CLONAL DEATH ASSOCIATED WITH THE NUMBER OF FISSIONS IN PARAMECIUM CAUDATUM

YOSHIOMI TAKAGI AND MICHIKO YOSHIDA
Department of Biology, Nara Women's University, Nara 630, Japan

SUMMARY

Contrary to an earlier report suggesting clonal immortality in Paramecium caudatum, the clones of the same species in this study terminated inevitably with a maximal life-span of 658 fissions and showed, prior to clonal death, decreased fission rate, increased probability of the appearance of non-dividing or dead cells after cell division, and increased frequency of morphological abnormality and of division asynchrony. Clonal senescence and death after a limited number of fissions was reproducible even if subclones derived from the original clone of a known fission age were examined again after a lag of 468 days. These results indicate that clones of P. caudatum are mortal and that they use fissions, not days, to measure life-span. Possible causes for the discrepancies between the earlier report and the present one are discussed.

INTRODUCTION

We have attempted to test the hypothesis that clones of Paramecium caudatum age and die. This hypothesis cannot be considered a priori correct. First, although the reality of clonal aging was demonstrated in Paramecium aurelia by Sonneborn (1954) and has been suggested in P. bursaria (Jennings, 1944; Siegel, 1967) and P. jenningsi (Miyake & Miyake, 1967), senescence in other paramecia species remains still to be studied. Second, some species of Tetrahymena, except some genetic strains, are regarded as essentially immortal (Nanney, 1959, 1974; Nanney & McCoy, 1976). There are some other species, such as Uroleptus mobilis (Calkins, 1919), Euplotes crassus (Heckmann, 1967), E. patella (Katashima, 1971) and E. woodruffi (Kosaka, 1974), in which clonal senescence was suggested. Third, more directly, old work by Galadjieff & Metalnikow (1933) on P. caudatum suggested a limitless capacity for cell division, and this claim has remained unchallenged.

The present study indicates, if not proves, the reality of clonal aging terminated by death in P. caudatum and suggests the importance of fission age rather than calendar age in the determination of clonal life-span. The results have partly been reported in abstracts (Takagi & Yoshida, 1977; Yoshida & Takagi, 1978).
MATERIALS AND METHODS

Stocks

Stocks Ksy-i (mating type V), dN14a (VI), 84 (V) and 89 (VI) of Paramecium caudatum, syngen 3 were the sources of the clones studied. Several hundred clones were obtained from Ksy-i x dN14a and from 84 x 89, but those derived through macronuclear regeneration and those dying soon after conjugation were discarded. Among the rest, 6 vigorous clones that reached the 150th fission were used to estimate the clonal life-span. Of these 6, clones 7a, 16a and 17a were derived from the cross Ksy-i x dN14a, and clones D6a, D6b and M18a were derived from the cross 84 x 89. D6a and D6b were sister clones originating from a pair of exconjugants. All of the clones except 17a were mating type V.

Culture conditions

The culture medium was 2% lettuce juice in 2 mM sodium phosphate buffer solution, pH 6.8, inoculated 1 day before use with Aerobacter aerogenes. The temperature was kept at about 25 °C throughout the study unless otherwise stated.

Maintenance of cell lines

Cell lines were maintained in serial reisolation cultures: single cells were put in slide depressions (3 depressions per slide) containing 0.3-0.5 ml culture medium, the number of daughter cells was counted, and one of them was reisolated with a micropipette under a dissecting microscope into fresh medium. This procedure was repeated every alternate day in Experiment I, and daily in Experiment II.

Experiment I

Experiment I was designed to estimate the clonal life-span. Conjugation indicated zero time. At a given number of fissions after conjugation, 3 cells were selected randomly to expand 3 lines: this was done at the age of 5 or 6 fissions in 7a, 16a, 17a and M18a; at the age of 14 fissions in D6a and D6b. Lines were thereafter maintained by alternate-day isolations. The cells remaining after reisolation were supplied with food to give rise to surplus cultures consisting of about $10^3$ cells: they were used for replacement of lines, for testing mating reactivity to determine the onset of maturity, and for inspecting abnormal cells and selfers (intraclonal conjugation). To check selfers, not only were selfing pairs looked for under a dissecting microscope but also samples of 200-300 cells in stationary phase were stained with acetocarmine to determine whether cells with fragmented macronuclei characterizing the completion of selfing conjugation were included. Evidence for selfing was never detected in the surplus cultures throughout the experiment. The surplus cultures were, with intervals of about 50 fissions, further grown to mass cultures of about 1.6 x $10^4$ cells in 18-ml test tubes, which were kept at 17 °C as stock cultures and maintained by replacing half of the culture with fresh culture medium about every 3 weeks.

