers containing 5 ml of the cell suspension and 5 × 10⁻⁴ ml of the ethanolic toxin extract.

Assay of Cell Injury

Three different parameters were used to measure cell injury.

1. DYE UPTAKE: Differentiation between normal and injured E.A. cells after different times of exposure to *P. parvum* cytotoxin was based on the uptake of trypan blue dye (Allied Chemical & Dye Corp., New York) by the injured cells. At different time intervals, aliquots of toxin-treated cell suspensions were taken and mixed with trypan blue solution in physiological saline to give a final concentration of 0.5% (w/v) trypan blue in the test mixture. After 1-min exposure to the dye, the total cell number and the per cent of stained cells were enumerated in a hemacytometer. 250 to 400 cells served for each estimation.

2. SWELLING: Measurements of the kinetics of swelling of toxin-treated E.A. cells were carried out in an electronic particle counter (Model B, Coulter Electronics, Hialeah, Florida). This instrument has been used widely in the estimation of cell numbers and volume distribution of different cell types (Mattern, Brackett, and Olson, 1956; Brecher, Schneiderman, and Williams, 1956; Brecher et al., 1962) and in following the kinetics of swelling of cell populations (Shilo and Shilo, 1962). Aliquots of the toxin-treated cell suspensions were taken after different incubation times and diluted in Krebs-Ringer phosphate buffer (pH 7.4) to give 25 ml of a cell suspension containing 4 × 10⁴ (±5%) cells/ml. The cell numbers and volume distribution of the E.A. cell population were then measured in the particle counter with an orifice of 100 μ (instrument setting: amplification = ½; aperture current setting = ½; gain trim = 10). The temperature of the cell suspension during these measurements was kept at 22–25°. The volume of 50% of toxin-treated cells after any given incubation time was expressed in relative units with the initial volume of 50% of the cells = 1.

3. RELEASE OF INTRACELLULAR MACROMOLECULES: The appearance of cellular macro-

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Photomicrographs of typical toxin-treated Ehrlich ascites tumor cells (37°, pH 7.4) at different incubation times. Photographed under oil immersion with phase illumination (Zetopan, Reichert). × 2000.

FIGURE 1   Normal untreated Ehrlich ascites tumor cell.

