

# Biosynthesis and Turnover of Cell Wall Glycoproteins during the Vegetative Cell Cycle of *Chlamydomonas reinhardtii*

Jürgen Voigt

Institut für Allgemeine Botanik und Botanischer Garten, Universität Hamburg,  
Ohnhorststraße 18, D-2000 Hamburg 52, Bundesrepublik Deutschland

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Biosynthesis and turnover of the different cell wall components have been studied during the vegetative cell cycle of *Chlamydomonas reinhardtii* by pulse-labelling with [<sup>3</sup>H]proline and [<sup>35</sup>S]methionine and by pulse-chase experiments. Two phases of biosynthesis of insoluble cell wall material could be distinguished:

1. *de novo* synthesis of the daughter cell walls during cytokinesis and
2. cell wall enlargement during cell growth.

During the cell enlargement period, a turnover of the insoluble wall component was observed. The released fragments were found to be accumulated in the culture medium.

The LiCl-soluble cell wall glycoproteins were found to be precursors of the insoluble cell wall layer. Biosynthesis of the LiCl-soluble cell wall glycoproteins was observed mainly during the time period between cytokinesis and the end of the following cell enlargement period. Labelling of all the cell wall components was found to be strongly reduced during the time period between the end of the growth phase and cytokinesis.

During cytokinesis, labelling of the insoluble cell wall material preceded the incorporation of radioactive precursors into the LiCl-soluble wall fraction.

## Introduction

The cell walls of higher plants contain – in addition to cellulose and other polysaccharides – hydroxyproline-rich insoluble cell wall glycoproteins (extensins), which comprises 2–10% of the primary cell wall [1]. Extensins appear to be important for wall strength [2] and disease resistance [3]. They accumulate in plant cells upon wounding [4] and pathogenic attack [5] and become insolubilized with time [6]. The salt-extractable monomeric precursors secreted by the cells have been isolated and chemically analyzed [6–10]. Recently, the full amino acid sequence of the main carrot extensin has been derived indirectly via determination of the DNA-sequences of cloned cDNAs [11].

Although many data have been accumulated in the last few years concerning with the chemical structure of extensins, their significance with respect to the structure and function of the cell wall is still unclear. Early work suggested that extensins might serve as a covalent cross link between wall polysaccharides [12, 13]. The observation that extensins remained insoluble after chemical deglycosylation [14] indicates that extensin monomers are cross-linked to themselves – possibly via isodityrosine formation [6, 9, 12, 15].

This finding apparently contradicts the model that extensins act as cross linker of cell wall polysaccharides [12, 13].

The main problem with respect to an understanding of the structure and function of the cell wall of higher plants is due to the relative large number of different cell wall components. A comparison of the structures and functions of the cell walls of higher and lower plants might therefore help to get a better insight into structure and function of cell wall of higher plants.

The volvocales, which apparently have to be located within the stem of the phylogenetic tree of the plant kingdom lack cellulose and other cell wall polysaccharides [16]. The most intensively studied organism of the volvocales is *Chlamydomonas reinhardtii*. The cell wall of *C. reinhardtii* only consists of several layers of hydroxyproline-rich glycoproteins [17–21]. Part of these cell wall layers can be solubilized by chaotropic agents or sodium dodecylsulfate, whereas other parts of the cell wall remain insoluble [19–26]. The insoluble cell wall component and the cell wall glycoproteins, which can be solubilized by aqueous NaClO<sub>4</sub> or LiCl, are very similar with respect to their amino acid and sugar compositions [20]. Some contradictory results exist with respect to the localization of soluble and insoluble wall components: On the basis of their results, Roberts and coworkers have

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concluded that the innermost wall layer (W1) is the insoluble cell wall component [21]. Comparing the ultrastructure of *C. reinhardtii* cells prior to and after treatment with aqueous LiCl or NaClO<sub>4</sub>, it was found that part of the central tripllett (W2–W6) is resistant against these chaotropic agents ([27]; Voigt, Wachholz, Manshard and Mix, unpublished results).

