Macromolecules released into the culture medium during the vegetative cell cycle of the unicellular green alga *Chlamydomonas reinhardtii*

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The culture medium of growing *Chlamydomonas reinhardtii* cells contains hydroxyproline-rich glycoproteins, which are mainly liberated during release of the zoospores from the mother-cell wall. Pulse-labelling studies with [^3H]proline and [^{35}S]methionine have been performed in order to detect the protein components released by synchronously growing cells at different stages of the cell cycle. When either [^3H]proline or [^{35}S]methionine were applied during the phase of cell growth, radioactive label appeared in the released macromolecules after a lag period of 40 min, whereas incorporation into the insoluble part of the cell wall was delayed only by 20 min. When applied at the end of the growth phase, e.g. 13 h after beginning of the illumination period, the radioactive amino acids were incorporated into the cell wall, but radioactive labelling of macromolecules released into the culture medium could not be detected before the zoospores were liberated from the mother-cell wall. Maximal incorporation of [^3H]proline and [^{35}S]methionine into the insoluble part of the cell wall was observed during cell division, but essentially no radioactively-labelled macromolecules were released into the culture medium during this time period. Analysis of the macromolecules, which were liberated during cell enlargement, by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed distinct radioactive bands, which were differentially labelled with [^3H]proline and [^{35}S]methionine. Among the macromolecules released into the culture medium during cell growth, a component of an apparent M_r 35 000 was preferentially labelled with [^3H]proline. This component was also detected after labelling with [^{35}S]methionine, but components of an apparently higher M_r were more prominent after labelling with [^{35}S]methionine. Macromolecules released during the cell-enlargement period of synchronously growing cultures in the presence of [^3H]proline contained radioactively-labelled hydroxyproline in addition to proline. These results show that, during cell-wall growth, specific protein components are released into the culture medium and that at least one of these components contains large amounts of proline and hydroxyproline. At least some of these macromolecules seem to be constituents of the cell wall, because during pulse-chase experiments radioactively-labelled macromolecules appeared in the culture medium mainly during the time period when the specific radioactivity of the insoluble inner-cell-wall layer decreased.

In growing cultures of *Chlamydomonas reinhardtii*, large amounts of glycoproteins are accumulated in the medium. Most of these macromolecules are hydroxyproline-rich glycoproteins that are derived from the cell wall (Lang & Chrispeels, 1976). When cultures are synchronized by light/dark cycling, these glycoproteins are primarily

liberated in the last quarter of the cell cycle, after cell separation has occurred, but release of macromolecules into the culture medium by synchronously growing wild-type cells was also observed during the cell-enlargement period (Lang & Chrispeels, 1976). It is not yet known whether or not these macromolecules liberated during cell enlargement of wild-type cells are related to intrinsic cell-wall constituents. Other protein components

Abbreviation used: SDS, sodium dodecyl sulphate.

are also released into the culture medium in a cell-cycle-dependent manner. At the time when the daughter cells are liberated from the sporangia, lytic factors are released into the culture medium, which degrade the mother-cell wall but not the cell wall of the zoospores (Schlösser, 1966; Mihara & Hase, 1975; Schlösser, 1976; Mergenhagen, 1980). These lytic factors are able to release zoospores from sporangia that have not yet reached the stage to produce their own lytic factors (Schlösser, 1966, 1976; Mihara & Hase, 1975). Furthermore, phosphatases are released into the culture medium by *Chlamydomonas reinhardtii* cells when their cell wall is affected (Loppes, 1976). This finding is not surprising, since at least one phosphatase is located in the cell wall and in the space between the wall and the plasmalemma (Matagne *et al.*, 1976).

Since all the macromolecules present in the culture medium that have been studied so far are released at the time when the zoospores are liberated, I focused my attention to the question of whether or not specific protein or glycoprotein components are also released during the cell-enlargement period. To this end I studied the release of macromolecules into the culture medium by synchronously growing *Chlamydomonas reinhardtii* cells at different stages of the cell cycle by pulse-labelling with [³H]proline and [³⁵S]methionine. Since the cell-wall glycoproteins contain large amounts of hydroxyproline and proline, but low amounts of methionine (Roberts, 1974, 1979; Hills *et al.*, 1975; Catt *et al.*, 1976), a comparison of the labelling patterns obtained should be an indication whether or not a released macromolecule could be related to an intrinsic cell-wall constituent.