FIGURE 2   E.A. cell after 5-min incubation.
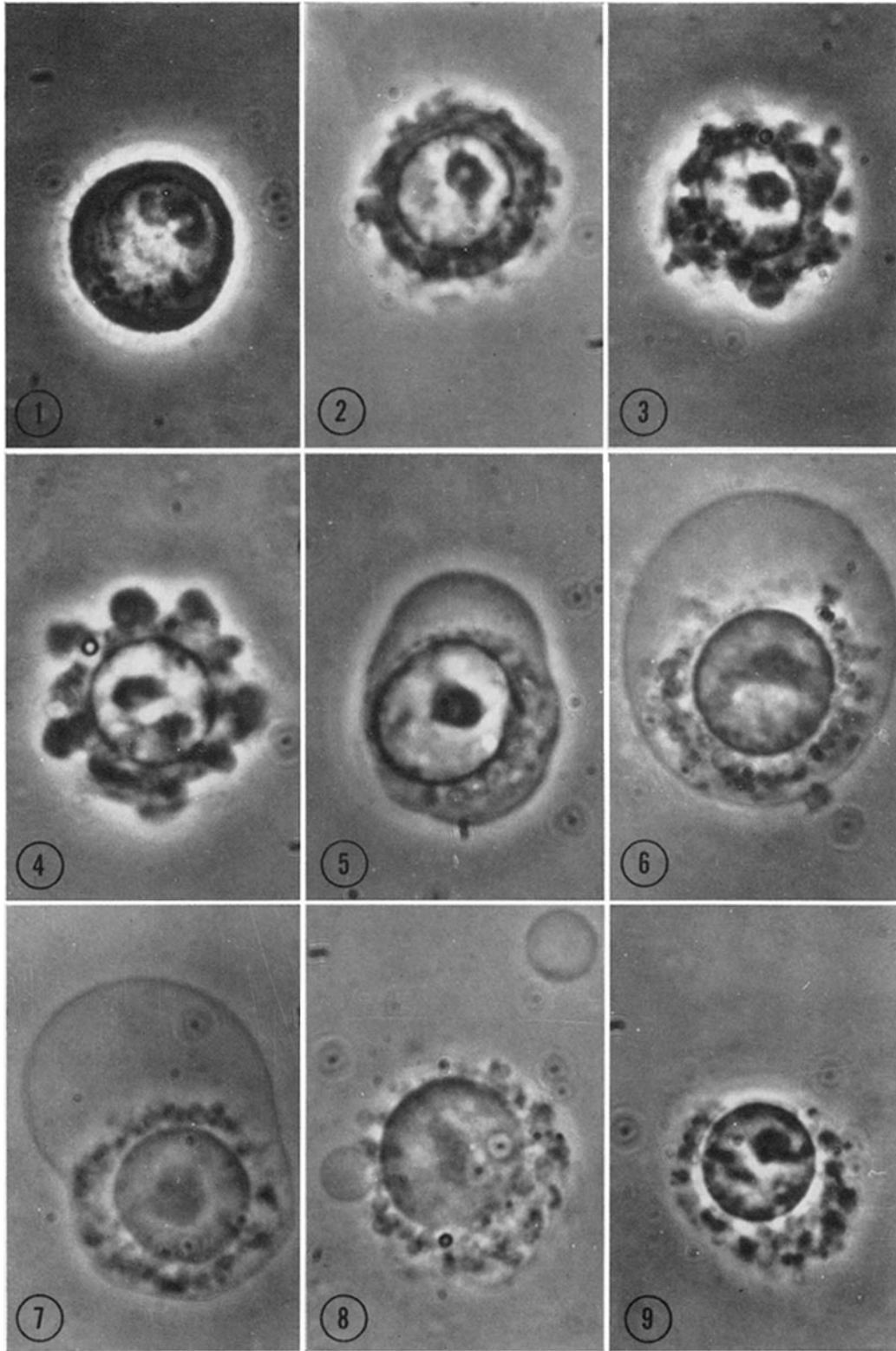
FIGURE 3   After 10-min incubation.

FIGURE 4   After 15-min incubation.

FIGURE 5   After 30-min incubation.

FIGURE 6   After 45-min incubation.

FIGURES 7 and 8   After 50- to 60-min incubation.

FIGURE 9   After 120-min incubation.

molecular constituents in the suspension medium after incubation with *P. parvum* cytotoxin was measured spectrophotometrically and by chemical estimation. 5-ml aliquots of cell suspension taken after different incubation times and control cell suspensions were filtered through Whatman No. 1 filter paper, and the filtrate collected for assaying. In cases in which the filtrate remained turbid, it was centrifuged at 3,000 *g* for 8 min in the cold, and the cleared supernatant was employed in assays. Untreated cell suspensions (5 ml) lysed by addition of 0.05 ml of ethanolic digitonin (USP XVI, Fluka AG, Buchs SG, Switzerland) solution (1% w/v) served as the standard for total lysis in these assays.

Spectrophotometry: The absorption spectrum of the cell-free filtrate of toxin-treated cells in the range of 230 to 390 mμ consistently showed the appearance of a single sharp peak at the 260-mμ wave length. In our experiments, the optical density at 260 mμ of the cell-free filtrate was measured in a spectrophotometer (Perkin-Elmer, Model 137 UV), with Krebs-Ringer phosphate buffer solution serving as blank.

DNA Assay: DNA in the cell-free filtrate was measured using a modification of Burton's (1956) method. Diphenylamine reagent (1 g of diphenylamine (A.R. Reidel-de-Haen AG, Seelze-Hannover) in 100 ml of glacial acetic acid containing 2.75 ml of concentrated

H_2SO_4 without addition of acetylaldehyde) was prepared immediately before use. The reaction mixture was incubated at 30° for 16 to 18 hr, and the optical density determined using a Klett Summerson colorimeter (filter 60).

RNA Assay: RNA in the cell-free filtrate was determined using a modification of Drury's (1948) method. The orcinol reagent was prepared by dissolving 1 g of orcinol (British Drug Houses, Ltd., Poole, Dorset, England) in 100 ml of HCl (concentrated) containing 0.1 g of ferric ammonium sulfate · 12 H_2O.

