MICROSPECTROPHOTOMETRIC AND AUTORADIOGRAPHIC STUDY OF THE TIMING AND DURATION OF PRE-MEIOtic DNA SYNTHESIS IN PARAMECIUM CAUDATUM

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SUMMARY
Timing and duration of pre-meiotic G1, S and G2 phases in Paramecium caudatum have been clarified by microspectrophotometry of DNA content and autoradiography with [3H]thymidine ([3H]dThd) and [3H]deoxyuridinemonophosphate ([3H]dUMP). Microspectrophotometric measurement of DNA content showed that cells in stationary phase have a G1 micronucleus, and that pre-meiotic DNA synthesis begins with the micronuclear swelling and ends immediately before the nucleus enters meiotic prophase. The results indicate that the micronucleus is in G1 during the first 1-5 h after onset of mating reaction at 25°C and in pre-meiotic S phase during the following 1 h, and that G2 phase is very short or lacking. Therefore, in P. caudatum, the transition point from pre-meiotic interphase to meiotic prophase is at or very close to the end of pre-meiotic DNA synthesis.

Autoradiographic study showed that although [3H]dThd and [3H]dUMP were well utilized in pre-mitotic DNA synthesis, only [3H]dUMP was well utilized in pre-meiotic DNA synthesis. [3H]dThd was utilized very little in pre-meiotic DNA synthesis even if the precursor was taken into the cell, suggesting that the activity of thymidine kinase is low in these cells.

INTRODUCTION
The ciliate Paramecium caudatum is a suitable experimental organism for studying the regulation of meiosis, and especially the mechanism of the induction of meiosis, because cells in the stationary phase can easily be switched to meiosis synchronously by mixing them with cells of complementary mating type to induce conjugating pairs. Meiosis is completed within 10–11 h after mixing cells of complementary mating types at 25°C. During that time all nuclear changes can easily be observed in living cells (Wichterman, 1940; Fujishima, unpublished data), as well as in fixed and stained cells, by the ordinary light microscope. Moreover, the cells are relatively large, so that micromanipulation techniques such as the injection of the micronucleus (Fujishima & Hiwatashi, 1978, 1981; Fujishima, 1980; Fujishima & Watanabe, 1981; Karino & Hiwatashi, 1981), cytoplasm or soluble substances (Miwa, Haga & Hiwatashi, 1975; Miwa, 1979a,b; Haga & Hiwatashi, 1981a,b) can be used.

Morphological nuclear changes during conjugation of P. caudatum have been reported previously (Calkins & Cull, 1907; Wichterman, 1940; Sugai, 1976). However, detailed correspondence of the observed nuclear changes to those of classical stages in pre-meiotic interphase, i.e. pre-meiotic G1, S and G2 phases, is not clear. In the present experiments, the timing and duration of each stage in pre-meiotic interphase

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were clarified by microspectrophotometry of DNA contents and by autoradiography with \[^3H\]thymidine and \[^3H\]deoxyuridinemonophosphate. Furthermore, using the autoradiographic study, it was found that pre-meiotic DNA synthesis differs from pre-mitotic DNA synthesis in the method of deoxythymidylic acid formation.

**MATERIALS AND METHODS**

**Strains and culture conditions**

*P. caudatum* syngen 3, mating type V (strains \(d^m-1\) and \(d^m-21\)) and mating type VI (strains 17B and \(d^m-23\)) were used. The culture medium was 1-25 % (w/v) fresh lettuce juice in Dryl’s (1959) solution, pH 7.0, inoculated with a non-pathogenic strain of *Klebsiella pneumoniae* 1 day before use (Hiwatashi, 1968). Several hundred cells were inoculated into 2 ml of culture medium in a test tube, and then fresh medium of 4 ml, 10 ml and 10 ml was added on successive days. One or two days after the final feeding, mating reactivity increased to the maximum intensity. The cultures were kept at 25 °C.