Materials and methods

L-[2,3,4,5-³H]Proline (sp. radioactivity 115 Ci/mmol) and L-[³⁵S]methionine (sp. radioactivity 800–1100 Ci/mmol) were purchased from NEN Chemicals (Dreieich, Germany). All other chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany).

Strains

The wild-type of *Chlamydomonas reinhardtii* used in the present experiments was strain 137C (mating type +) isolated by Dr. R. P. Levine (Harvard University, Cambridge, MA, U.S.A.). The mutant strain 'ls' (mating type –) was isolated by Mergenhagen (1980).

Media and growth conditions

Stock cultures were maintained on yeast/acetate agar slants at 15°C and illuminated (4000 lx) using

a 12h-light/12h-dark cycle. Cells of both strains were grown autotrophically on high-salt medium (Sueoka *et al.*, 1967) at 25°C. The cultures were constantly bubbled with filtered air and constantly agitated by means of magnetic stirring bars. For asynchronous growth the cells were continuously illuminated from the sides using a combination of white lamps (Osram type 36 W/25) and 'daylight' fluorescent lamps (Osram type 36 W/11). The light intensity was 10000 lx at the level of the cultures. Synchronous growth was achieved by the method of Surzycki (1971), a 12h-light/12h-dark illumination cycle being used. Cell concentrations were determined by duplicate haemocytometer counting.

Incorporation of radioactive precursors

Studies on the incorporation of radioactive amino acids into cell-derived proteins and proteins in the medium were performed under continuous light to avoid experimental artefacts due to changes in the uptake of the radioactive precursors (Howell *et al.*, 1977). Synchronized cultures continued to divide synchronously when exposed to continuous illumination for an additional 24 h, i.e. one entire cell-cycle period (Bernstein, 1964; Mihara & Hase, 1971).

Separation of cells and culture medium

The culture was rapidly cooled to 0°C and centrifuged at 10000 g for 10 min. The culture medium was decanted immediately after centrifugation.

Cell fractionation

To obtain the soluble components, the cells were extracted with a buffer consisting of 8 M-urea, 2% (w/v) SDS, 10 mM-EDTA, 200 mM-2-mercaptoethanol and 20 mM-Tris/HCl, pH 7.5, for 16 h at room temperature. The suspension was centrifuged for 30 min at 10000 g and the supernatant stored at –20°C. The pellet containing the crude inner-cell-wall layer was re-extracted several times with the same buffer. The orange-coloured inner-cell-wall-layer preparation was further purified by extraction with a buffer consisting of 2 M-NaCl, 3% (w/v) sodium deoxycholate, 3% (v/v) Triton X-100, 2 mM-EDTA and 20 mM-Tris/HCl, pH 7.5, until the resulting pellet was completely colourless. Finally the cell-wall preparation was washed several times with twice-distilled water.

Determination of radioactivity

Incorporation of radioactive precursors into cellular and medium macromolecules was determined by adding trichloroacetic acid [final concn. 10% (w/v)] to aliquots and collecting the denatured components by centrifugation. The pellets

were washed twice with 1 ml of a solution of 1 mM non-radioactive precursor, followed by washing with 1 ml of twice-distilled water and redissolved overnight in 0.1 ml of 8M-urea/2% SDS/1 mM-EDTA/200 mM-2-mercaptoethanol/20 mM-Tris/HCl, pH 7.5. The solution was mixed with 5 ml of Aqualuma (Baker Chemicals) and radioactivity was measured in a liquid-scintillation counter (Tri-Carb 300, Packard). Incorporation of the label into the inner-cell-wall layer was measured after dissolving the insoluble material in Lumasolve (Baker Chemicals). A 0.2 ml portion of cell-wall suspension was incubated with 1 ml of Lumasolve overnight at room temperature. After addition of 10 ml of Lipoluma (Baker Chemicals), the radioactivity in the sample was measured in a liquid-scintillation counter.