Each clone was, as a rule, maintained in a set of 3 reisolation lines. Cells for reisolation were usually selected at random; but, if the number of fission-products deviated from $2^n$ (n: integer) or if visibly abnormal cells were present, the most rapidly dividing and visibly normal cell was selected. Lines which became extinct were replaced whenever possible by isolating cells from other lines of the set or from the most recent surplus culture. Isolations from the surplus cultures were done only when the surplus cultures were still in logarithmic phase of growth. The number of lines per set was increased to 6 by replicating the set of 3 lines when replacements were needed frequently in later stages of clonal life. The time when this was done differed from clone to clone. The sum of the number of fissions in the longest-lived line of the set was taken as the life-span of the clone.

Experiment II

Experiment II was designed to make the closed life history revealed in Experiment I more certain. From one of the stock cultures of M18a, which had been kept at 17 °C for 468 days
Clonal death in Paramecium

since the 178th fission, 10 cells were isolated randomly and allowed to divide twice to initiate 10 subclones each consisting of a set of 3 lines (30 daily reisolation lines in total). The number of fissions during the stock culture was estimated to be 60 from growth curves of 20 serial subcultures produced for 468 days. Thus the fission age when 30 daily reisolation lines were initiated was estimated to be 240 (178 fissions during the period from conjugation to the transfer to the stock culture, plus 60 fissions during the stock culture at 17 °C for 468 days, plus 2 fissions for the expansion to 3 lines).

Ten subclones or 30 lines were halved and maintained by Y. T. and M. Y., independently. In Experiment II, the total number of reisolation lines was fixed to 15 for each person; the number of lines of some subclones was increased from 3 to 6, 9 and so on, after the other subclones died out.

Other experimental procedures were the same as in Experiment I. Again, no selfing was observed in the surplus cultures throughout Experiment II.

Evaluation of division potential

As shown diagrammatically below, daily reisolation lines are composed of branch lines produced by the expansion or replacement (in this diagram, 3 daily reisolation lines are composed of 6 branch lines).

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X = dead or non-dividing cell
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Evaluation of division potential was made in 2 ways. First, the frequency of non-dividing or dead cells in the set of lines was designated as % replacement; the number of reisolations (arrows) ending in X was divided by the number of all the reisolations during a given interval (5/30 or 16.7% in 10-day period of this diagram). Second, the longevity of each branch line measured in fissions was summed and averaged during a given interval.

RESULTS

Estimate of clonal life-span

In Experiment I, clonal life-span was estimated with 6 clones derived from 2 crosses (Table 1). Although the clonal life-spans varied greatly from clone to clone, progeny clones from S4 x S9 lived longer than those from K8-1 x dN14a. M18a lived longest; the clonal life-span was 478 fissions if represented by a set of 3 lines or 527 fissions if represented by a set of 6 lines.

Changes in mean fission rates and in the frequency of replacements for successive 10-day periods throughout the life history in these clones are shown in Fig. 1.

The first 10-day means of fission rate were generally low and were followed by a rapid or gradual increase to the peak level. A fission rate at near the peak was maintained for a long time in 16a, D6a and D6b, while the peak of fission rate was followed by a sudden or gradual decline in 7a, 17a and M18a.

Replacement of lines was not common during about the first 100 fissions, but was necessary continuously and increasingly during about the last 100 fissions of the
life history. It was, however, continuously necessary throughout the life cycle of clone 17a.

The first appearance of visibly abnormal cells in 7a and 16a was at 87 and 152 fissions, respectively. In the other clones, it was roughly the same as that of the first replacement.