**Feulgen microspectrophotometry**

Two days after the final feeding, the cells of strains \(d^m-1\) and 17B in tube cultures were strained through four layers of fine cheese-cloth to remove gross debris. Then the cells were harvested by centrifugation for 3 min at 600 rev./min in an oil centrifuge with 100 ml centrifuge tubes, and then the cell density was adjusted to 2500 cells/ml by adding Dryl’s solution. About 0.4 ml of cell suspension from each of the complementary mating types were mixed together in depression slides and kept in a moist chamber at 25 °C. The preparations for microspectrophotometric measurements were made every 1 h for 8 h after the complementary mating types were mixed. The cells were harvested by centrifugation in a hand-operated centrifuge, air-dried on clean microscope slides, fixed in an ethanol/acetic acid (3: 1) mixture for 10 min, and transferred to 70 % ethanol for storage until stained. The slides were then passed through an ethanol series to water, and hydrolysed for 8 min in 1 N-HCl at 60°C. The cells were stained for 2 h in Schiff’s reagent, bleached, dehydrated, cleared in xylene and mounted with Caedax (Merck). Cells were also fixed and stained before mixing with the complementary mating type. In each experiment, all slides were hydrolysed and stained simultaneously, and aged for 1–2 weeks prior to measurement.

Relative DNA contents of the micronuclei were measured microspectrophotometrically by using C-Zeiss UMSP-1. Absorption at 560 nm was measured by scanning with a 1 μm monochromatic beam at a magnification of ×1000. Background readings were also taken and automatically subtracted from the nuclear value.

**Autoradiography**

Two days after the final feeding, cells of complementary mating types, \(d^m-21\) and \(d^m-23\), were strained through four layers of fine cheese-cloth to remove gross debris. Then the cells were centrifuged for 3 min at 600 rev./min in 100 ml tubes in an oil centrifuge and washed with Dryl’s solution using the same conditions of centrifugation. Cell density was adjusted to 2500 cells/ml by adding Dryl’s solution. Ten ml of the cell suspension from each of the complementary mating types were mixed with an equal volume of Dryl’s solution, which contained \(3 \times 10^7\) polystyrene Ratex particles (Difco, diameter 0.81 μm) and either 20 μCi/ml \[^3H\]thymidine (\[^3H\]dThd) (Daichki Pure Chemicals; sp. act. 11-6 Ci/mmol, radioactive concentration 1.0 μCi/ml) or 20 μCi/ml \[^3H\]deoxyuridinemonophosphate (\[^3H\]dUMP) (The Radiochemical Centre; sp. act. 12.7 Ci/mmol, radioactive concentration 1.0 μCi/ml). As a result, the cells were suspended in a solution of 1.5 × 10^7 polystyrene particles/ml and either of the radioactive DNA precursors at a cell density of 2500 cells/ml. This suspension was kept for 1 h at 25 °C. During this period the cells formed many food vacuoles containing the particles. The polystyrene particles were used with the expectation that incorporation of external radioactive DNA precursor into the cells might be enhanced by this method, because the precursor might be taken up into the cells not only from cell membrane but also from food vacuoles ingesting external medium together with the particles. Mating reactivity was
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not decreased even after 4 h incubation with the polystyrene particles. After 1 h incubation, the cells were washed by three centrifugations in Dryl's solution to remove the radioactive DNA precursor and polystyrene particles in the medium. One ml of the cell suspension of mating type V was transferred to a test tube, 2 ml culture medium were added, and it was kept for 11 h at 25°C. The cells were then fixed and stained for autoradiography. On the other hand, the remaining cells were mixed with cells of the complementary mating type in a 30 ml flask, kept at 25°C for several hours, and then fixed and processed for autoradiography. Utilization of the DNA precursor in pre-mitotic and pre-meiotic DNA syntheses was examined in the former and the latter autoradiograms, respectively.

Preparations for autoradiography were made as follows. Cells were harvested by hand-operated centrifugation and dried on clean microscope slides and fixed in an ethanol/acetic acid (3:1) mixture for 10 min. The slides were extracted three times (5 min each) in cold (4°C) 5 % (w/v) trichloroacetic acid to remove the non-specific label and then rinsed in distilled water. Subsequently, all slides were subjected to 0.02 % (w/v) RNase (Worthington Biochemical Co., in 10 mM-phosphate buffer, pH 6.9) digestion for 7 h at 35°C, and several slides were subjected to 0.01 % (w/v) DNase (Worthington Biochemical Co., in 5 mM-MgSO₄, 10 mM-phosphate buffer, pH 6.9) digestion for 7 h at 35°C to check the specificity of the incorporation of the tracer into DNA. These slides were hydrolysed in 5 n-HCl for 30 min at room temperature and stained by the Feulgen reaction. Dried slides were dipped in Sakura liquid-type nuclear emulsion (NR-M2), allowed to dry, and stored in a light-tight box for 15 days for [³H]dThd labelling and 34 days for [³H]dUMP labelling, respectively, at 4°C. The slides were processed in Rendol (Fuji) developer for 5 min and in Fuji Fix for 15 min, and then rinsed in distilled water. They were dehydrated through a graded ethanol series, cleared in xylene and mounted with Caedax (Merck). Grain counts were made in an area over the micronucleus and compared with grains in an area of the cytoplasm of the same size as that over the micronucleus.