Using the cell-wall-defect mutant CW-2, which sheds its cell wall into the culture medium [28], Lang and Crispeels [29] have shown that the production of cell wall material is cell-cycle dependent. Cell wall fragments are accumulated in the culture medium of the mutant CW-2, which apparently contain all the cell wall layers found in the cell wall of wild-type cells [28] and all the salt-soluble high-molecular-weight cell wall glycoproteins found in wild-type cells [30]. Although Lang and Crispeels [29] have studied the biosynthesis of total cell wall material by pulse-labeling and pulse-chase experiments throughout the vegetative cell cycle, there are no data concerning the individual cell wall components. With respect to the turnover of the cell wall components, there are only data about the degradation of the mother cell walls during liberation of the zoospores by the 'lytic factors' [29, 31–35].

Our recently developed procedures to isolate the different cell wall components from different cell cycle stages of *Chlamydomonas reinhardtii* [25, 26, 36] enabled us to study biosynthesis and turnover of the different cell wall components during the vegetative cell cycle of *Chlamydomonas reinhardtii* wild-type.

## Materials and Methods

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

## Strains

The wild-type strain of *Chlamydomonas reinhardtii* used in the present experiments was strain 137C (mating type +) obtained from Dr. R. P. Levine (Harvard University, Cambridge, Mass.). The mutant strain "ls" (mating type –) was isolated from wild-type strain 137C by Mergenhagen [37].

## Media and growth conditions

Stock cultures were maintained on yeast/acetate agar slants at 15 °C and illuminated (4000 lx) using a 12 h light – 12 h dark cycle. Cells of both strains were grown autotrophically on high salt minimal medium [38] at 25 °C. The cultures were constantly bubbled with filtered air and constantly mixed by stirring bars. For asynchronous growth the cells were continuously illuminated from the sides by a combination of white lamps (Osram type 36W/25) and "daylight" fluorescent lamps (Osram type 36W/11). The light intensity was 10000 lx at the level of the cultures. Synchronous growth was achieved by the method of Surzycki [39] using a 12 h light – 12 h dark illumination cycle. Cell concentrations were determined by doublet hemocytometer counting.

## Incorporation of radioactive precursors

Studies on the incorporation of radioactive amino acids into cellular and medium proteins were performed under continuous light to avoid experimental artefacts due to changes with respect to the uptake of the radioactive precursors [40].

Synchronized cultures continued to divide synchronously when exposed to continuous illumination for an additional 24 h, *i.e.* one entire cell cycle period [32, 41].

## Cell fractionation

The cultures were rapidly cooled to 0 °C and centrifuged at 6000 × *g* for 10 min. The culture medium was decanted immediately after centrifugation.

The cells were washed twice with high salt medium and resuspended in high salt medium to a final concentration of 1 × 10<sup>8</sup> cells/ml. Extraction of the extracellular polypeptide components was performed by addition of the same volume of 8 M LiCl in high salt medium, incubation for 10 min at 0 °C and centrifugation at 15000 × *g* for 20 min [26]. The supernatant was collected and the LiCl-extracted cells submitted to extraction with 8 M urea – 2% (w/v) SDS – 10 mM EDTA – 200 mM 2-mercaptoethanol – 20 mM Tris-HCl, pH 7.5. The urea-SDS extracts were collected and the crude insoluble wall layer purified by extractions with 2 M NaCl – 3% (w/v) sodium deoxycholate – 3% (v/v) Triton X-100 – 2 mM EDTA – 20 mM Tris-HCl, pH 7.5, and finally washed with bidistilled water [25, 36].