Protein Assay: Protein in the cell-free filtrate was measured by the method of Lowry et al. (1951).

RESULTS

Kinetics of Swelling and Mortality of Ehrlich Ascites Tumor Cells Induced by P. parvum Cytotoxin

Microscopic observations under phase contrast illumination (Figs. 1 to 9) show that cells exposed to the action of cytotoxin promptly underwent a series of morphological changes. Initially, cytoplasmic swelling and focal pouching with periph-
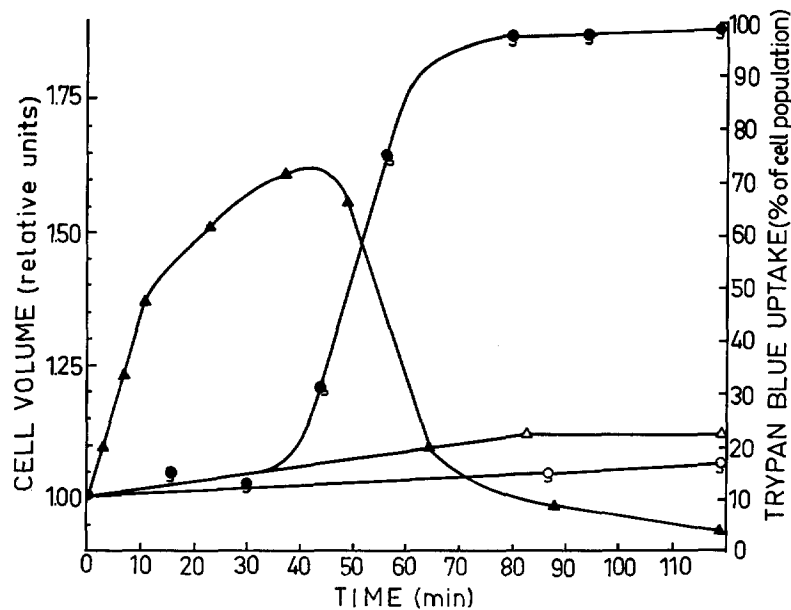

FIGURE 10 Effect of *P. parvum* cytotoxin on E.A. cell volume and trypan blue uptake. Incubation mixtures (*see* Methods) were kept up to 120 min at pH 7.4, 37°. Estimations of cell volume and the trypan blue uptake are as described in Methods. Triangles, cell volume; circles with tails, trypan blue uptake; solid symbols represent toxin-treated cells; open symbols, untreated control cells.

eral extrusions were observed (Figs. 2 and 3). Later, the pseudopod-like extrusions grew (Fig. 4) and became confluent, with uniform involvement of a large part of the cell circumference (Figs. 5 to 7). The uniformly clear cytoplasmic space became progressively lighter during this stage of swelling (Fig. 5 in contrast to Fig. 6 and Fig. 7), while the nucleus was surrounded by clusters of cytoplasmic granules. Finally, the swollen cells burst, liberating cytoplasmic spheres (Fig. 8) and leaving naked nuclei and cellular debris (Fig. 9).

The kinetics of swelling (measured in the Coulter counter) and mortality (measured by the loss of ability to exclude dyes) of E.A. cells treated with *P. parvum* cytotoxin at pH 7.4, 37°, are shown in Fig. 10. The increase in cell volume commenced immediately upon addition of the cytotoxin without any lag period, reaching a maximum value

followed by a rapid decline. During this stage of cell volume increase, the E.A. cells underwent the morphological sequence shown in Figs. 2 to 6. No trypan blue uptake occurred during the period of swelling, but, with the onset of the decline in cell volume and the morphological appearance of lysis (Figs. 7 to 9), there was a concomitant increase in cell stainability.