RESULTS

Comparison of DNA content between the micronuclei of cells in stationary phase and G₁ micronuclear standards

In P. tetraurelia (formerly syngen 4 of P. aurelia) (Woodard, Gelber & Swift, 1966), P. trichium (Pieri, Vaugien & Trouillier, 1968) and P. bursaria (Golikova, 1974), it has been reported that pre-meiotic DNA synthesis takes place during the conjugation process. These reports indicate that mating-reactive cells in stationary phase have a G₁ micronucleus. In other words, these paramecia enter conjugation with a G₁ micronucleus. On the other hand, it is well known that Tetrahymena already has a G₂ micronucleus before entering conjugation, since DNA synthesis is completed soon after cell division (McDonald, 1962). Therefore, in Tetrahymena, the micronuclear changes observed early in the conjugation process should be those of meiotic prophase. In P. caudatum, however, there has been no work published to clarify the stage of the micronucleus immediately before conjugation, i.e. in stationary phase cells.

In order to identify the micronuclear stage of P. caudatum in stationary phase, relative DNA content was compared between micronuclei of cells in stationary phase and G₁ micronuclear standards by microspectrophotometric measurement. Cells having a G₁ micronucleus were obtained as follows. Dividing cells in early stationary phase were transferred to Dryl's solution and kept at 25°C. Cells within 1 h after cell division were used as cells having a G₁ micronucleus, because Rao & Prescott (1971) have reported that cells within 3 h after cell division have a G₁ micronucleus when the
cell cycle time is 8 h. Since the cell cycle time of strains d^m^-1 and 17B used in this experiment was also about 8 h at 25 °C, cells within 1 h after cell division were expected to have a \( G_1 \) micronucleus. Because the cells in this phase had few food vacuoles compared with the cells in log phase, it was possible to measure the nuclear DNA content by microspectrophotometry without contamination from bacterial DNA in the food vacuoles. The results are summarized in Table 1. No significant difference in DNA content between the micronuclei of cells in stationary phase and the \( G_1 \) micronuclear standard was shown. Thus, it becomes clear that cells in stationary phase have a \( G_1 \) micronucleus and pre-meiotic DNA synthesis occurs some time after the beginning of the mating reaction.

**Measurement of micronuclear DNA content early in the conjugation process**

Morphological changes in micronuclei in cells within 6 h after mixing complementary mating types at 25 °C have been classified into five stages and reported, with their time course (Fujishima & Hiwatashi, 1981). The process is summarized schematically in Fig. 1. These nuclear events are basically the same as the observation by Calkins & Cull (1907). Stage I micronucleus is observed in cells during the mating reaction and those of conjugating pairs united at their anterior ends (holdfast union). Microscopically, the micronuclei in these cells could not be distinguished from those of cells in stationary phase, the diameter of which is about 5 \( \mu \text{m} \). About 1.5–2.5 h after the beginning of the mating reaction, a stage II micronucleus is observed in cells of conjugating pairs united at the regions of the cytostom (paroral union). In this stage, the micronucleus swells to a diameter of about 7–10 \( \mu \text{m} \) and the chromatin, which was condensed in stage I, is dispersed uniformly throughout the nucleus. The swollen micronucleus begins to elongate, becomes rod-shaped and accumulates condensed chromatin in granules at one side of the nucleus. This micronucleus was designated stage III. This stage is very short, and is observed in conjugating cells about 2.5–3.0 h after the beginning of the mating reaction. Then chromatin threads appear and arrange themselves parallel to the long axis of the elongated micronucleus. After a while, the chromatin threads become obscure following further nuclear elongation. These micronuclei were designated stage IV, and are observed in conjugating cells about 3.0–5.5 h after the beginning of the mating reaction. As a consequence of this nuclear elongation, the micronucleus curves to form a ‘crescent’, which is a characteristic shape of prezygotic nuclear division in many holotrich ciliates. This is stage V, which

