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# How *Paramecium* cells die under a cover glass?

Yoshiomi TAKAGI[1,*], So KITSUNEZAKI[2], Tae OHKIDO[1,$] and Rie KOMORI[1,#]

[1]*Department of Biological Sciences, Nara Women's University, Nara 630-8506, Japan,*
[2]*Department of Physics, Nara Women's University, Nara 630-8506, Japan*

**SUMMARY**

The dying process of *Paramecium tetraurelia* cells under a cover glass without supporting pillars was described. The initiation of the process was defined by the cessation of swimming, and the termination by cell rupture. The process required 6 min on average, ranging from 1 to 11 min. The first symptom of death was the formation of a small bleb, a local swelling of the outer cell membrane. The number of blebs increased, each bleb grew, and neighboring blebs fused to form a larger bleb. By supplying water, the blebs disappeared only when their size and numbers were relatively small, indicative of a commitment point to death. Finally the outer membrane and then the inner membrane were broken, and the cytoplasmic contents flew out. Until the last moment of the cell rupture, and sometimes even after that, ciliary beating was observed somewhere locally on the cell membrane. Among the cilia, those at the cytopharynx were the first to stop beating so that food vacuole (FV) formation stopped somewhat earlier than the cellular disorganization. Cessation of the contractility of contractile vacuoles (CVs) occurred a little bit later on average than the cessation of FV formation. Anterior and posterior CVs were not related to each other in the timing of loss of contractility and in the preceding pulsating cycles. Finally, we calculated the physical pressure to burst the cell at about 950 atm and estimated the actual pressure to be at 470~2,000 atm.

*Corresponding author
Tel: 075-791-6846, E-mail: takagi@cc.nara-wu.ac.jp

Present addresses:
[*]1-1 Kitadacho, Kamitakano, Sakyo-ku, Kyoto 606-0036
[$]2-277 Mozu-Honmachi, Sakai, Osaka 591-8036
[#]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, Shido, Kagawa 769-2193

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## INTRODUCTION

People used to say that biology is the science of living organisms, not dead substances. Items destined for death or extinction such as the tail of a tadpole during metamorphosis, the cuticle of an insect pupa during molting, or the macronucleus of the ciliate during sexual process were not of interest until the concept of *apoptosis* (Kerr et al., 1972) became widely accepted. That caused a revolutionary change in biology by introducing death as an essential part of the living system. However, there are still few investigations on the dying process of protozoan cells, although the dying process in apoptotic cells has been described abundantly, and the mechanism of genetically controlled cellular death is now one of the most fascinating biological themes.

We found ourselves unable to answer the question of "how *Paramecium* cells die when they are at an extremity of senescence", although we had long engaged in examining aging and lifespan in *Paramecium* (Takagi and Yoshida, 1980; Takagi and Kanazawa, 1982; Takagi et al., 1987a, 1987b; Takagi, 1988, 1999; Komori et al., 2004, 2005). We noticed that we had little knowledge about the dying process of *Paramecium,* not only at senescence but also at more commonly occurring accidental death from physical, chemical and biological causes, although we had frequently met death of *Paramecium* caused by competition for survival (Maruyama et at., 1996, 2001; Maruyama and Takagi, 1997), by UV (Yamamoto et al., 1997, 2005), and by toxins contained in a component of culture medium (Tokusumi and Takagi, 2000; Mizobuchi et al., 2003).

We have noticed so far that there are 2 forms of death: (a) death leaving the shape of the dead cell, and (b) death not leaving the shape of the dead cell. A typical death form of (a) is death by desiccation, which is identical to the familiar process of making permanent cell-preparations, and that of (b) is death by heat or pressure. It remains uncertain whether (a) and (b) are fundamentally different phenomena or not. It is natural, among living organisms, that a dead body will eventually disappear (even cells killed by desiccation cannot be a permanent preparation unless treated with chemical fixative). For example, when we studied death by chemicals, we would observe many dead cells some hours after the treatment, but no corpses on the next day. It should be asked, therefore, how cells die and how long the dead cells keep their shape.

The length of time during which we can observe dead cells varies according to the situation. But we have no answer to such questions as "Are there any relationships between the cause of death and the process of death, between the kind of chemicals and the process of death, and between the concentration of chemicals and the persistence time of dead cells?".