Analyses of cell supernatants for intracellular macromolecular constituents after different times of incubation with toxin showed (Fig. 11) that 260-mμ absorbing material, protein, and RNA were released from the injured cells but that there was no detectable release of DNA. Similar to dye uptake by injured cells, release of cytoplasmic macromolecules was low during the stage of cellular swelling but increased rapidly after the onset of the lytic phase and the decline in cell volume.
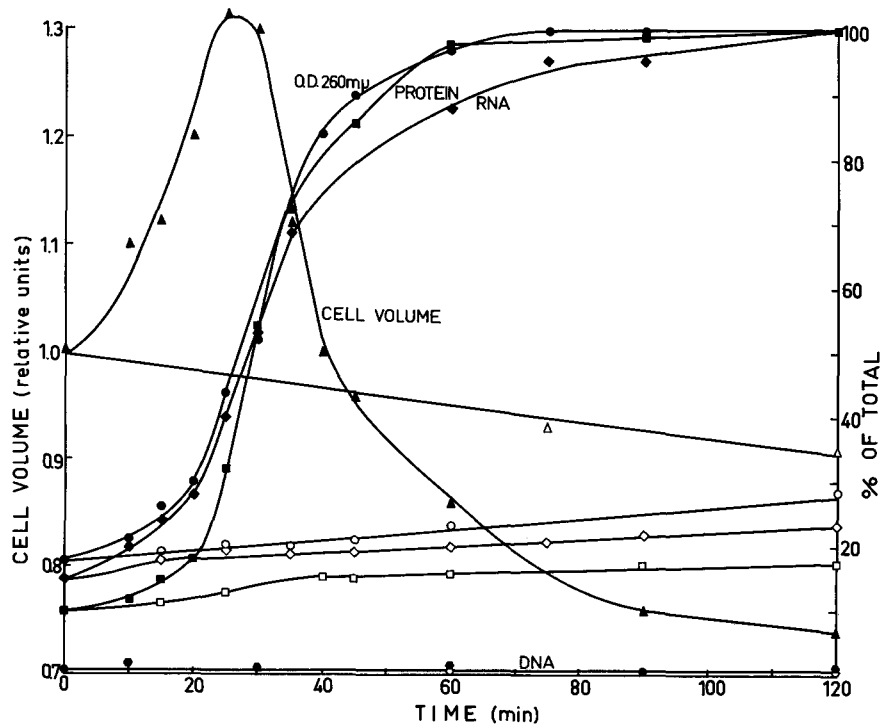

FIGURE 11 Effect of *P. parvum* cytotoxin on E.A. cell volume and release of intracellular macromolecules into the suspension medium. Incubation mixtures were kept up to 120 min at pH 7.4 and 37°. Measurements of cell volume are as in Fig. 10. OD at 260 mμ and RNA, protein, and DNA content of treated and control cell-free filtrates (prepared as described in Methods) are given as % of values obtained in the cell-free filtrate of the totally digitonin-lysed E.A. cell suspension (*see* Methods). Triangles, cell volume; circles, OD at 260 mμ; squares, protein; diamonds, RNA; hexagons, DNA. Solid symbols represent toxin-treated suspensions; open symbols, untreated controls.

The Effect of Incubation Temperature and pH on the Different Parameters of the P. parvum Cytotoxic Activity

The results described in Figs. 10 and 11 clearly show that the swelling of the E.A. cells preceded all the other manifestations of the cytotoxic action measured. The effect of different environmental conditions on the swelling as compared to their effect on the other cytopathological phenomena in E.A. cells was, therefore, studied. At a pH of 6.4 (Fig. 12) or at a temperature of 27° (Fig. 13), E.A. cells swelled but no significant leakage of macromolecules (as 260-mμ absorbing materials) or uptake of trypan blue occurred. Cells already swollen in the presence of *P. parvum* cytotoxin under conditions of lowered pH (Fig. 12) or temperature (Fig. 13) were lysed (as expressed by dye uptake, release of intracellular macromolecules, and decline in cell volume) when the pH was returned to 7.4 or the temperature raised to 37°.

Fig. 14 shows the swollen condition of an E.A.

cell treated with *P. parvum* cytotoxin at pH 6.4, and Fig. 15 shows its subsequent lysis after the pH was raised to 7.4. Similar morphological changes were observed when the incubation temperature of toxin-treated E.A. cells was raised from 27° to 37°.

Cells incubated with toxin at pH 7.4 and at 37° were transferred at various times to conditions of lower pH (Fig. 16) or lower temperature (Fig. 17). The volume decline and leakage of macromolecules inevitable at 37° and pH 7.4 could be arrested at any stage of the lytic sequence by lowering the pH or the temperature. Even at a period when a large portion of the cells in the suspension were already lysed, the remaining highly swollen cells could be protected from lysis by lowering the pH or temperature of the incubation mixture.