SDS/polyacrylamide-gel electrophoresis

Cell-derived macromolecules and those in the medium were precipitated by addition of trichloroacetic acid (final concn. 10%, w/v) in the cold and the precipitates collected by centrifugation. The pellets were washed with 1 ml of twice-distilled water and redissolved in 60 μ l of a buffer consisting of 8M-urea, 2% SDS, 200 mM-2-mercaptoethanol and 20 mM-Tris/HCl, pH 7.5. After addition of 20 μ l of sample buffer (Laemmli, 1970) containing Bromophenol Blue as tracking dye, the samples were analysed by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970), 15%-(w/v)-polyacrylamide gel slabs being used. Fluorography of the gel slabs was performed as described by Laskey & Mills (1975).

Determination of hydroxyproline

Culture medium obtained from synchronously growing cultures of *Chlamydomonas reinhardtii* that were labelled with [3 H]proline was adjusted to 10% (w/v) trichloroacetic acid. The precipitated macromolecules were collected by centrifugation and hydrolysed with 6M-HCl containing 10 mM-2-mercaptoethanol at 120°C for 20 h. After removal of HCl, the residue was submitted to paper chromatography on Whatman 3MM paper (solvent: butan-1-ol/acetic acid/water, 15:3:5, by vol.). The radioactive material that co-migrated with L-proline and L-hydroxyproline was determined and the relative amount of hydroxyproline calculated.

Results

Time course of radioactive labelling with [3 H]proline and [35 S]methionine of cellular proteins, the insoluble inner-cell-wall layer and of the macromolecules released into the culture medium

The time courses of the incorporation of [3 H]proline and [35 S]methionine into cellular pro-

teins, the insoluble inner-cell-wall layer and the macromolecules released into the culture medium were studied in synchronously growing cultures of *Chlamydomonas reinhardtii*. When the radioactive precursor was applied 8 h after the beginning of the light period, i.e. during the cell-enlargement period, accumulation of radioactively-labelled macromolecules in the culture medium was observed after a delay of more than 40 min (Fig. 1c), whereas incorporation into cellular proteins proceeded without an appreciable lag period (Fig. 1a). The incorporation into the insoluble inner layer of the cell wall was found to be delayed by about 20 min in the experiments with [3 H]proline as well as in those with [35 S]methionine (Fig. 1b). Similar

Fig. 1. Pulse-labelling with [3 H]proline (—) or [35 S]methionine (----) of cellular macromolecules (a), those in the medium (c) and those in the insoluble inner cell-wall layer (b) of *Chlamydomonas reinhardtii* during the phase of cell enlargement

[3 H]Proline (1 mCi) or [35 S]methionine (250 μ Ci) was added to a 1000-ml culture of synchronized cells (cell density 1×10^6 cells/ml) 8 h after beginning of the light period. Samples (100 ml) were taken at indicated times. After 2 h the labelled cells were collected by centrifugation, washed and resuspended in fresh medium. Cell fractionation was performed as described in the Materials and methods section. The arrow indicates the time of cell division.

results were obtained with asynchronously growing cultures (results not shown). When the radioactive precursors were applied 13 h after beginning of the light period, incorporation into cytoplasmic proteins again proceeded without delay (Fig. 2a), whereas labelling of macromolecules released into the culture medium did not occur before the liberation of the zoospores from the mother-cell walls (Fig. 2c). Slight labelling of the insoluble inner layer of the cell wall was observed after a lag period of about 30 min (Fig. 2b). Under these conditions, incorporation of the radioactive precursors into the cell wall was observed mainly at the time of cell division.