### Determination of radioactivity

Incorporation of radioactive precursors into cellular and medium macromolecules was determined by adding TCA (final concentration 10%) to aliquots and collecting the denaturated components by centrifugation. The pellets were washed twice with 1 ml of a solution of 1 mM nonradioactive precursor followed by washing with 1 ml bidestilled water and redissolved overnight in 0.1 ml 8 M urea – 2% SDS – 1 mM EDTA – 200 mM 2-mercaptoethanol – 20 mM Tris-HCl, pH 7.5. The solution was mixed with 5 ml Aqualuma (Baker Chemicals) and measured in a liquid-scintillation counter (Tri-Carb 300, Packard). Incorporation of the label into the insoluble wall layer was measured after dissolving the insoluble material in Lumasolve (Baker Chemicals). 0.2 ml Cell wall suspension was incubated with 1 ml Lumasolve overnight at room temperature. After addition of 10 ml Lipoluma (Baker Chemicals) radioactivity of the sample was measured in a liquid-scintillation counter.

### SDS-polyacrylamide gel electrophoresis

LiCl-extracted macromolecules were precipitated by addition of TCA (final concentration 10%) in the cold and the precipitates collected by centrifugation. The pellets were washed with 1 ml bidestilled water and redissolved in 60 µl buffer consisting of 8 M urea – 2% SDS – 200 mM 2-mercaptoethanol – 20 mM Tris-HCl, pH 7.5. After addition of 20 µl sample buffer [42] containing bromophenolblue as tracking dye, the samples were analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli [42] using 15% polyacrylamide gel slabs. Fluorography of the gel slabs was performed as described by Laskey and Mills [43].

## Results

Pulse-labelling and pulse-chase experiments with [ $^3\text{H}$ ]proline and [ $^{35}\text{S}$ ]methionine have been performed to study biosynthesis and turnover of the different cell wall components during the vegetative cell cycle of *Chlamydomonas reinhardtii*. Cell wall glycoproteins contain high proportions of hydroxyproline and proline but low proportions of methionine [20, 21, 29]. Therefore, biosynthesis and turnover of hydroxyproline-rich cell wall components can be estimated by comparing the incorporation of [ $^3\text{H}$ ]proline

and [ $^{35}\text{S}$ ]methionine into the different subcellular fractions and by investigating the fate of the radioactive label under chase conditions. Cell fractionation was performed as depicted in Fig. 1. Since it has been observed that macromolecules are released into the culture medium not only during liberation of the zoospores from the sporangia but also during the phase of cell enlargement [36], labelling of the macromolecules released into the culture medium was routinely analyzed. The salt-soluble glycoprotein components of the cell wall were obtained by extraction of intact cells with aqueous LiCl [26]. Intracellular proteins were obtained by extraction of LiCl-pre-treated cells with a buffer containing urea (8 mol l $^{-1}$ ) and SDS (2%, w/v). The crude insoluble wall component obtained after extraction of intact cells with the

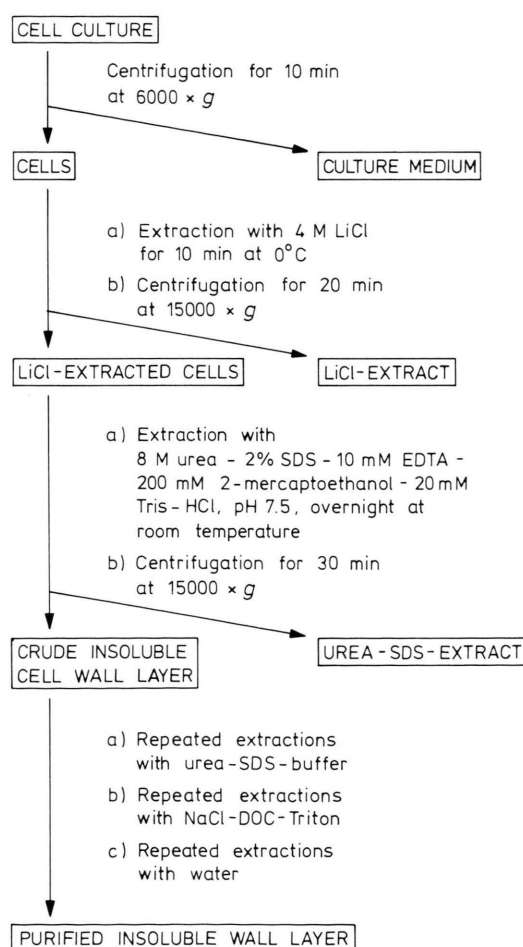


Fig. 1. Isolation of cell wall components from cultures of *Chlamydomonas reinhardtii*.

urea-SDS buffer was purified by repeated extractions with urea-SDS buffer, NaCl-DOC-Triton X-100 buffer and water [25]. A phase contrast micrograph of the insoluble wall component isolated by this procedure is shown in Fig. 2.