<table>
<thead>
<tr>
<th>Micronuclei measured</th>
<th>Relative DNA content*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( d^m^-1 ) (mating type V)</td>
</tr>
<tr>
<td>Mating reactive cells in stationary phase</td>
<td>37.8 ± 0.6 (28)</td>
</tr>
<tr>
<td>( G_1 ) standard</td>
<td>35.3 ± 1.5 (15)</td>
</tr>
</tbody>
</table>

* Mean value ± 95% confidence limit in arbitrary units.
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Fig. 1. Schematic representation of micronuclear changes early in the conjugation process of P. caudatum. The micronuclear changes of stages I–V can be observed during the first 6 h after the onset of the mating reaction at 25 °C.

is observed in conjugating cells about 5.5–6.5 h after the beginning of the mating reaction. This crescent later retracts to form an ellipsoid. Immediately after the beginning of retraction, thin chromatin threads appear again and the threads become obvious chromosomes. These nuclear changes are observed between about 6.5 and 8.0 h after the beginning of the mating reaction. Metaphases of the first and second meiotic divisions then follow at about 8 and 10 h, respectively, after mixing complementary mating types. It can be assumed that stage III is the beginning of meiotic prophase, because chromatin condensation starts at this stage, the condensed chromatin granules develop into chromatin fibres in stage IV, and such continuous nuclear changes lead eventually to the first meiotic metaphase. During the stages from stage III to the first meiotic metaphase, there is no stage that can be assumed to be pre-meiotic G2 phase.

In each of the stages I–V, micronuclear DNA content was measured by microspectrophotometry. The results are shown in Fig. 2, and the data presented in Fig. 2 are summarized in Table 2. As a standard of G1 micronuclear DNA content,
Fig. 2. Micronuclear DNA contents in cells early in the conjugation process in *P. caudatum*. Relative DNA contents were measured microspectrophotometrically with Feulgen-stained preparations. (●) Stage I; (○) stage II; (▲) stages III, IV; (△) stage V. The micronuclear stages correspond to those in Fig. 1.

### Table 2. DNA content of micronuclei in stationary phase cells and in cells early in the conjugation process

<table>
<thead>
<tr>
<th>Stages of micronuclei measured</th>
<th>N</th>
<th>Relative DNA content*†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating-reactive cells in stationary phase</td>
<td>19</td>
<td>41.3 ± 0.9 (38.6–46.0)</td>
</tr>
<tr>
<td>Cells early in the conjugation process†</td>
<td>30</td>
<td>42.7 ± 0.8 (37.0–46.1)</td>
</tr>
<tr>
<td>Stage I</td>
<td>29</td>
<td>75.8 ± 4.6 (55.7–93.7)</td>
</tr>
<tr>
<td>Stage II</td>
<td>13</td>
<td>83.4 ± 2.8 (80.5–91.0)</td>
</tr>
<tr>
<td>Stage V</td>
<td>8</td>
<td>84.5 ± 8.9 (62.8–101.0)</td>
</tr>
</tbody>
</table>

Micronuclear DNA content in cells of early conjugating process were summarized on the basis of the data in Fig. 4.

* Mean value ±95% confidence limit in arbitrary units.
† Numbers in parentheses are the minimum and maximum values in arbitrary units.
† Stages I–V correspond to those in Fig. 1.
micronuclei in mating-reactive cells of the complementary mating types just before mixing were also measured (Table 2). As shown in the table, the DNA content of stage I micronuclei showed no significant difference from that of the G1 micronuclei. Stage II micronuclei showed various values of DNA content between the value and twice the value of G1 micronuclei. Stages III–V micronuclei contained about twice the DNA content of the G1 nuclei. Thus, it appears that the micronucleus of stage I is in G1 and that of stage II is in pre-meiotic S phase, in which DNA synthesis starts and is completed. These results strongly support the assumption that the micronucleus of stage III is the beginning of meiotic prophase, suggesting that G2 phase is very short or lacking. Therefore, in P. caudatum, the transition point from pre-meiotic interphase to meiotic prophase is at or very close to the end of pre-meiotic DNA synthesis.