DISCUSSION

Ehrlich ascites tumor cells have been used widely as a model system in studying the mechanisms underlying immune cytolysis (Green and Goldberg, 1960) as well as illuminating the nature of
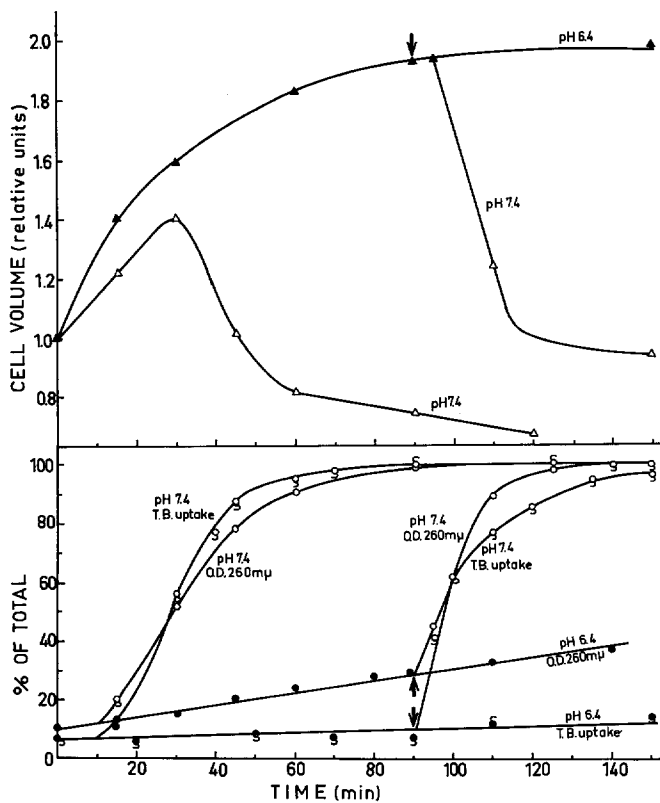

FIGURE 12 Effect of pH of incubation milieu on E.A. cell volume, dye uptake, and release of macromolecules. Incubation mixtures were kept up to 150 min at 37° and pH 7.4 or 6.4. Arrow (↓) indicates change of pH of incubation mixture from 6.4 to 7.4 by titration with 0.5 N NaOH. Assay methods are as described for Figs. 10 and 11. triangles, cell volume; circles with tails, trypan blue uptake; circles, OD at 260 mμ. Solid symbols represent pH 6.4; open symbols represent pH 7.4.

the damage inflicted upon cells by various in-jurious agents, including plant hormones (Schultz and Norman, 1965) and bacterial toxins (Ginz-burg, 1959; Eaton, Scala, and Jewell, 1959). The morphological changes occurring in the cells (Easty and Ambrose, 1957; Flax, 1956; Goldberg and Green, 1959; Bickis, Quastel, and Vas, 1959; Ross, 1957; Bitensky, 1963), swelling of injured cells (Flax, 1956; Ellem, 1957; Green and Silver-blatt, 1960; Ross, 1957), loss of the ability to exclude dyes (Pappenheimer, 1917; Gorer and O'Gorman, 1956; Eaton, Scala, and Jewell, 1959; Reif and Norris, 1960), and the leakage of cell constituents into the suspension medium (Ellem, 1957, 1958; Colter et al., 1957; Green, Barrow, and Goldberg, 1959; Goldberg and Green, 1960) are common effects of most of these cytotoxic agents. The cytotoxic activity of *P. parvum* extracts on these cells showed many simi-larities to the effects of the other injury-inducing agents.

Different single criteria usually served, in the above-mentioned studies, as the measure of cell injury by cytotoxic agents, thus limiting the perspective of their interpretations of the mode of action of the cytotoxic agents. In our investiga-tions, four criteria for following the cytotoxic ac-tion (dye exclusion, release of intracellular macro-molecules, change in volume of the injured cells, and microscopically observed morphological changes) were compared. Different stages in the lytic sequence seem to be separable and are af-fected variously by different environmental con-ditions. Only by the simultaneous use of different parameters was it possible to assess the relation-ship of the stages to one another.