Before answering such questions, we should know the death process in detail. We here paid attention to the simple occasion of death by pressure under a cover glass. We observed more than 100 samples of dying *Paramecium* cells under a cover glass without supporting pillars, depicted the sequence of the death process, and estimated the pressure required for cell rupture.

## MATERIALS AND METHODS

The wild-type stock 51 of *Paramecium tetraurelia* was used.

Cells were cultured in 0.5% phosphate-buffered wheat grass powder (Pines Int., Inc., Lawrence, KS) infusion supplemented with 0.5 mg/l stigmasterol and inoculated with *Klebsiella pneumoniae* 2 days before use (Mizobuchi et al., 2003). A small sample of cells at the stationary phase of growth were transferred to SMBIII (Miyake, 1981), a saline solution, in wells of de-

pression slide for washing.

A 10 μl drop of the saline solution including a definite number of less than 10 cells was put on a clean slide glass, and a cover glass of $(1.8\ \text{cm})^2$ in area and 0.1 g in weight was put on the drop without pillars. A single cell was chased until it died and the dying process was observed, paying special attention to cell morphology, ciliary beating, food vacuole (FV) formation and contractile vacuole (CV) activity. We observed more than 100 dying cells in all. Video-recordings of cells were used for the observation of CVs.

Handling and observation were performed at room temperature, which was usually in the range of 22~28ºC.

## RESULTS

### Sequence of death

*Paramecium* cells which were swimming actively under a cover glass without pillars stopped moving before long. Although the time to the stoppage varied greatly among cells because of such factors as inconsistent vigor of each cell, variable rates of loss of water, contamination by debris that supported the cover glass and so on, the period between the cessation of swimming and the cell rupture was 6 min on average, ranging from 1 to 11 min.

The first symptom of death was the formation of a small bleb, a local swelling of the outer cell membrane. The number of blebs increased, each bleb grew, and neighboring blebs fused to form a larger bleb. Cessation of ciliary beating at the cytopharynx (buccal cavity), which means the cessation of FV formation, tended to occur somewhat earlier than the cessation of the contractility of CVs. Ciliary beating was observed until the last moment of the cell rupture at least somewhere locally on the cell surface. Finally the outer cell membrane and then the inner cell membrane were broken, and the cytoplasmic contents flew out.

### Dying process in detail

**<Cell morphology>**

After the cells stopped swimming, a small bleb, an extrusion of the outer cell membrane was formed as the first symptom of the death process. The region where the first bleb was formed was not fixed; every region on the cell surface appeared to have an equal chance to form a bleb. The number of blebs increased and small neighboring blebs fused to form a larger bleb. A typical morphological change is shown in Fig. 1. If water was supplied from a cover glass edge when blebs were smaller in size and fewer in number, the blebs disappeared: the cell restored its normal shape and began to swim normally. The point of no-return or the commitment point appeared to exist somewhere during the bleb-growing process. The cells at stages B and C in Fig. 1 would recover, but thereafter would not. Cilia were regularly located on the surface of the blebs, i.e., on the outer membrane. Trichocyst-discharging was rarely observed during the dying process except at the moment of cell rupture. When observed, it was outside the blebs.

Concomitant with the enlargement of blebs, enlargement of intracellular vacuoles occurred. We were unable to identify each vacuole enlarged except CVs which we will mention later.

**<Ciliary beating >**

*Paramecium* cells stopped swimming, although cilia all over the cell surface continued to beat. Sometimes cells that stopped forward or backward swimming rotated at the resting position. This made it easy to observe the subsequent processes leading to death. Cilia on a part of the cell surface continued to beat until the moment of cell rupture, and sometimes continued to beat after the cytoplasm began to flow out (Fig. 1F).