These data were obtained from several slides because the cells were fixed at different times before or after mixing complementary mating types, but all the slides were hydrolysed and stained at the same time to minimize the difference in staining intensity between the slides. As shown in Fig. 2 and Table 2, however, stage V micronuclei showed a fluctuation in their DNA contents compared with those in other stages. The variability may be due partly to errors in measurement, but largely to difference in degrees of nuclear compaction (Garcia, 1970). Such a variability in micronuclear DNA content has also been observed in other studies (Doerder & De Bault, 1975; Mikami & Koizumi, 1979).

**Autoradiography with [3H]thymidine**

Autoradiography is a powerful technique for cell cycle analysis. In order to identify pre-meiotic S phase of P. caudatum by autoradiography, each of the complementary mating types in stationary phase was incubated with 10 μCi/ml [3H]thymidine ([3H]dThd) for 1 h at 25 °C; they were then mixed with each other and allowed to proceed to the conjugation process in the presence of [3H]dThd. In most cells, however, no significant incorporation of the labelled precursor into the micronucleus was observed, despite the fact that the nucleus should have passed through the pre-meiotic S phase (Fujishima, unpublished). Rao & Prescott (1967) have reported the utilization of [3H]dThd in vegetative DNA synthesis in both the micro- and the macronucleus of P. caudatum. Therefore, the inability to use [3H]dThd in pre-meiotic DNA synthesis seems to be caused either by the fact that these cells could not take the external [3H]dThd into the cell, or that they could take it up but could not use it for pre-meiotic DNA synthesis. In order to solve this problem, the following experiments were performed. Cells of complementary mating types in stationary phase were incubated in Dryl's solution containing [3H]dThd and polystyrene Latex particles (see Materials and Methods). After 1 h of incubation, the cells were washed with Dryl's solution, and then a sample of the cell suspension was transferred to culture medium and cultivated for 11 h at 25 °C. During this time some cells divided once but never twice. Though one cell cycle time of the strains used was about 8 h at 25 °C, the time up to the first division took more than 8 h when cells from a starved culture were inoculated into fresh culture medium. After 11 h of cultivation, the
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dividing cells that had a furrow and were just about to separate into two daughter cells were harvested from the culture and then fixed and processed for autoradiography. The micronucleus in these dividing cells had already divided into two daughter nuclei as the result of micronuclear mitosis. This means that the micronucleus had passed through a pre-mitotic S phase during cultivation, because the micronucleus in cells before transferring to culture medium was in G1 phase, as shown in Table 1. Therefore, if [3H]dThd was taken into the cell during 1 h of incubation with the labelled DNA precursor, numerous grains would be expected to appear on the micronucleus as the result of utilization of the precursor in pre-mitotic DNA synthesis, because, as mentioned above, [3H]dThd has been reported to be used in pre-mitotic DNA synthesis in *P. caudatum* (Rao & Prescott, 1967). For pre-meiotic DNA synthesis, the remaining cells of the complementary mating types were mixed together, kept at 25 °C for 4 h and then fixed and processed for autoradiography. During this time, the cells formed conjugating pairs and their micronuclei proceeded to meiotic prophase, stage IV. Therefore, if the cells could utilize [3H]dThd in pre-meiotic DNA synthesis, numerous grains should appear on the stage IV micronucleus.

The results are shown in Fig. 3 and Table 3. It is evident that [3H]dThd was taken into the cells and utilized in pre-mitotic DNA synthesis of the micro- and the macronucleus (Fig. 3A). However, no significant incorporation of the precursor was observed in most cells in meiotic prophase, stage IV (Fig. 3B). In Table 3, this phenomenon is very clearly shown by the data of the grain counts. In this table, the

![Fig. 3](image)

**Fig. 3.** Autoradiographs with [3H]thymidine. A. Micro- (*mi*) and macronucleus (*ma*) in a dividing cell. Note that numerous grains appear on both nuclei. B. Micro- (*mi*) and macronucleus (*ma*) in conjugating cells. The micronucleus is in meiotic prophase, stage IV. Note that the number of grains in these nuclei is not significantly different from those in the cytoplasm. ×2300.
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Table 3. $[^3]H$thymidine incorporation during pre-mitotic and pre-meiotic DNA syntheses in micronuclei*