Table 1. Life-spans of 6 clones studied

<table>
<thead>
<tr>
<th>Cross</th>
<th>Clone</th>
<th>Mating type</th>
<th>Maximal life-span in fissions when the clone is represented by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 lines</td>
</tr>
<tr>
<td>Ksy × dN14a</td>
<td>7a</td>
<td>V</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>16a</td>
<td>V</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>17a</td>
<td>VI</td>
<td>157</td>
</tr>
<tr>
<td>84 × 89</td>
<td>D6a</td>
<td>V</td>
<td>402</td>
</tr>
<tr>
<td></td>
<td>D6b</td>
<td>V</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td>M18a</td>
<td>V</td>
<td>478</td>
</tr>
</tbody>
</table>

Each clone was cultivated in a set of 3 lines of serial alternate-day isolation cultures. The number of lines per set was later increased to 6 by replicating the initial set of 3 lines. Replacements were allowed within the set of lines if some lines failed to continue. The total number of fissions from conjugation to death in the longest-lived line in the set was taken as the life-span of the clone.

Reproducibility of the fission life-span

In Experiment II, one of the 6 clones, M18a, was examined again regarding longevity. Ten subclones each consisting of a set of 3 lines were started at the parental age of 240 fissions after the surplus culture of the 178th fission had been kept at 17 °C for 468 days.

The results are summarized in Table 2. Residual life-spans in fissions of 30 lines ranged from 10 to 230 with a mean of 141.9 (A2) or total life-spans ranging from 250 to 470 with a mean of 381.9 (A2). The mean total life-span of 10 subclones was 422.9 fissions if represented by the longest-lived line in the non-replaceable set of 3 lines (A2); 478.1, 543.4 and 626 fissions if represented by the longest-lived line in the replaceable set of 3 (B), 6 (C) and 15 lines (D), respectively. The difference of the life-span between subclones maintained by Y.T. (Y1–Y5) and those maintained by M.Y. (M1–M5) was insignificant, even if comparisons were made with A2 data (380.6 ± 60.5 vs. 383.3 ± 56.3) or A3 data (431.4 ± 22.4 vs. 414.4 ± 37.5) or B data (489.0 ± 50.8 vs. 497.2 ± 56.7). This suggests that the termination of lines reflects not a technical artefact but some intrinsic process. Although the maximal life-span of M18a was finally lengthened to 658 fissions, the initially estimated value of 478 fissions or 527 fissions (p. 179) was reproducible, if comparisons were made in the corresponding scale of the set of lines: 478 vs. 478.1 ± 52.0 or 527 vs. 543.4 ± 21.2. This good agreement indicates that clones of P. caudatum use fissions or something correlated with fissions, not days, to measure duration of life history.
Fig. 1. Changes in mean fission rates (solid line) and percent replacements (dotted line) for successive 10-day periods throughout the life history of 6 clones: A, 7a; B, 16a; C, 17a; D, D6a; E, D6b; F, M18a. Each clone was subcultured to 6 lines, which were maintained in alternate day-isolation cultures and allowed to replace one another if some line became extinct. The clone was represented by the longest-lived line. Every 100th fission and the fission age at the onset of maturity are marked off by arrows and open arrows, respectively.
Table 2. Life-spans of 10 subclones or 30 lines in fissions

<table>
<thead>
<tr>
<th>Subclone Line</th>
<th>(A₁)</th>
<th>(A₂)</th>
<th>(A₃)</th>
<th>3 lines (B)</th>
<th>6 lines (C)</th>
<th>15 lines (D)</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y₁ a</td>
<td>(72)</td>
<td>312</td>
<td>470</td>
<td>513</td>
<td>519</td>
<td>—</td>
<td>380·6 ± 60·5</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>(230)</td>
<td>470</td>
<td></td>
<td></td>
<td></td>
<td>431·4 ± 22·4</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>(79)</td>
<td>319</td>
<td></td>
<td></td>
<td></td>
<td>489·0 ± 50·8</td>
</tr>
<tr>
<td>Y₂ a</td>
<td>(125)</td>
<td>365</td>
<td>427</td>
<td>530</td>
<td>530</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>(187)</td>
<td>427</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>(54)</td>
<td>294</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y₃ a</td>
<td>(30)</td>
<td>270</td>
<td>412</td>
<td>435</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>(88)</td>
<td>328</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>(172)</td>
<td>412</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y₄ a</td>
<td>(185)</td>
<td>425</td>
<td>425</td>
<td>534</td>
<td>572</td>
<td>594</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>(173)</td>
<td>413</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>(185)</td>
<td>425</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y₅ a</td>
<td>(169)</td>
<td>409</td>
<td>423</td>
<td>433</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>(183)</td>
<td>423</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>(177)</td>
<td>417</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Ten subclones (30 lines) were initiated from the clone M18a 240 fissions old. They were halved and maintained by Y. T. (Y1–Y5) and M. Y. (M1–M5) independently. Each line was cultivated in daily reisolating cultures until termination without any replacement; the number of fissions during this period is shown in A1; the total number of fissions since conjugation (A1 + 240) in A1; the largest number of fissions in each set of 3 lines in A1. After termination of any one of the lines, replacements were allowed within the set of 3 lines. The number of lines per subclone was increased to 6–15 in later periods. The life-span of subclones is also shown as the total number of fissions since conjugation in the longest-lived line in a set of 3 (B) or 6 (C) or 15 lines (D).