The detailed analysis of immune cytolysis of E.A. cells by Green and Goldberg (1960) has led these investigators to propose a mechanism in which primary irreversible damage is inflicted upon the cells by antibody in the presence of complement. They assumed the formation of functional "holes" in the cell membrane large enough to permit rapid exchange of inorganic cations and small molecules, but not of macro-molecules. With disturbance in the K^+ and Na^+ equilibrium, the resulting osmotic imbalance
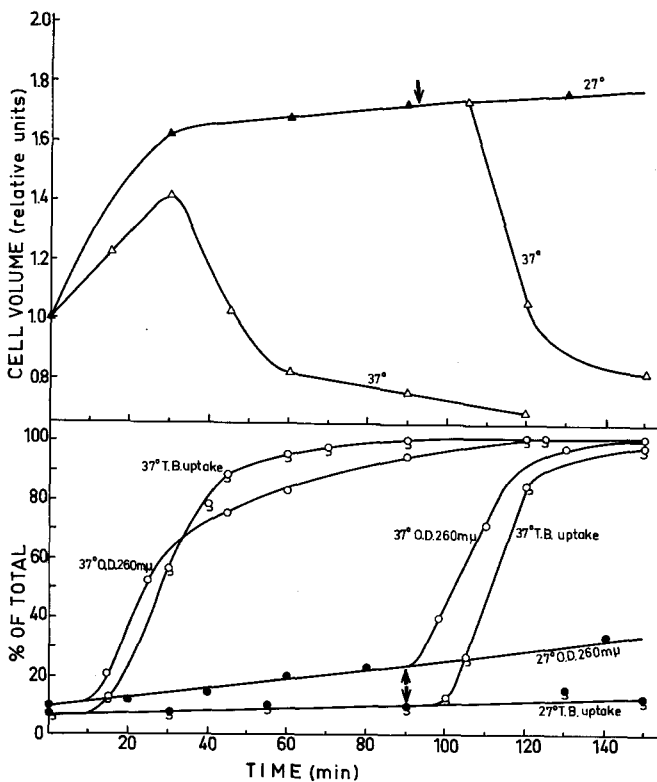

FIGURE 13 Effect of temperature of incubation mixture on E.A. cell volume, dye uptake, and release of macromole-cules. Incubation mixtures were kept up to 150 min at pH 7.4 and at 37° or 27°. Arrow (↓) indicates change of tempera-ture from 27° to 37°. Triangles, cell volume; circles with tails, trypan blue uptake; circles, OD at 260 mμ. Solid symbols represent 27° incubation tem-perature; open symbols represent 37°.
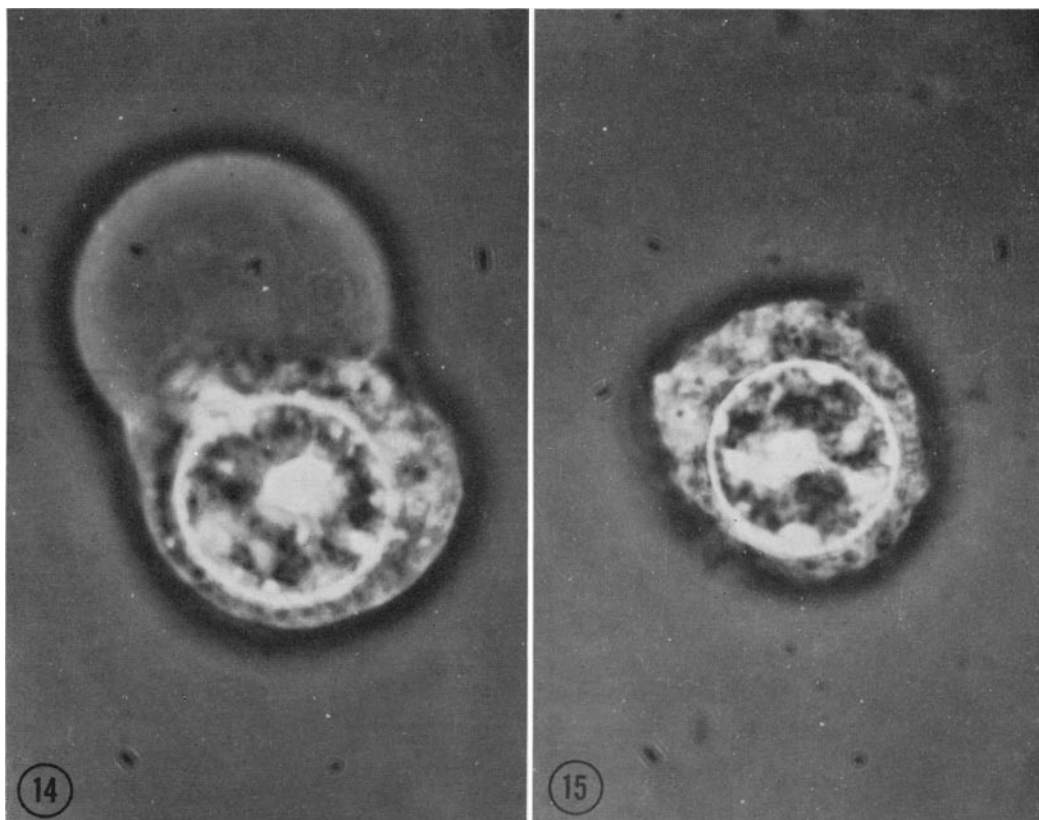
Photomicrographs of an Ehrlich ascites cell treated in toxin incubation mixture (37°) before and after change in pH from 6.4 to 7.4. Photographed under oil immersion with anoptral phase illumination. × 2000.