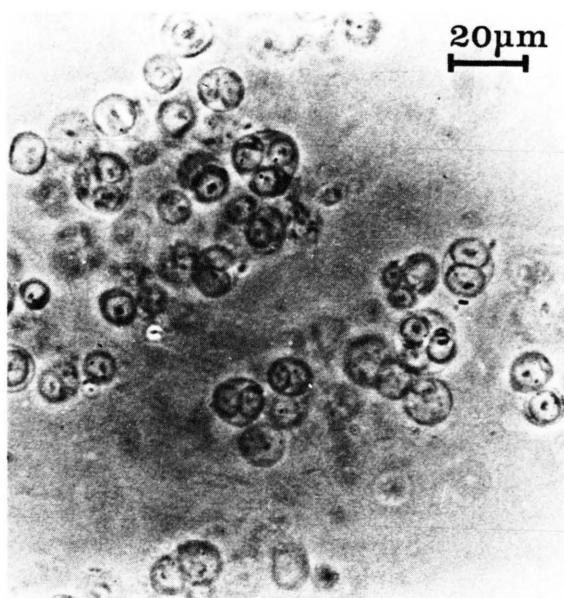


Fig. 2. Phase contrast micrograph of the insoluble cell wall component of *Chlamydomonas reinhardtii* purified as shown in Fig. 1. Bar = 20  $\mu\text{m}$ .

Since it has been found that the cell wall glycoproteins are not the only hydroxyproline-rich glycoproteins of *C. reinhardtii* [44], the patterns of labelled macromolecules extracted by aqueous LiCl were routinely investigated by SDS/polyacrylamide-gel electrophoresis and fluorography of the gels. As shown in Fig. 3, the pattern of macromolecules labelled with [ $^{35}\text{S}$ ]methionine largely corresponds to the pattern of polypeptides observed after staining of the gels with Coomassie Brilliant Blue (slots a, b). The fluorograph of the gels containing LiCl-extracted macromolecules from cells labelled with [ $^3\text{H}$ ]proline, however, differed considerably from the pattern of polypeptides observed after staining with Coomassie (slots c, d) and agrees much better with the pattern of carbohydrate-containing macromolecules observed after staining with periodic acid/Schiff's.



Fig. 3. Differential labelling with [ $^{35}\text{S}$ ]methionine and [ $^3\text{H}$ ]proline of macromolecules present in the LiCl-extract of intact cells of *Chlamydomonas reinhardtii*. Macromolecules extracted by aqueous LiCl from  $5 \times 10^7$  cells labelled for 2 h with either 25  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]methionine (a, b) or 100  $\mu\text{Ci}$  [ $^3\text{H}$ ]proline (c, d) were submitted to SDS/polyacrylamide-gel electrophoresis on slab gels containing 15% acrylamide. The gels were stained with Coomassie Brilliant Blue and photographed (a, c). Thereafter, the gels were completely destained, prepared for fluorography according to Laskey and Mills [43] and exposed to Kodak X-Omat X-AR5 film at  $-70^\circ\text{C}$  (b, d).