s.d. = standard deviation.
On the basis that clonal life-span varied greatly among lines and could be lengthened by an increase in the number of lines, a question may be raised whether an indefinite elongation of clonal life history may be possible. One will never be able to answer to this question strictly, since it is impossible to follow the fate of all the cell lines produced during 658 fissions or more. But indirect evidence against clonal immortality is given in Fig. 2, which shows that replacements in later stages resulted in branch

![Diagram](image)

Fig. 2. Longevity in fissions of branch lines of the subclone Y4, which was derived from M18a 240 fissions old, here corresponding to 0 fission, and maintained in daily reisolation cultures.

Table 3. Mean longevity in fissions of branch lines produced at different ages of subclones

<table>
<thead>
<tr>
<th>Subclone</th>
<th>Clonal age when branch lines were made</th>
<th>No. of branch lines</th>
<th>Longevity of branch lines, mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y4</td>
<td>240</td>
<td>3</td>
<td>181 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>400-500</td>
<td>12</td>
<td>32 ± 26.7</td>
</tr>
<tr>
<td></td>
<td>501-550</td>
<td>29</td>
<td>12 ± 9.9</td>
</tr>
<tr>
<td></td>
<td>551-560</td>
<td>11</td>
<td>56 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>561-570</td>
<td>12</td>
<td>3.9 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>571-580</td>
<td>19</td>
<td>3.7 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>581-590</td>
<td>39</td>
<td>1.4 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>591-600</td>
<td>3</td>
<td>0.5 ± 8.8</td>
</tr>
<tr>
<td>Collectively*</td>
<td>240</td>
<td>27</td>
<td>137.6 ± 59.0</td>
</tr>
<tr>
<td></td>
<td>250-300</td>
<td>3</td>
<td>88.3 ± 66.5</td>
</tr>
<tr>
<td></td>
<td>301-400</td>
<td>29</td>
<td>37.0 ± 45.7</td>
</tr>
<tr>
<td></td>
<td>401-450</td>
<td>58</td>
<td>10.2 ± 15.7</td>
</tr>
<tr>
<td></td>
<td>451-500</td>
<td>65</td>
<td>8.8 ± 15.1</td>
</tr>
<tr>
<td></td>
<td>501-550</td>
<td>129</td>
<td>6.2 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>551-600</td>
<td>122</td>
<td>4.0 ± 4.8</td>
</tr>
</tbody>
</table>

* All the subclones except Y4 are included.
Clonal death in *Paramecium* lines with shorter longevity than replacements in earlier stages. This was not specific for the subclone Y₄, but a general trend in all the subclones as summarized in Table 3.

Some characteristics of clonal senescence

In addition to a decrease in division potential with age (Fig. 2, Table 3), some age-correlated deteriorative changes were observed.

The starting age of 240 fissions in the 10 subclones can be considered to have been at the period of peak fission rate (Fig. 1F). Gradual decline in fission rate from the peak level was again observed in the 10-day means of fission rates of 30 lines (Fig. 3). Mean fission rate of 30 lines was higher than 3 (fission/day) for the first two 10-day periods but decreased to less than 2 after the 7th 10-day period: the difference is statistically highly significant (t-test; \( P < 0.01 \)).

![Fig. 3. Changes in mean fission rates for successive 10-day periods in 30 lines of 10 subclones, which were derived from M18a 240 fissions old and maintained in daily reisolation cultures. Standard deviations are indicated by vertical bars.](image)

Since the number of daughter cells produced from every reisolated cell was recorded in Experiment II, the proportion of each daughter cell-number was calculated every 50 fissions in all the reisolation lines. Changes in the frequency distribution of the daughter cell-number with age are shown in Fig. 4. During the first 50 fissions, a majority of the daughter cell-number was 8. But with age the proportion of 8 and those larger than 8 decreased gradually and, instead, the proportion of 2, 3, and 4 increased gradually. Fig. 4 shows also a gradual increase in frequency of non-dividing and dead cells (black areas) with age.