**<Food vacuole (FV) formation>**

Although FVs under formation were not al-

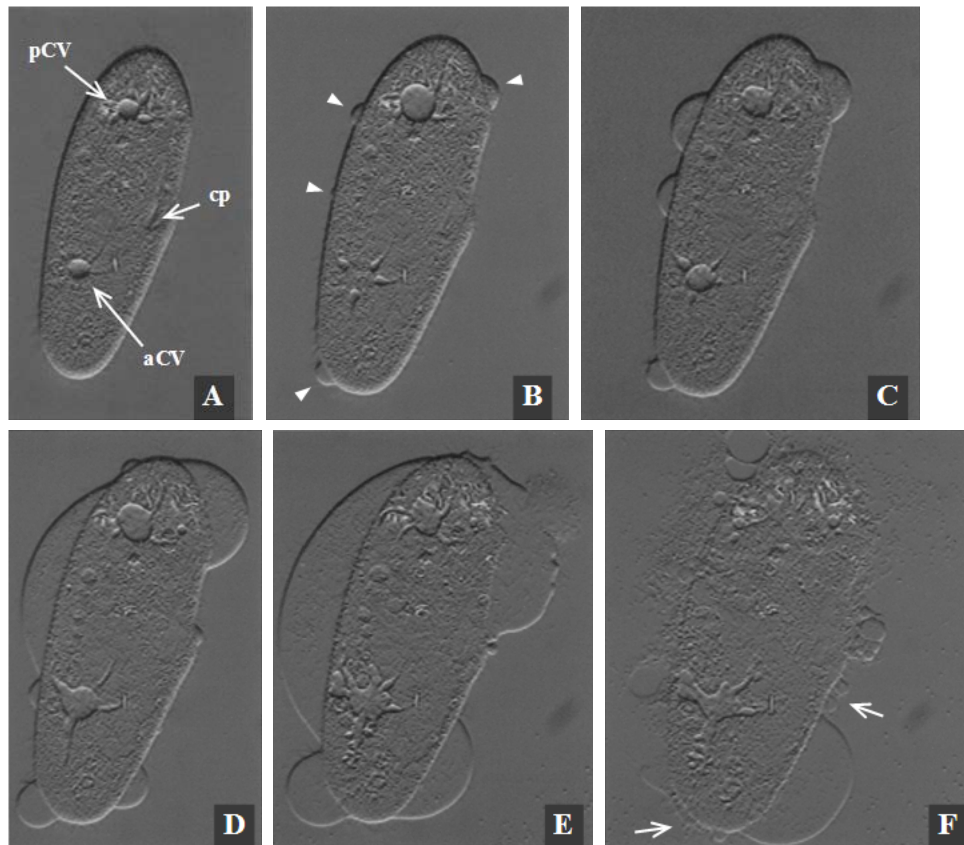


Fig. 1. Video-images of a typical dying cell under a cover glass without pillars. A: Normal cell that stopped swimming. aCV: anterior contractile vacuole, pCV: posterior contractile vacuole, cp: cytopharynx. B: Four small blebs (arrow heads) were formed. C: The blebs grew. D: Some blebs fused to form an enlarged bleb and new blebs were formed. CVs also enlarged. E: The blebs grew to a single outer cell membrane. CVs were somewhat deformed. F: The outer cell membrane at the posterior half ruptured. Cilia at the regions indicated by arrows were still beating.

ways discernible, cells would have the ability of FV formation as long as the ciliary beating along the cytopharynx (buccal cavity) continued. The cilia on this region were the first to stop beating. FVs that were present in the cytoplasm tended to enlarge prior to death.

**<Contractile vacuoles (CVs)>**

The CV complex is an osmoregulatory organelle composed of many vesicles (Allen and Fok, 1988; Allen, 2000). We here call the easily visible complex of central vesicle and radial arms the CV. We recorded dying cells in a video recorder and counted the pulsation cycles of the anterior CV (aCV) and the posterior CV (pCV) until the cells collapsed. Three representative examples are shown in Fig. 2.

In the cell of Fig. 2A, aCV pulsated more slowly and 3 times less frequently than pCV during the observation period. The pulsating cycle of 9~10 s in aCV and that of 6~8 s in pCV remained for a time before death. The last pulsating cycle of aCV was 12 s, and that of pCV was 17 s. The total time of pulsation was 131 s for aCV and 125 s for pCV. The pCV, therefore, lost contractility earlier