<table>
<thead>
<tr>
<th>DNA synthesis in micronuclei</th>
<th>Mean no. of grains/area of micronucleus†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Micronucleus</td>
</tr>
<tr>
<td>Pre-mitotic DNA synthesis</td>
<td>34.74 ± 5.10</td>
</tr>
<tr>
<td>Pre-meiotic DNA synthesis</td>
<td>14.64 ± 3.50</td>
</tr>
</tbody>
</table>

* The slides for autoradiography were exposed for 15 days in the dark at 4°C. † The number of grains/area of micronucleus ±95% confidence limit. ‡ The number of background grains in an adequate area of cytoplasm, surrounding the micronucleus, was converted into the number of grains in an area of the same size as that of micronucleus in each cell, and then the mean value of the background grains was calculated.

value for pre-mitotic DNA synthesis represents the mean number of grains per two daughter micronuclei, to be comparable with those of pre-meiotic DNA synthesis. These data indicate that $[^3]H$dThd was taken into the cells and utilized in pre-mitotic DNA synthesis, but was utilized very little in pre-meiotic DNA synthesis.

Autoradiography with $[^3]H$deoxyuridinemonophosphate

It is well known that thymidine is converted to deoxythymidinemonophosphate (dTMP) in many organisms by phosphorylation with thymidine kinase, and that dTMP is formed also by methylation of deoxyuridinemonophosphate (dUMP) with thymidylate synthetase (Kit, 1970). These pathways for deoxythymidylic acid formation are called the salvage and de novo pathways, respectively. In order to examine whether the cells in pre-meiotic S phase use the de novo pathway for obtaining dTMP, $[^3]H$dUMP was used for the DNA precursor instead of $[^3]H$dThd.

Cells grown for 11 h in culture medium after 1 h of incubation with $[^3]H$dUMP showed numerous grains on the micro- and macronucleus (Fig. 4A). This result shows that $[^3]H$dUMP is taken into the cells during 1 h of incubation with the labelled precursor, and that the cells in the vegetative cycle use the de novo pathway as well as the salvage pathway for obtaining dTMP in DNA synthesis.

On the other hand, cells that were allowed to enter into the conjugation process were fixed every 1 h for 6 h after mixing complementary mating types and testing for the incorporation of $[^3]H$dUMP by autoradiography. The results are shown in Fig. 4B–E and in Table 4. In stage I, no significant difference was observed between the mean number of grains on the micronucleus and that on the cytoplasm. In stage II (pre-meiotic S phase), however, the number of grains on the micronucleus increased significantly. As a result of the incorporation of the labelled precursor in stage II, many grains were also observed on the micronuclei in stage III–V, but there was no significant difference between the mean number of grains on stage III–IV micronuclei and the mean number on the stage V micronucleus. These results are well in accordance with the results of microspectrophotometry presented in Table 2.
Fig. 4. Autoradiographs with $[^3]$Hdeoxyuridinemonophosphate. A. Micro- (mi) and macronucleus (ma) in a dividing cell. Note that numerous grains appear in both nuclei. B–E correspond to stage I, II, IV and V micronuclei, respectively, in conjugating cells. Note that there are no grains in stage I micronucleus, while many grains appear in stage II–V micronuclei. X2300.

Table 4. $[^3]$Hdeoxyuridinemonophosphate incorporation into the micronuclei of cells early in the conjugation process

<table>
<thead>
<tr>
<th>Stages of the micronucleus†</th>
<th>N</th>
<th>Mean no. of grains/area of micronucleus‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Micronucleus</td>
</tr>
<tr>
<td>Stage I</td>
<td>12</td>
<td>0.50 ± 0.42</td>
</tr>
<tr>
<td>Stage II</td>
<td>27</td>
<td>6.37 ± 1.76</td>
</tr>
<tr>
<td>Stage III, IV</td>
<td>41</td>
<td>16.20 ± 1.91</td>
</tr>
<tr>
<td>Stage V</td>
<td>20</td>
<td>19.80 ± 3.05</td>
</tr>
</tbody>
</table>

* The slides for autoradiography were exposed for 34 days in the dark at 4°C.
† Stages I–V correspond to those in Fig. 1.
‡ Mean no. of grains/area of micronucleus ±95% confidence limit.
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Unlike the former experiment with \[^3H\]dThd, \[^3H\]dUMP was well utilized in both pre-meiotic and pre-mitotic DNA synthesis. The results show that in pre-meiotic DNA syntheses, dTMP is mainly formed through the de novo pathway, suggesting that the activity of thymidine kinase is low in cells early in the conjugation process. It should be noted that although the incorporation of the labelled precursor was observed in both micro- and macronuclei in vegetative cells (Fig. 4A), it was observed only in micronuclei in cells early in the conjugation process (Fig. 4c).