Among the spectra of the daughter cell-numbers from 3 to 16, the numbers 4, 8 and 16 may be regarded as the products of synchronous cell divisions. If their proportion is regarded as a tentative index of division synchrony, the index decreases linearly with fission age (Fig. 5).

One of the most conspicuous properties of aged clones was the increased appearance of morphologically abnormal cells: cells with cytoplasmic protrusions or vacuoles; fat or round or tiny or twiggy cells; cells resulting from delay or complete failure of cell divisions, which produced a chain of 2-8 cells, or L-shaped cells, or various kinds of amorphous monsters, etc. Some abnormalities appearing in aged surplus cultures older than 350 fissions are shown in Fig. 6. Uncoupling of cytokinesis with karyokinesis was often observed (e.g. Fig. 6c, e). Typical abnormal cells rarely, if ever, appeared in surplus cultures of 30 lines by about 350 fissions and, if they did,
Fig. 4. Changes in the frequency distribution of daughter cell-numbers for successive 50-fission periods in 30 lines of 10 subclones, which were derived from Mr8a 240 fissions old, here corresponding to a fission, and maintained in daily reisolation cultures. All the reisolated cells were classified according to the number of daughter cells produced in a day; the frequency of each daughter cell-number is represented by the fraction area. Black fractions indicate the frequencies of the number of 0 and 1, i.e. the frequency of dead and non-dividing cells.

Fig. 5. Changes in a tentative index of division synchrony for successive 50-fission periods. On the basis of the data in Fig. 4, the percent fractions of 4, 8 and 16 cells among those of more than 3 cells was regarded tentatively as an index of division synchrony.

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they could produce normal cells after cell divisions. But subsequently they appeared increasingly and produced either abnormal or dead cells by cell divisions with increasing probability with age. At last almost all the cells in surplus cultures were more or less morphologically abnormal.

**DISCUSSION**

In order to estimate clonal life-span, the process of asexual reproduction must never be interrupted by the occurrence of sexual reproduction. The classical work by Woodruff (see Wichterman, 1953), which was interpreted as showing that clones of *P. aurelia* have an unlimited life-span, was demonstrated to be erroneous by the discovery of periodic autogamy (Sonneborn, 1954). The constant presence of excess food in daily reisolation cultures was the method used to avoid occurrence of autogamy and conjugation in *P. aurelia*. In *P. caudatum*, the method of reisolation on alternate days appears to be adequate for this purpose, since fission rate is less than two thirds of that in *P. aurelia* and there is no natural autogamy. The possibility of occurrence of the sole sexual process, i.e. selfing, can be ruled out in the present study, since (1) replacements either from the set of lines or from surplus cultures were made only when the cultures were still in the logarithmic phase of growth, and (2) selfing was in fact never detected in any surplus cultures used for replacements.

Galadjieff & Metalnikow (1933) reported the occurrence of 8704 fissions without conjugation during the course of 22 years and 5 months in *P. caudatum*. This report has remained unchallenged although there are now reasons to question its validity. The study was performed before the discovery of mating type in *Paramecium* (Sonneborn, 1937a) and before the establishment of modern concepts concerning various sexual processes. At that time, endomixis was believed to occur in *P. caudatum* as well as in *P. aurelia* (see, for example, Sonneborn, 1937b, pp. 484–485). Although originally described as a nuclear reconstruction process without meiosis, endomixis was later demonstrated cytologically (Diller, 1936) and genetically (Sonneborn, 1939, 1947) to be autogamy. Present knowledge excludes the occurrence of both endomixis and natural autogamy in *P. caudatum* (Hiwatashi, 1969). To examine their results we may suppose that Galadjieff and Metalnikow isolated cells from the surplus cultures containing exconjugants originated from selfing conjugation. They would undoubtedly regard the cells as vegetative products, since nuclear changes in single cells, if detected, would be regarded as endomixis. A careful reading of their report led us to suspect that they made an oversight of selfing that could occur in the starved surplus cultures they used for replacements.