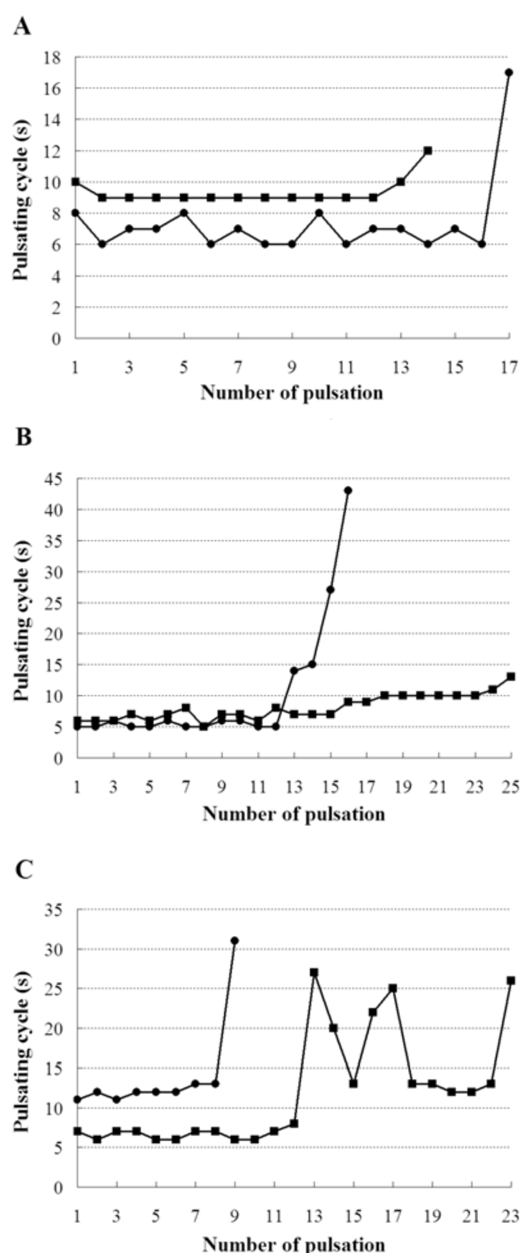


Fig. 2. Contractility of aCV (rectangles) and pCV (circles) in 3 representative cells (A~C) when they were to die. Abscissa: Number of pulsations until the last moment of death. Start of the counting was arbitrary. Ordinate: Pulsating cycles in s.

than aCV, and aCV pulsated 1 time more after pCV stopped pulsation.

In the cell of Fig. 2B, the pulsating cycle was almost identical in both CVs: 5~8 s in aCV and 5~6 s in pCV. However, pCV suddenly increased the cycle and lost contractility earlier than aCV: the total time of pulsation was 202 s for aCV and 163 s for pCV. The last pulsating cycle of aCV was 13 s, and that of pCV was 43 s. The aCV pulsated 9 times more than pCV during the observation period, and 4 times more after pCV stopped pulsation.

In the cell of Fig. 2C, pCV was pulsating more slowly and lost contractility earlier than aCV: the total time of pulsation was 276 s for aCV and 127 s for pCV. The abrupt increase in the pulsating cycle in pCV was a symptom of the loss of contractility as in other cases, whereas contractility was restored in aCV after an abrupt increase in the pulsating cycle. The last pulsating cycle of aCV was 26 s, and that of pCV was 31 s. The aCV pulsated 14 times more than pCV during the observation period, and 9 times more after pCV stopped pulsation.

In all of the above 3 cases, pCV stopped pulsation earlier than aCV; 6 s earlier in A, 39 s earlier in B and 149 s earlier in C. This was, however, not a general rule as shown in Table 1, in which we show the difference of pulsation in time and in frequency between aCV and pCV during the observation period. Twelve cells, including those in Fig. 2, were from video-recordings used to successfully count pulsating cycles in both CVs. Positive numbers mean that aCV pulsated for a longer time and more frequently than pCV, and negative numbers mean the reverse. The results show no correlation between aCV and pCV in their pulsation when the cells are at death; aCV (or pCV) pulsated for a longer time and more frequently than pCV (or aCV) in some cells, while aCV (or pCV) pulsated for a longer time but less frequently than pCV (or aCV) in other cells. In 2

Table 1. Discrepancy in pulsation between aCV and pCV.

| Cell | Difference of pulsation in time and in frequency between aCV and pCV during the observation period | |
|---|---|---|
| | Time in s | The number of pulsation |
| No. 1 | 154 | 10 |
| No. 2 | 149 | 14 |
| No. 3 (aCV-1) | 87 | 0 |
| No. 3 (aCV-2) | -18 | 1 |
| No. 4 | 39 | 9 |
| No. 5 (pCV-1) | 21 | 5 |
| No. 5 (pCV-2) | -37 | 4 |
| No. 6 | 18 | 4 |
| No. 7 | 6 | -3 |
| No. 8 | 3 | 1 |
| No. 9 | -20 | 1 |
| No. 10 | -36 | -2 |
| No. 11 | -47 | -2 |
| No. 12 | -215 | -1 |