FIGURE 14   Typical toxin-treated E.A. cell after incubation at pH 6.4 for 90 min.

FIGURE 15   Typical E.A. cell 15 min after the pH of the incubation mixture was changed from pH 6.4 to pH 7.4.

causes swelling of the cells and stretching of the damaged membrane, at which stage macromolecules leak out.

With *P. parvum* cytotoxin at pH 7.4 and at a temperature of 37°, the morphological sequence of lysis is similar to that observed in immune cytolysis. However, when incubation with *P. parvum* cytotoxin was carried out at pH 6.4 or at a temperature of 27°, the cells swelled considerably without any leakage of intracellular macromolecules. It thus appears that, in this case, even the highly stretched membrane did not become permeable to macromolecules. Moreover, these swollen cells did not lose their ability to exclude dye

(trypan blue) and did not lyse. No irreversible damage seems to be inflicted upon the toxin-treated cells, even when markedly swollen, until a period very close to lysis. This is borne out by the fact that even when part of the toxin-treated cell population already is lysed, the highly swollen cells remaining intact can be protected from the inevitable lysis by changing the pH from 7.4 to 6.4 or by lowering the temperature from 37° to 27° (see Figs. 16 and 17). Thus, while in immune cytolysis the antibody and complement-dependent damage is irreversible and all later stages of the lytic sequence are predetermined, the lytic activity of *P. parvum* cytotoxin can easily be dis-
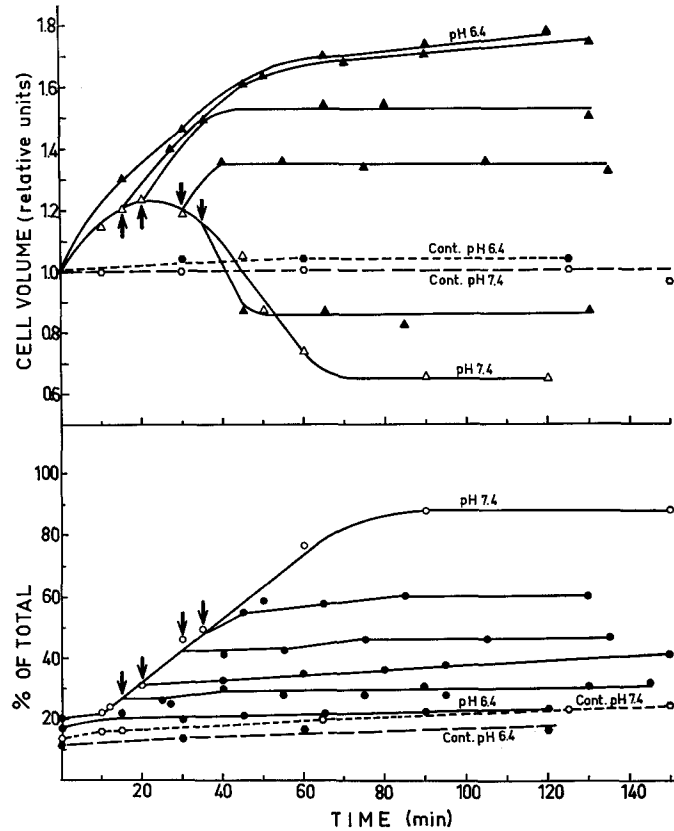
FIGURE 16 Interruption of lytic sequence in incubation mixtures of E.A. cells by lowering of pH. Incubation mixtures were initially incubated at pH 7.4 and at 37°. Arrow (↓) indicates time of change of pH to 6.4 by titration with 0.5 N HCl. Measurements of cell volume and OD at 260 mμ given as in Fig. 11. Triangles, cell volume; circles, OD at 260 mμ; hexagons, untreated control; open symbols represent incubation at pH 7.4; solid symbols represent incubation at pH 6.4.