Macromolecules released into the culture medium during cell enlargement contain hydroxyproline and are differentially labelled by [^3H]proline and [^{35}S]methionine

To ascertain that the trichloroacetic acid-insoluble radioactivity accumulated in the growth me-

dium during pulse-labelling is not caused by incomplete sedimentation of the cells, the labelled macromolecules of the medium and cellular proteins were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. As shown in Fig. 3, striking differences exist between the labelling patterns of cellular proteins and those of the medium. However, in the labelling experiments with [^3H]proline the most prominent band within the cellular macromolecules as well as those of the medium was a component of an apparent M_r 35000 (Fig. 3, slots a and b). This component was also detected in both fractions after labelling with [^{35}S]methionine (Fig. 3, slots c and d), but incorporation of [^{35}S]methionine into this component was much less striking than incorporation of [^3H]proline. The most prominent radioactive bands observed within the macromolecules of the medium after labelling with [^{35}S]methionine (Fig. 3d) were found to be components with apparent M_r values of 44000 and 49000. These bands were much less prominent after labelling with [^3H]proline (Fig. 3b). Other components of the macromolecules of the medium and the cellular proteins were

Fig. 2. Pulse-labelling with [^3H]proline (—) and [^{35}S]methionine (---) of cellular proteins (a), macromolecules released into the culture medium (b) and the insoluble inner-cell-wall layer (c) at the end of the cell-enlargement period (13 h after beginning of the light period)

For details, see Fig. 1. The first (solid) arrow indicates time of cell division; the second (open) arrow indicates release of zoospores from the mother-cell walls.

Fig. 3. Labelling with [^3H]proline or [^{35}S]methionine of the cellular proteins and macromolecules released into the culture medium

Cellular proteins (a, c) and released macromolecules (b, d) were isolated from synchronously growing cultures of *Chlamydomonas reinhardtii* labelled for 2 h with [^3H]proline (a, b) or [^{35}S]methionine (c, d) during the cell-enlargement period. Samples (500000 c.p.m.) were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

also found to be differentially labelled by [^3H]proline and [^{35}S]methionine.

In algae and higher plants, polypeptide chains with high proportions of proline are often modified after translation by hydroxylation of proline residues and subsequent glycosylation of the resulting hydroxyproline residues (Fincher *et al.*, 1983). In the case of *Chlamydomonas reinhardtii*, hydroxyproline-rich glycoproteins have been found in the cell wall and on the flagellar membrane of gametes (Roberts *et al.*, 1972; Roberts, 1974; Hills *et al.*, 1975; Catt *et al.*, 1976; Cooper *et al.*, 1983). We therefore studied the relative amounts of labelled proline and hydroxyproline present in the macromolecules that are released into the culture medium during the cell-enlargement period. Synchronously growing cultures of *Chlamydomonas reinhardtii* were labelled with [^3H]proline during the cell-enlargement period and the radioactively-labelled macromolecules were isolated from the culture medium and analysed for the presence of radioactive proline and hydroxyproline after hydrolysis with 6M-HCl. Chromatography of the hydrolysed samples on Whatman 3MM paper (solvent: butan-1-ol/acetic acid/water, 15:3:5, by vol.), and measurement of the radioactivity profiles revealed several peaks. The two main peaks, however, co-migrated with proline and hydroxyproline, respectively, run on the same paper strips (results not shown). The radioactive material co-migrating with hydroxyproline corresponded to 40% and the material co-migrating with proline corresponded to 30% of total radioactivity.

Time course of the labelling by [^3H]proline and [^{35}S]methionine of individual macromolecules released into the culture medium during the cell-enlargement period

When [^3H]proline was added to synchronously growing cultures of *Chlamydomonas reinhardtii* during the cell-enlargement period, the first radioactively labelled macromolecules could be detected in the culture medium after about 1 h. Analysis of the individual labelled macromolecules by SDS/polyacrylamide-gel electrophoresis and subsequent fluorography revealed that radioactive components with apparent M_r values of 35000, 44000, 49000, 57000 and more than 100000, as well as very small components, were present in the culture medium 1 h after the addition of [^3H]proline (Fig. 4d). None of these bands was predominant at this time period. After a further 1 h (Fig. 4e), the 35000- M_r component as well as material with apparent M_r values of more than 100000 and material running in front of the gel became predominant. Additional bands were observed, with apparent M_r values of 41000 and 66000, and