#### *Cell-cycle-dependent incorporation of [ $^3\text{H}$ ]proline and [ $^{35}\text{S}$ ]methionine into the different subcellular fractions of *Chlamydomonas reinhardtii**

Incorporation of [ $^3\text{H}$ ]proline and [ $^{35}\text{S}$ ]methionine into intracellular proteins was found to be almost constant throughout the cell cycle. The values depicted in Fig. 4B were calculated on the basis of the initial cell number and not corrected for the increase of the cell number during liberation of the zoospores from the sporangia (compare Fig. 4A). In contrast to the intracellular proteins, the labelling of the LiCl-extracted macromolecules varied during the vegetative cell cycle (Fig. 4C). Labelling with [ $^3\text{H}$ ]proline was found to be high during cell growth, but decreased 12 h after beginning of the light phase when the cells became nonmotile. During cell division, the labelling of the LiCl-extracted components was reduced to about 30% of the value measured during cell enlargement. After apparent cytokinesis an increased incorporation of [ $^3\text{H}$ ]proline into the LiCl-soluble components was found. With [ $^{35}\text{S}$ ]methionine no decrease but a slight increase was observed with respect to the radioactive labelling of the LiCl-extracted macromolecules during cell division. Incorporation



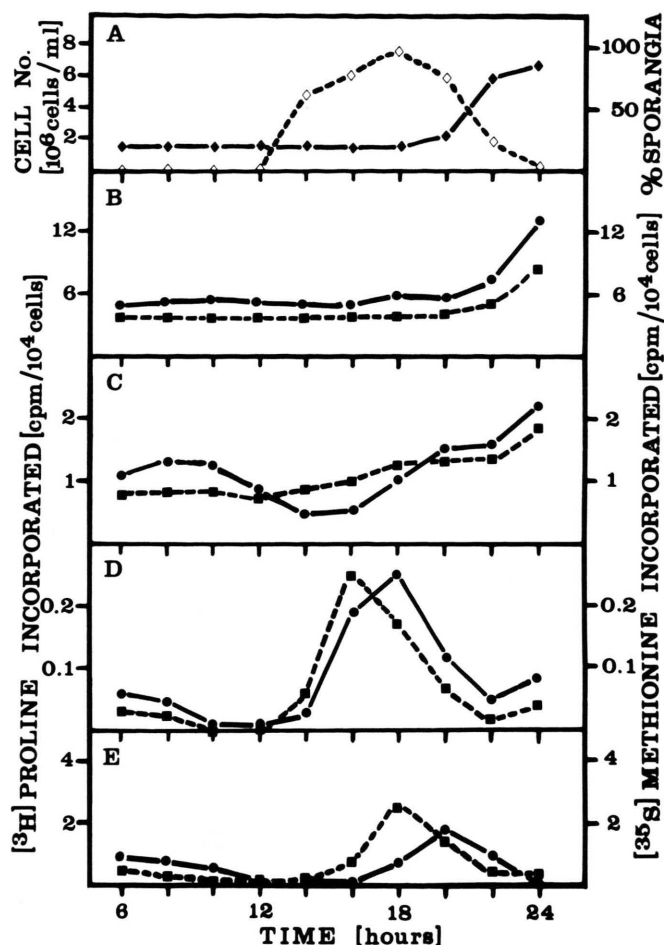


Fig. 4. Incorporation of  $[^3\text{H}]$ proline (—) and  $[^{35}\text{S}]$ methionine (---) into the subcellular fractions during the vegetative cell cycle of *Chlamydomonas reinhardtii*. Portions (40 ml) of a 1-litre culture synchronized by 12 h-light/12 h-dark were pulse-labelled for 90 min in the light with 100  $\mu\text{Ci}$  of  $[^3\text{H}]$ proline or 25  $\mu\text{Ci}$  of  $[^{35}\text{S}]$ methionine. Cells were withdrawn every 2 h, labelled and analysed as described in the Materials and Methods section. (A) Cell density; (B) incorporation into intracellular protein; (C) incorporation into protein components present in the LiCl-extract; (D) incorporation into the insoluble cell wall component; (E) incorporation into macromolecules released into the culture medium. The amount of radioactivity incorporated per cell was calculated on the basis of the initial cell number and not corrected for the increase of the cell density during liberation of the zoospores.

of radioactive precursors into the insoluble wall material was maximal during apparent cytokinesis but could also be measured during cell growth (Fig. 4D). It has to be mentioned that labelling of the LiCl-extracted components with  $[^3\text{H}]$ proline always increased at the end of apparent cytokinesis, e.g. delayed with respect to the increase of incorporation of labelled precursors into the insoluble wall layer. Macromolecules released into the culture medium were pulse-labelled during the cell enlargement period and around the time period when the zoospores are released from the mother cell wall (Fig. 4E), as already reported [36].