Positive numbers indicate that aCV pulsated for a longer time and more frequently than pCV during the observation period, and negative numbers indicate the reverse. In No. 1 cell, for example, aCV pulsated 154 s longer and 10 times more than pCV. No.3 and No. 5 cells had 2 aCVs and 2 pCVs, respectively. In No. 5 cell, for example, aCV pulsated 21 s more than pCV-1 but 37 s less than pCV-2, and aCV pulsated 5 and 4 times more than pCV-1 and pCV-2, respectively. A, B and C cells in Fig. 2 are identical to No. 7, 4 and 2 cells in Table 1, respectively.

of 12 cells, 1 of the CVs, aCV in 1 case and pCV in the other case, duplicated during the death process, and the resulting CVs resided close to each other. Even in such a case, the 2 CVs pulsated independently.

CVs enlarged and deformed prior to the cell rupture, maintaining the ability of pulsation.

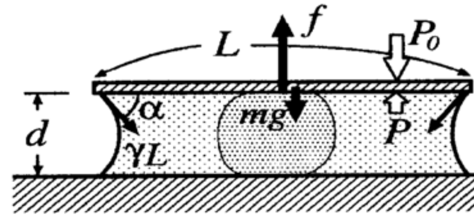


Fig. 3. Schematic depiction of a cell in water that supports the cover glass showing possible forces in arrows. *f*: force supported by paramecia, *mg*: weight of cover glass, *P*: hydrostatic pressure of water, $P_0$: atmospheric pressure, *L*: side length of cover glass, *d*: depth of water or the pressed width of the cell, *α*: contact angle between water and a cover glass, *γ* : surface tension of water.

## Estimation of pressure at death

As water under a cover glass decreases due to desiccation, *Paramecium* cells bear the force arising from the pressure difference between the water and the atmosphere as well as the weight of the cover glass. We here assume that the cover glass is supported evenly by *N* cells of paramecia. Fig. 3 is a schematic depiction of a cross section, where the thickness of water is represented by *d* that is equal to the pressed width of the cell, and the cover glass is rectangular with mass *m* and length of a side *L*.

As water begins to evaporate from the sides of the cover glass, the lateral interface of water becomes concave to the atmosphere. A cross-section of the interface is approximated as a circular arc because the thickness *d* is sufficiently smaller than the capillary length in general. Using the contact angle between water and a glass plate, *α*, the average curvature of the interface *κ* is expressed as $\kappa = 2\cos\alpha / d$. The difference between the hydrostatic pressure of water *P* and the atmospheric pressure $P_0$ is derived from Laplace's theorem as

$$P - P_0 = \gamma\kappa = 2\gamma\cos\alpha / d, \qquad (1)$$

where *γ* is the surface tension of water (de

Gennes et al., 2004).

The vertical force supported by $N$ paramecia, $f$, is caused by the weight of the cover glass $m$, the pressure difference $P$ - $P_0$ and the surface tension acting on the 4 sides of the cover glass, $4\gamma L$. Representing the acceleration due to gravity by $g$, we obtain the equation

$$f = mg + L^2 (P - P_0) + 4\gamma L \sin\alpha. \quad (2)$$

Substituting Eq. (1), we find that the third term is negligibly small compared with the second term in the case of $d << L$. The equation is approximated as

$$f \approx mg + 2\gamma L^2 \cos\alpha / d. \quad (3)$$

We represent a typical contact area between each *Paramecium* and the cover glass by $S$, and assume the intracellular pressure of paramecia as $P + P_*$. $P_*$, the difference between the inside and outside pressures, works to burst paramecia. Regarding each *Paramecium* as a fluid cell surrounded by a soft membrane, the inside pressure is uniform everywhere in the cell. The equilibrium equation of forces acting on the contact areas is expressed by $f = NSP_*$, where the surface tension of the membranes is negligible because the contact areas are approximately flat. Therefore $P_*$ is estimated by the equation