Fig. 4. Analysis by SDS/polyacrylamide-gel electrophoresis of liberated macromolecules labelled with [^3H]proline during cell enlargement

[^3H]Proline (1 mCi) was added to a 1000-ml culture of synchronized cells (cell density 1×10^6 cells/ml) 8 h after beginning of the light period. Samples (100 ml) were taken immediately (a), 20 min (b), 40 min (c), 60 min (d) and 2 h (e) after addition of [^3H]proline. At 2 h after addition of the radioactive label, the remaining cells of the labelled culture were collected by centrifugation, washed and resuspended in the same volume of fresh medium and further incubated in the light. Samples (100 ml) were taken 3 h after zero time (= 1 h after removal of the radioactive precursor; slot f) and 4 h (g), 6 h (h), 8 h (i) and 10 h (j) after zero time. Labelled cells were removed from the 100 ml samples by centrifugation and the macromolecules present in the culture medium precipitated by addition of trichloroacetic acid (final concn. 10%) and 30 μg of lysozyme as carrier. The precipitates were collected by centrifugation, washed with water and redissolved as described in the Materials and methods section. Labelled macromolecules released per 10^8 cells were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

minor components, with M_r values below 35000. After removal of the radioactive precursor by centrifugation, washing of the cells and subsequent addition of fresh culture medium, the 35000- M_r component and the material with M_r values of more than 100000 or less than 15000 respectively were accumulated in the culture medium (Figs. 4f–4k). In addition, the molecules with apparent M_r values of 41000, 44000, 48000, 55000 and 66000 could be found as minor components, in addition to material running faster than the 35000- M_r component.

In the labelling experiments performed with [^{35}S]methionine (Fig. 5), the radioactive components with apparent M_r values of 35000, 44000, 48000, 55000, 66000 and >100000 were accumu-

Fig. 5. Analysis by SDS/polyacrylamide-gel electrophoresis of liberated macromolecules labelled with [^{35}S]methionine during cell enlargement

A portion of [^{35}S]methionine (250 μCi) was added to a 1000-ml culture of synchronized cells (cell density 1×10^6 cells/ml) 8 h after beginning of the light period (= zero time). Samples (100 ml) were taken immediately after addition of the radioactive label (a) and 20 min (b), 40 min (c), 60 min (d) and 120 min (e) after zero time. At 2 h after addition of [^{35}S]methionine the labelled cells of the remaining culture were collected by centrifugation, washed and resuspended in the same volume of fresh medium. Samples (100 ml) were then taken 3 h after zero time (f = 1 h after removal of [^{35}S]methionine) and 4 h (g), 6 h (h), 8 h (i) and 10 h (j) after zero time. The macromolecules released into the culture medium were isolated and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography as described in the legend of Fig. 4.

lated in the culture medium with about the same kinetics as in the experiments with [^3H]proline. The relative intensities, however, were completely different. The component with apparent M_r 35000, predominantly labelled in the [^3H]proline series, was only a minor component in the experiments with [^{35}S]methionine. On the other hand, the radioactive bands with apparent M_r values of 44000 and 48000 were more intense after labelling with [^{35}S]methionine than in the experiments with [^3H]proline. Furthermore, the amount of radioactive material running in front of the gel was higher after labelling with [^{35}S]methionine than after labelling with [^3H]proline. Very similar results were obtained with asynchronously grown cultures (results not shown).

When labelling with [^3H]proline or [^{35}S]methionine was performed at the end of the cell-enlargement period, radioactive macromolecules could not be detected in the culture medium before release of zoospores from the mother-cell wall (Fig. 6). The patterns of radioactively labelled macro-

Fig. 6. Analysis by SDS/polyacrylamide-gel electrophoresis of radioactive macromolecules released into the culture medium by synchronously growing *Chlamydomonas reinhardtii* cells, which were labelled with [^3H]proline (a-c) or [^{35}S]methionine at the end of the cell-enlargement period