When the LiCl-extracted macromolecules that were pulse-labelled with  $[^{35}\text{S}]$ methionine at different cell cycle stages were analyzed by SDS/polyacrylamide-gel electrophoresis and subsequent fluorogra-

phy (Fig. 5A), reduced amounts of labelled components were found during early stages of the phase of cell division (slots c–d). During and after apparent cytokinesis (slots e and f) and particularly after release of the zoospores (slots g and h) increased labelling of all the different components was observed. The most striking variation of the labelling patterns throughout the cell cycle was observed with respect to the polypeptides of the apparent molecular weights of 47 000, 52 000 and 58 000. At the end of the cell enlargement period, the  $M_r$  58 000 component was found to be more prominent than the components of the  $M_r$ -values of 52 000 and 47 000 (slots a–c). After cytokinesis, the components of the  $M_r$ -values of 52 000 and 47 000 became more prominent than the  $M_r$  58 000 component (slots f–h). Changes of the labelling patterns were also observed with re-

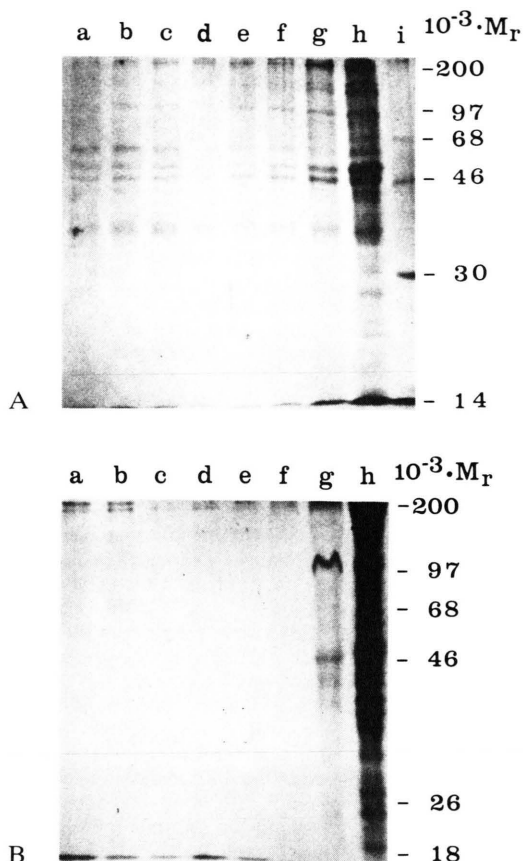


Fig. 5. Analysis by SDS/polyacrylamide-gel electrophoresis and fluorography of LiCl-extractable protein components that are pulse-labelled with [ $^{35}\text{S}$ ]methionine (A) or [ $^3\text{H}$ ]proline (B) at different stages of the vegetative cell cycle. Portions (40 ml) of a 1-litre culture synchronized by a 12 h-light/12 h-dark illumination cycle were pulse-labelled for 90 min in the light with either 25  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine (A) or 100  $\mu\text{Ci}$  of [ $^3\text{H}$ ]proline (B). Radioactively labelled cells were washed, extracted with aqueous LiCl, and the LiCl-extracts processed for SDS/polyacrylamide gel electrophoresis as described in the Materials and Methods section. Samples were taken and pulse-labelled 8 h (a), 10 h (b), 12 h (c), 14 h (d), 16 h (e), 18 h (f), 20 h (g) and 24 h (h) respectively after beginning of the light period. The total amount of LiCl-extracted protein components was submitted to electrophoresis and fluorography.

spect to the high-molecular-weight cell wall subunits of the  $M_r$ -values of 97000, 150000 and 160000. At the end of the cell enlargement period, during cytokinesis and release of the zoospores, the 97000 component was found to be more strongly labelled than the glycoproteins of the  $M_r$ -values of 150000 and 160000 (slots a–g). At the beginning of the new cell

enlargement period (slot h), labelling of the larger components was found to be more increased than the labelling of the  $M_r$ -97000 component.