continued even in advanced phases of the lytic sequence.

The observed effects of pH and temperature, which clearly separate the stages of swelling from those leading to lysis (concomitant with release of macromolecular cellular constituents and uptake of trypan blue), indicate that the *P. parvum* cytotoxin induces lysis in consecutive sequential steps which are separable by means of environmental conditions. Another possibility, however, which cannot be ruled out in accounting for the observed facts is that the toxic principle responsible for swelling is unrelated to that involved in the process of lysis and differs in the environmental requirements for its activity.

The earliest recognizable effect of *P. parvum* ichthyotoxin on the intact fish is the loss of the selective permeability of the gill tissue, allowing entrance of trypan blue and labeled I^{125} and macromolecules such as radioiodinated human serum albumin I^{131} into the gill tissue (Ulitzur and Shilo, 1966). This resemblance to the cytotoxic effect in Ehrlich ascites tumor cells indicates that the principles involved may be closely related and that an elucidation of the mode of cytotoxic action could shed light on the mechanism of action of the toxin upon the intact fish.
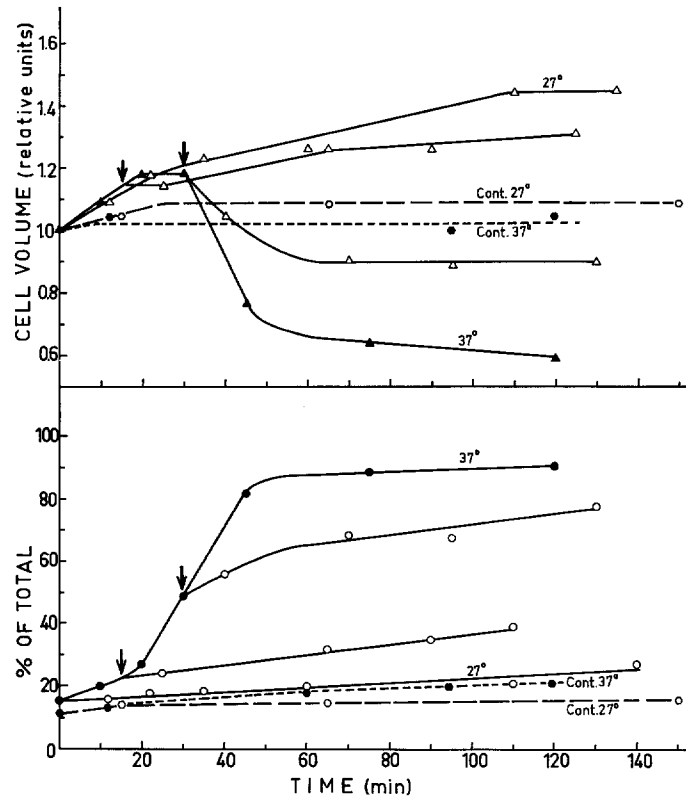
FIGURE 17   Interruption of lytic sequence in incubation mixtures of E.A. cells by lowering of temperature. Incubation mixtures were initially incubated at 37°, pH 7.4. Arrows (↓) indicate time of change of temperature to 27°. Measurements of cell volume and OD at 260 mμ given as in Fig. 11. Triangles, cell volume; circles, OD at 260 mμ; hexagons, untreated control; solid symbols represent incubation at 37°; open symbols represent incubation at 27°.

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