Extraction with DNase

In order to examine the specificity of the incorporation of tracer into DNA, several slides were digested with DNase (see Materials and Methods). By this treatment, a majority of the grains on the nuclei, such as those shown in Figs 3A, 4A and 4c–e, were removed; whereas the grains in the cytoplasm were largely resistant to the treatment, suggesting that the majority of the grains on the nuclei were due to incorporation of tracer into DNA, but that those in the cytoplasm might be due to non-specific incorporation.

DISCUSSION

The results presented here demonstrate that the micronucleus of stage I is in G1 and that of stage II is in pre-meiotic S phase, and that G2 phase is very short or lacking in Paramecium. Therefore the transition point from interphase to meiotic prophase in Paramecium is at or very close to the end of the pre-meiotic DNA synthesis. Although the micronuclei in stages III–V were assumed, morphologically, to be in meiotic prophase, detailed correspondence of these stages to those of the classical stages in meiotic prophase, i.e. leptotene, zygotene, pachytene, diplotene and diakinesis, are not clear, because the chromosomes are very thin, long and closely packed together even in metaphases I and II. Furthermore, the number of chromosomes is so numerous that the identification of each chromosomes and observation of homologous chromosome pairing are very difficult in Paramecium. In ciliates, a typical synaptonemal complex (SC) has been observed only in Blepharisma americanum (Jenkins, 1973), though the exact stage at which the SC was formed is not clear. In P. primaurelia (formerly syngen 1 of P. aurelia), Stevenson (1972) has found ‘ill-defined, synaptonemal, complex-like material’ in the stage prior to crescent formation. This structure resembles the SC found in Chlamydomonas reinhardtii (Storms & Hastings, 1977). In Tetrahymena thermophila (formerly syngen 1 of T. pyriformis), Wolfe, Hunter & Adair (1976) suggested that the micronuclear elongation should not be identified as meiotic prophase and that meiotic prophase occurs after the elongation stage. Although a typical SC has not been observed in those ciliates, the stages of nuclear elongation early in the conjugation process can be assessed to be meiotic prophase, because in the fully elongated and curved stage of T. thermophila, light microscopic observation has confirmed that pairing of homologous chromosomes has already been accomplished (Ray, 1956; Sugai & Hiwatashi, 1974), suggesting that the pairing has started at a much earlier stage. The facts that pre-meiotic DNA synthesis...
is completed during stage II and chromatin condensation starts at stage III micronucleus, strongly support the idea that the stage III micronucleus is the beginning of meiotic prophase in *P. caudatum*.

Previously, Rao & Prescott (1967) have reported the duration of each stage (*G*₁, *S*, *G*₂ and *M*) in the mitotic cell cycle of *P. caudatum*. By pulse-labelled autoradiography with [³H]dThd at 27 °C, they confirmed that one cell cycle time was about 8 h and the pre-mitotic *S* phase in the micronucleus was 3–3.5 h. In the present study, in which the temperature was 25 °C, one cell cycle time in log phase culture was also about 8 h. Since stage II is the pre-meiotic *S* phase and lasts for about 1 h at 25 °C, both the results of Rao & Prescott (1967) and the present work agree, in that the duration of pre-meiotic *S* phase is much shorter than that of the pre-mitotic *S* phase. This tendency is also the case in *P. tetraurelia* (formerly syngen 4 of *P. aurelia*), in which the duration of the pre-mitotic *S* phase was 40 min (Woodard, Gelber & Swift, 1961) and that of the pre-meiotic one was 15–25 min (Woodard *et al.* 1966). The fact that the pre-meiotic *S* phase is shorter than the pre-mitotic one in *Paramecium* is contradictory to the general concept that the duration of pre-meiotic *S* phase is considerably longer than that of the pre-mitotic one, as was shown in yeast (compare Williamson & Scopes, 1961 & Simchen, Salts & Piñon, 1973), mouse (Monesi, 1962; Lima-de-Faria & Borum, 1962; Kofman-Alfaro & Chandley, 1970), wheat (Bennett, Chapman & Riley, 1971), *Triturus* (Callan, 1972) and *Lilium* (unpublished data, cited by Stern & Hotta, 1974). Furthermore, various aspects of differences in the pre-meiotic *S* phase have been found in *Triturus vulgaris* (Callan, 1972), *Lilium* (Hotta & Stern, 1971; Roth & Ito, 1967), yeast *Saccharomyces cerevisiae* (Simchen, Piñon & Salts, 1972) and *Schizosaccharomyces pombe* (Egel & Egel-Mitani, 1974) and the Basidiomycete, *Coprinus lagopus* (Lu & Jeng, 1975). The prolonged duration of pre-meiotic *S* phase in those organisms may be connected with a physiologically special state that is necessary for readiness to undergo meiosis. However, the evidence in *Paramecium* shows that such prolonged duration in pre-meiotic *S* phase is not a common feature of meiotic cells.