Different labelling patterns were observed when the LiCl-extracted proteins that were pulse-labelled with [ $^3\text{H}$ ]proline at different cell-cycle stages were analyzed (Fig. 5B). At the end of the cell enlargement period, the protein components with the apparent molecular weights of 68000, 150000, 160000 and more than 200000 were predominantly labelled (slots a and b). During cell division, reduced labelling of the different components was observed (slots c–e). When the zoospores were released from the mother cell walls, the 97 kD and the 47 kD components were found to be strongly labelled (slot g). Labelling with [ $^3\text{H}$ ]proline of the other components of the LiCl-extract of intact cells was found to be strongly increased after beginning of the next cell enlargement period (slot h).

#### *Biosynthesis of the different cell wall components during cell enlargement*

Cell wall growth is a prerequisite of cell enlargement. To study the mechanism of cell wall growth, the time-course of incorporation of radioactively labelled amino acids into the different cell fractions has been studied during the cell enlargement period (Figs. 6 and 7). Labelling of intracellular proteins occurred without measurable lag-phase (Fig. 6B) as compared to the time-course of the uptake of radioactive amino acids (Fig. 6A). A lag-phase of about 10 min was observed with respect to the labelling of the LiCl-extracted macromolecules (Fig. 6C), whereas labelling of the insoluble wall component could be measured after a lag-phase of 20 min (Fig. 6D). No significant differences could be observed with respect to the time-courses of the incorporation of [ $^3\text{H}$ ]proline and [ $^{35}\text{S}$ ]methionine into the subcellular fractions. However, the relative amounts of [ $^3\text{H}$ ]proline and [ $^{35}\text{S}$ ]methionine incorporated into the different subcellular fractions varied considerably. The relative amount of incorporated [ $^3\text{H}$ ]proline to incorporated [ $^{35}\text{S}$ ]methionine increased in the following order: intracellular protein < LiCl-extract < insoluble wall layer < macromolecules released into the culture medium.

The time-course of labelling of the individual macromolecules present in the LiCl-extract was