$$P_* = f/NS \approx ( mg + 2\gamma L^2 \cos\alpha / d) / NS. \quad (4)$$

As an example, we calculated $P_*$ assuming that $N = 10$, $S \approx 3.0 \times 10^{-5}$ cm$^2$ and $d \approx 15$ μm = $1.5 \times 10^{-3}$ cm: A *P. tetraurelia* cell was considered as a rotatory ellipse with length of 100 μm and width of 30 μm; when it was pressed to half, the projection area of the original ellipse would be expanded; the contact area of the cell to the cover glass ($S$) was assumed to be similar to the projection area of the original ellipse. We used $m \approx 10^{-1}$ g, $g \approx 980$ cm/s$^2$, $\gamma \approx 70$ dyn/cm and $L \approx 1.8$ cm. Adopting $\alpha \approx 20°$ as a typical value of the contact angle, although it depends on the state of the glass surfaces, we obtained an estimation of $P_*$ as

$$P_* \approx 9.5 \times 10^8 \text{ dyn/cm}^2 \approx 9.5 \times 10^2 \text{ atm}. \quad (5)$$

In this case, the pressure caused by the weight of the cover glass, $mg/NS \approx 0.3$ atm, is very small compared with this value.

## DISCUSSION

By carefully observing death process of *Paramecium* cells under a cover glass without supporting pillars, we found that ciliary beating continued until the last moment of cell rupture or even after the cytoplasm began to flow out. This may not be surprising, since ciliary beating has been shown to continue in non-living cell models such as the Triton model (Naitoh and Kaneko, 1972).

Cessation of CV pulsation tended to occur later than the cessation of FV formation. Pulsation of CVs continued until immediately before the cell rupture. Enlarged and deformed CVs could still pulsate. In contrast to the ciliary beating, however, CV pulsation was never observed after the cytoplasm began to flow out, although autonomous pulsation of CV could occur in vitro (Tani et al., 2001). We found that aCV and pCV pulsated independently with different cycles and frequencies in the cells at death, although the 2 CVs appeared to pulsate with regular intervals in intact cells. It has been suggested that no fibrous network system surrounds the CV (Naitoh et al., 1997; Tani et al., 2001). This means that the independency between aCV and pCV pulsation is of an original nature, and is not due to disconnection at death. Also found was an occasional occurrence of an abrupt duplication of CV during the dying process. Both aCV and pCV were duplicated, and the resulting

CVs pulsated independently (Table 1).

We estimated that the *Paramecium* cell membrane was broken only when the difference between the outside and inside pressures of the cell was as high as ~950 atm. The value of $P_*$ calculated by Eq. (4) would change if the assumption of *N, S* and *d* differed. For the evaluation of *S*, the most puzzling value, we assumed that *S* would be similar to the projection area (A) of the original ellipse with long axis of 100 μm and short axis of 30 μm. We calculated A ($2.4 \times 10^{-5}$ $cm^2$), corrected by computer-aided calculation of the surface area of photographed cells ($4.3 \times 10^{-5}$ $cm^2$), and adopted the value of $S = 3.0 \times 10^{-5}$ $cm^2$. However, the actual contact area of the cell to the cover glass might be in the range of $2S$~$S/2$ so that the actual value of $P_*$ might be in the range of 470~2,000 atm.

*N* is also a factor that affects $P_*$. Although we assumed that the pressure was supported evenly by *N* cells, debris of similar size, which was not completely eliminated, would also support the pressure. We therefore consider that $P_*$ with the range of 470~2,000 atm would be reasonable.

In classic studies of the effects of hydrostatic pressure introduced by Wichterman (1953), *Paramecium* cells became immobile at 600 atm but they could recover even after exposure to 800 atm. A recent experimental study by Dr. Y. Taniguchi of Ritsumeikan University showed that *P. multimicronucleatum* cells could endure hydrostatic pressure of ~1,000 atm (Takagi, personal communication). Such information suggests that *P. tetraurelia* cells in this study were exposed to 600~800 atm when they stopped swimming at the beginning and to more than 1,000 atm when they were ruptured. It should be emphasized, however, that $P_*$ is different from the hydrostatic pressure.

The present observations on the death process are a typical case of accidental death which takes place in a short time. It is of interest to know how cells die in other cases such as death by physiological chemicals at higher concentrations and death by starvation, which occur more slowly than the present case.

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