The ciliates have two functionally different nuclei in a cell, the germinal micronucleus and the somatic macronucleus. The former is usually diploid, but the latter is usually highly polyploid. In *P. caudatum*, the macronuclear/micronuclear DNA ratio is about 50:1 when both nuclei are in *G*₁ phase (Fujishima, unpublished data). On the other hand, in *P. tetraurelia*, the ratio is about 860:1 for the majority of cells (Woodard *et al.* 1966). In these ciliates, the pre-mitotic *S* phase of the micronucleus begins almost simultaneously with macronuclear DNA synthesis, and ends before the macronuclear synthesis is completed (Rao & Prescott, 1967; Woodard *et al.* 1961), whereas in pre-meiotic *S* phase, DNA synthesis occurs exclusively in the micronucleus, as was shown by autoradiography in the present work (Fig. 4c, d). Therefore, in pre-mitotic *S* phase the micronucleus has to compete with the macronucleus for a DNA precursor within the cell, but this does not occur in pre-meiotic *S* phase. This may be a cause of the shortened duration of pre-meiotic *S* phase in *Paramecium*, although other possibilities cannot be ruled out.

Autoradiographs of vegetative cells showed a relatively large number of grains in
the cytoplasm compared with those of conjugating cells. These grains in the cytoplasm were very resistant to treatment with DNase, RNase and 5 % trichloroacetic acid, whereas the grains on the nuclei could be removed by treatment with DNase. Such non-specific grains in the cytoplasm have been reported by Kimball & Perdue (1962) in *P. tetraurelia*, when $[^3]H$Thd was introduced directly from the medium. Afterwards, it was found that the grains in the cytoplasm could be reduced if the $[^3]H$Thd was introduced into the cells through ingested bacteria into which the labelled precursor had been previously incorporated (Berger & Kimball, 1964). In the experiment with conjugating pairs, however, this method cannot be used, because feeding of bacteria makes cells unable to form conjugating pairs and thus prevents the cells from entering the meiotic cycle.

Autoradiographic study showed that deoxythymidylic acid is formed by both the *de novo* and salvage pathways in pre-mitotic DNA synthesis, but mainly by the *de novo* pathway in pre-meiotic DNA synthesis. Namely, in pre-mitotic DNA synthesis $[^3]H$dThd and $[^3]H$dUMP were well utilized, but in pre-meiotic DNA synthesis $[^3]H$Thd was utilized very little even if the precursor had been taken into the cell. This result indicates that the activity of thymidine kinase is low in cells early in the conjugation process. It is not clear when the activity of thymidine kinase becomes low. It may be at the time when the growth of the cells reaches the stationary phase, or when the cells enter the conjugation process.

In *P. caudatum*, the micronucleus enters into meiotic prophase immediately after the pre-meiotic S phase. Therefore, up to the end of the pre-meiotic S phase, cells have to be in a state ready to enter meiotic prophase. Although it is still not clear whether pre-meiotic and pre-mitotic S nuclei are in different states for meiosis, this question could be answered by using the nuclear transplantation technique. As recently reported (Fujishima & Hiwatashi, 1981), pre-meiotic S micronuclei transplanted into cells in early meiotic prophase enter meiotic prophase. If pre-mitotic S nuclei were used as donor nuclei and it was found that they cannot enter meiotic prophase in the recipient cells, it would make clear the difference between pre-meiotic S nuclei and pre-mitotic S nuclei, and could prove that pre-meiotic DNA synthesis is indeed a necessary step leading to meiosis.

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