A portion of [^3H]proline (1 mCi) or [^{35}S]methionine (250 μCi) was added to a 1000-ml culture of synchronized cells (cell density 1×10^6 cells/ml) 13 h after beginning of the light period. After 2 h the labelled cells were collected by centrifugation, washed and resuspended in fresh medium (1000 ml). The radioactive supernatants were analysed for labelled macromolecules (a, d). Samples (400 ml) were taken from the cultures 4 and 8 h after resuspension in fresh medium and analysed for radioactive macromolecules released into the culture medium. At 4 h after resuspension in fresh medium (b, e), the cultures contained more than 90% sporangia; 8 h after resuspension in fresh medium (= 21 h after beginning of the light period; c, f), the zoospores were found to be released from the mother-cell walls. Radioactively-labelled macromolecules released into the culture medium by 4×10^8 cells were submitted to analysis by SDS/polyacrylamide-gel electrophoresis. For details, see the legend to Fig. 4.

molecules was completely different from that observed during the phase of cell enlargement (Figs. 4 and 5). The distinct radioactive bands observed in the culture medium during cell enlargement were missing and the differences between the labelling patterns obtained after labelling with [^3H]proline and [^{35}S]methionine during the period of cell growth could not be observed under these conditions.

Pulse-chase experiments

The observations that the macromolecules released into the culture medium during cell enlargement contained hydroxyproline and became radioactively labelled after a longer lag phase than the insoluble inner-cell-wall layer indicate that at least part of these components could be constitu-

ents of the cell wall. Pulse-chase experiments have been performed in order to examine this possibility. As shown in Fig. 7(a), the specific radioactivity of the cellular proteins that could be extracted with urea/SDS buffer from cells labelled for 1 h with either [^3H]proline or [^{35}S]methionine decreased without appreciable lag phase after addition of unlabelled proline and methionine respectively. The specific radioactivity of the insoluble cell-wall layer was found to be doubled during the first 1 h of the chase (Fig. 7b). After this time period, a decrease of radioactive label could be observed indicating that a turnover of the insoluble inner cell-wall layer takes place during cell enlargement. Release of labelled macromolecules into the culture medium was observed mainly

during the time period when the specific radioactivity of the insoluble cell-wall layer was found to decrease (Fig. 7c).

*Incorporation of [^3H]proline and [^{35}S]methionine into cellular proteins, macromolecules released into the culture medium and into the insoluble cell-wall layer during the vegetative cell cycle of *Chlamydomonas reinhardtii**

The incorporation of [^3H]proline and [^{35}S]methionine into cellular macromolecules and those in the medium and into the insoluble inner layer of the cell wall of *Chlamydomonas reinhardtii* was studied during the vegetative cell cycle (Fig. 8). Maximal labelling of the insoluble inner cell-wall layer was observed at about the time of cell division (Fig. 8c). Incorporation of both radioactive amino acids could be observed also during the

Fig. 7. Disappearance of radioactive label from soluble macromolecules (a) and the insoluble inner cell-wall layer (b) of *Chlamydomonas reinhardtii* cells pulse-labelled for 1 h with [^3H]proline (—) or [^{35}S]methionine (----) after addition of unlabelled amino acids and accumulation of radioactively labelled macromolecules in the culture medium (c).

A portion of [^3H]proline (1 mCi) or [^{35}S]methionine (250 μCi) was added to a 1000-ml culture of synchronized cells (cell density 1×10^6 cells/ml) 8 h after beginning of the light period. After 1 h the cells were collected by centrifugation, washed and resuspended in fresh culture medium containing 10 mM unlabelled proline and 10 mM unlabelled L-methionine respectively. Cell fractionation was performed as described in the Materials and methods section.

Fig. 8. Incorporation of [^3H]proline and [^{35}S]methionine during the vegetative cell cycle of *Chlamydomonas reinhardtii*.