Selfing in *P. caudatum* occurs seldom in the clone of odd-numbered mating type (Hiwatashi, 1968; Myohara & Hiwatashi, 1975). Although the present clones are, except 17a, all mating type V, we made a careful examination for selfing in various test-tube cultures or flask cultures raised from stock cultures of known ages. We found that selfing could take place in all the clones studied here under specific conditions, i.e. in stationary phase of growth in old clones. In M18a, for example, selfing was observed at a frequency of less than 1%, but only in a part of the cultures
that were older than 274 fissions. Judging from its low frequency and late expression, selfing in the odd-numbered mating type appears to be under a different control mechanism from selfing in the even-numbered mating type, which comes to expression in a high frequency at an early period of maturity (Myohara & Hiwatashi, 1975): the former phenomenon appears similar to that reported in *Tetrahymena canadensis* (Outka, 1961) or *Euplotes* (Heckman, 1967; Katashima, 1971), in which selfing occurs only in old clones. Anyway, this finding made us discard experimental data on 10 subclones (30 lines) of M18a reinitiated at the parental age of 450 fissions. In fact a discrepant distribution of total life-spans in these subclones suggested the occurrence of selfing in 4 subclones during the long period of periodic starvation in stock culture: 4 subclones showed a total life-span of 770 ± 70 fissions on average, ranging from 699 to 854, while the other 6 showed a total life-span of 546 ± 36 fissions on average, ranging from 478 to 579. Thus the former 4 subclones might involve 2 generations including selfing progeny that would live for at least 249–414 fissions (699 or 854 minus 450).

The maximum life-span revealed in this study was 658 fissions. However, this could be further lengthened by selection even if selfing was not allowed to occur. The possibility of selecting more vigorous cells will increase as the population from which cells are transferred to the next culture increases in size. This was shown in this study as an effect of lengthening the life-span by an increase in the number of lines from 3 to 6, 9 and so on. Even more effective selection would be realized in mass cultures. However, the fact that, among 45 stocks of *P. caudatum* collected in nature 30 years ago, only 1 stock which is a selfer has remained (K. Hiwatashi, personal communication) suggests that clonal death is inevitable even in mass cultures. We estimate that the maximum life-span of his stocks did not exceed 1000 fissions.

Changes associated with clonal senescence in *P. aurelia* include: (1) decline in fission rate and in viability after sexual and asexual reproduction (Sonneborn, 1954; Sonneborn & Schneller, 1960; Fukushima, 1975; Rodermel & Smith-Sonneborn, 1977); (2) drift of sexual pattern from conjugation to autogamy (Sonneborn, 1957); (3) increased frequency of cytological abnormality (Mitchison, 1955; Dippell, 1955; Sonneborn, 1957; Sonneborn & Dippell, 1960); (4) increased sensitivity to ultraviolet (Smith-Sonneborn, 1971) and caffeine (Smith-Sonneborn, 1974); and (5) reduction in DNA content (Schwartz & Meister, 1973; Klass & Smith-Sonneborn, 1976), RNA synthesis, DNA template activity (Klass & Smith-Sonneborn, 1976) and endocytic capacity (Smith-Sonneborn & Rodermel, 1976). In the present study, some of these changes were also encountered in old clones: decline in fission rate, increased probability of the appearance of non-dividing or dead cells after cell division and increased frequency of morphological abnormality and of division asynchrony.

The results in this study thus show it is very likely that clonal death in *P. caudatum* is inevitable, if selfing is avoided, after about 600–700 fissions (1000 fissions as a maximum) since the previous conjugation, although we cannot absolutely deny the exceptional existence of an immortal cell line.
An additional finding in this study is that clonal life-span appears to be related to number of fissions rather than to days elapsed. This association is consistent with the results in *P. aurelia* (Smith-Sonneborn & Reed, 1976). In paramecia fission age, not calendar age, is associated with the length of immaturity (Kroll & Barnett, 1968; Takagi, 1970, 1974; Miwa & Hiwatashi, 1970). Although it is not yet clear whether paramecia use a common cellular clock for both the timing of clonal maturity and clonal death, the following observations are suggestive: (1) Both duration of immaturity (Siegel, 1961) and life-span (Smith-Sonneborn, Klass & Cotton, 1974) can be shortened by increasing the parental age; and (2) both duration of immaturity (Takagi, 1974) and life-span (Fukushima, 1974) can be shortened by u.v. and X-ray, respectively. In this connexion, it would be interesting to study whether early maturity mutants in *P. caudatum* (Myohara & Hiwatashi, 1978) have a shorter life-span.

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