Portions (40 ml) of a 1-litre culture synchronized by 12 h-light/12 h-dark were pulse-labelled for 90 min in the light with 100 μCi of [^3H]proline or 25 μCi of [^{35}S]methionine. Cells were withdrawn every 2 h, incubated, and analysed as described in the Materials and methods section. (a) Cell density; (b) incorporation into cellular protein; (c) incorporation into the insoluble inner layer of the cell wall; (d) incorporation into macromolecules released into the culture medium.

phase of cell growth, but not during the time period preceding the cell division. Release of labelled macromolecules could be observed during cell enlargement and during release of zoospores from the mother cell walls, but not during the time period between the end of the cell-enlargement period and sporulation (Fig. 8*d*). The radioactively labelled macromolecules released into the culture medium during liberation of the zoospores cannot be fragments of the mother-cell wall, because it is very unlikely that radioactive precursors are incorporated into the mother-cell wall after cytokinesis. The radioactively labelled macromolecules released during liberation of zoospores should be the 'lytic factors' described by Schlösser (1966, 1976) and Mihara & Hase (1975), and other components released by secretion or exocytosis. Incorporation of [35 S]methionine into cellular proteins was almost constant during the cell cycle, whereas incorporation of [3 H]proline peaked at the end of the cell-enlargement period and increased again at about the time when zoospores were liberated from the mother-cell walls (Fig. 8*b*).

When the liberated proteins that were pulse-

Fig. 9. Analysis by SDS/polyacrylamide-gel electrophoresis and fluorography of radioactive macromolecules that are released into the culture medium by synchronously growing *Chlamydomonas reinhardtii* cells pulse-labelled with [3 H]proline at different stages of the cell cycle

Portions (40 ml) of a 1-litre culture synchronized by a 12 h-light/12 h-dark illumination cycle were pulse-labelled for 90 min in the light with 100 μ Ci of [3 H]proline. The radioactively-labelled macromolecules present in the culture medium of the samples were precipitated by addition of trichloroacetic acid and quantitatively submitted to analysis by SDS/polyacrylamide-gel electrophoresis and fluorography. For details see the legend to Fig. 4. Samples were taken and pulse-labelled 6 h (a), 8 h (b), 10 h (c), 12 h (d), 15 h (e), 18 h (f) and 21 h (g) respectively after beginning of the light period.

labelled with [3 H]proline at different cell cycle stages were analysed by SDS/polyacrylamide-gel electrophoresis and subsequent fluorography (Fig. 9), release of the 35000- M_r component was detected during the phase of cell enlargement, but not during cell division. During release of zoospores from the mother-cell wall, a component of a very similar M_r was sometimes observed (Fig. 9*f*), but usually it was covered by a smear of radioactive degradation products.

Discussion

The culture medium of growing *Chlamydomonas reinhardtii* cells contains protein components that are liberated not only during release of the zoospores from the mother-cell walls, but also during the cell-enlargement period. Both the macromolecules released during cell growth as well as those liberated during release of the zoospores from the mother-cell wall contain hydroxyproline. Distinct radioactive macromolecules were found in the culture medium after pulse-labelling with radioactive amino acids. The pattern of radioactively labelled components liberated during cell enlargement differed from that obtained at the time of release of zoospores from the mother-cell walls (compare Figs. 3, 4 and 6). Therefore the possibility can be excluded that the macromolecules released during cell enlargement could be sporangia wall fragments of cells that had escaped synchronous cell division. This conclusion is supported by the fact that no sporangia could be detected during this time period.

When synchronized cultures of *Chlamydomonas reinhardtii* were pulse-labelled with [3 H]proline or [35 S]methionine during the time period when the zoospores were released from the mother-cell walls, a large number of labelled macromolecules were liberated into the culture medium (Figs. 8 and 9). These radioactively labelled macromolecules cannot be fragments of the sporangial wall because it is very unlikely that precursors are incorporated into the mother-cell wall after cytokinesis. These radioactive macromolecules released during liberation of the zoospores should be the lytic factors (Schlösser, 1966, 1976; Mihara & Hase, 1975), and other components released by secretion or exocytosis.

All the protein or glycoprotein components that have been demonstrated to be released into the culture medium by synchronously growing *Chlamydomonas reinhardtii* cells are liberated during release of zoospores: fragments of the mother-cell wall (Lang & Chrispeels, 1976), lytic factors involved in sporangial-wall lysis (Schlösser, 1966, 1976; Mihara & Hase, 1975; Mergen-hagen, 1980),

and phosphatases (Matagne *et al.*, 1976). The question therefore arises as to what is the nature and possible function of the macromolecules released into the culture medium during the cell-enlargement period. The observation that these macromolecules contain hydroxyproline points to the possibility that at least one of these components is related to the cell wall. Since, however, it has been shown that a flagellar membrane protein of the gametes also contains hydroxyproline, this observation does not prove that these components are really constituents of the cell wall, since other proteins or glycoproteins not studied so far may also contain hydroxyproline. On the other hand, it is possible that a small portion of the subunits of the outer-wall layers dissociate from the cells and thereby accumulate in the culture medium during cell enlargement. This interpretation is, however, very unlikely, since labelling of the released macromolecules takes place after a considerably longer lag phase than labelling of the insoluble cell-wall layer, and during pulse-chase experiments the label appears in the released macromolecules during the time period when the radioactive label is lost from the insoluble cell-wall layer (Figs. 1 and 7). The latter findings indicate that at least some of the macromolecules released during cell enlargement are constituents of the insoluble inner cell-wall layer.

Cell growth requires cell-wall enlargement. Enlargement of the insoluble inner cell-wall layer requires cleavage of at least some cross-links between the glycoprotein subunits to permit extension of this wall layer and incorporation of additional glycoprotein subunits. Our pulse-chase experiments clearly demonstrate that a turnover of the insoluble inner cell-wall layer takes place during the cell-enlargement period, because the radioactive label present in this wall layer was strongly decreased during the chase (Fig. 7). Therefore glycoprotein components of the insoluble inner wall layer are released during cell-wall extension and seem to appear in the culture medium (Fig. 7c). It remains to be clarified whether a complete turnover of the insoluble inner wall layer takes place during the cell-enlargement period or whether parts of the inner cell-wall layer of the zoospores are still present in the insoluble wall layer of the mother-cell wall. Furthermore it needs to be clarified which of the macromolecules released into the culture medium during cell enlargement are intrinsic cell-wall constituents. Our observation that these macromolecules are differentially labelled with [^3H]proline and [^{35}S]methionine indicate that the released macromolecules are not exclusively cell-wall fragments. Since cell-wall glycoproteins contain high proportions of hydroxyproline and proline but low proportions of

methionine, the released macromolecules that are more strongly labelled with [^3H]proline than with [^{35}S]methionine are probably related to the cell wall, whereas the components that are more strongly labelled with [^{35}S]methionine, are presumably not related to the cell wall. Some of these components could be extracellular enzymes located in the cell wall or in the space between the wall and the plasmalemma. Studies on phosphatases of *Chlamydomonas* have shown that such enzymes are released into the culture medium in cell-wall-deficient mutants (Loppes, 1976). Turnover of the inner cell-wall layer therefore could result in release of enzymes attached to the wall. Before studying the nature and possible function of those protein components that are preferentially labelled with [^{35}S]methionine and therefore are presumably not related to the cell wall, it is necessary to unequivocally identify the cell-wall fragments released during cell enlargement. Unfortunately the published data on the cell-wall glycoproteins of *Chlamydomonas reinhardtii* are largely restricted to the sporangial walls accumulated in the culture medium (Roberts *et al.*, 1972, 1980; Roberts, 1974, 1979; Hills *et al.*, 1975; Catt *et al.*, 1976). Analysis of these cell-wall preparations do not necessarily reflect the cell-wall structure of a growing cell, particularly because these sporangial walls have been already affected by the 'lytic factors' (Schlösser, 1966, 1976; Mihara & Hase, 1975). Indeed, considerable differences have been observed with respect to the amino acid compositions of sporangial walls isolated from the culture medium (Roberts, 1974; Hills *et al.*, 1975) and cell walls isolated from growing cells (Voigt, 1984). Therefore analysis of the cell-wall structure of growing *Chlamydomonas reinhardtii* cells is a prerequisite for the identification of cell-wall fragments released into the culture medium during the cell-enlargement period.

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