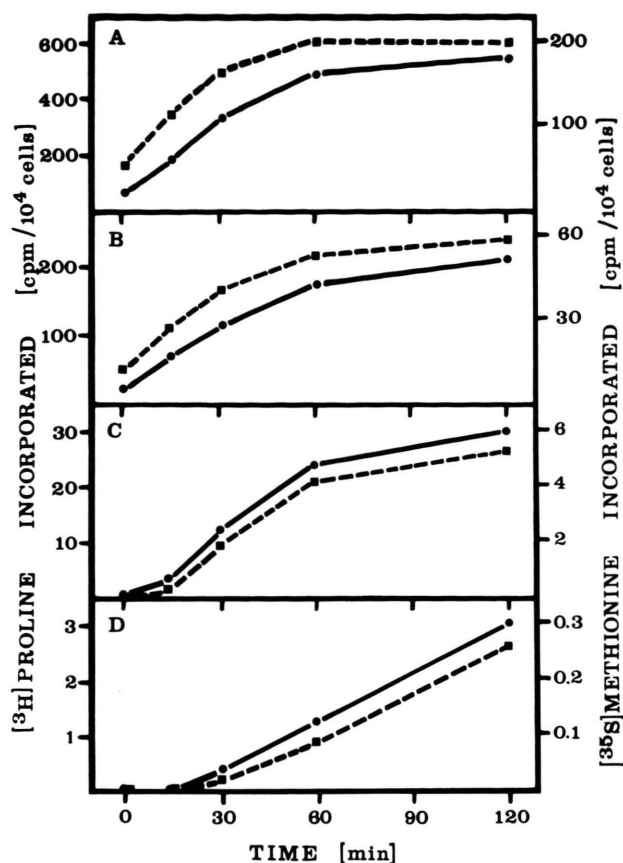
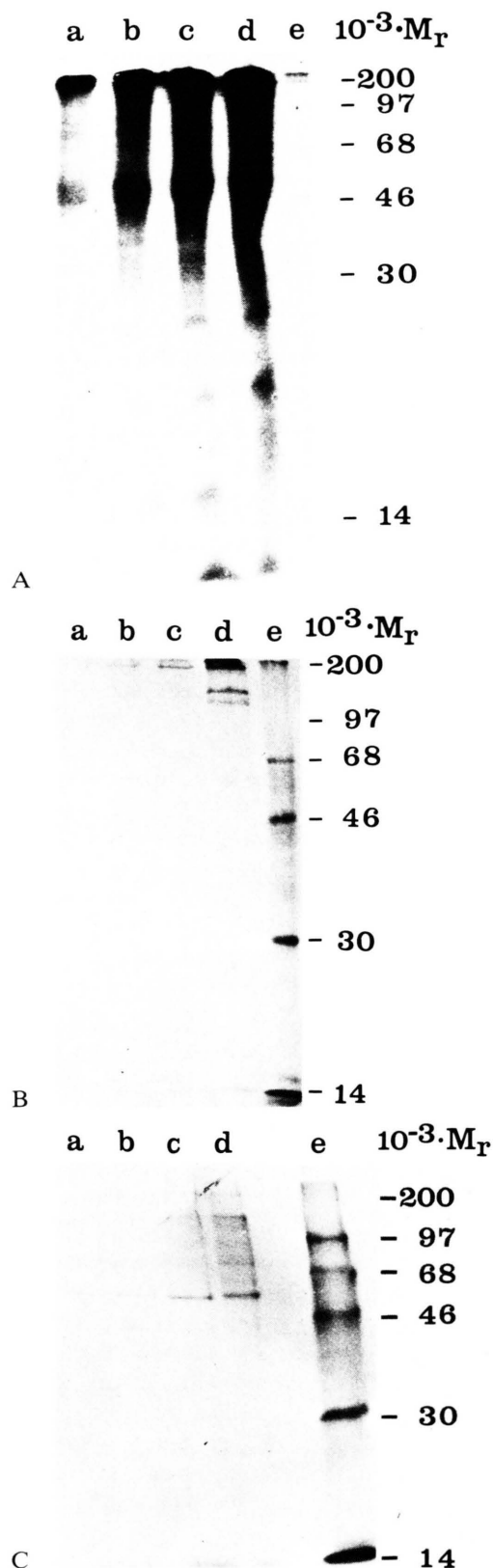


Fig. 6. Time-course of the uptake of radioactive amino acids (A) and the incorporation of [<sup>3</sup>H]proline (—) or [<sup>35</sup>S]methionine (---) into intracellular proteins (B), extracellular proteins (C) and the insoluble cell wall component of *Chlamydomonas reinhardtii* (D). [<sup>3</sup>H]proline (1 mCi) or [<sup>35</sup>S]methionine (250  $\mu$ Ci) was added to a 1000 ml culture of synchronized cells (cell density  $1 \times 10^6$  cells/ml) 6 h after beginning of the light period. Samples (100 ml) were taken at the indicated times. The cells were collected by centrifugation, washed and fractionated as described in the Materials and Methods section.

Fig. 7. Analysis by SDS/polyacrylamide-gel electrophoresis of LiCl-extractable protein components pulse-labelled with [<sup>35</sup>S]methionine (A) or [<sup>3</sup>H]proline (B and C) during cell enlargement. [<sup>35</sup>S]methionine (125  $\mu$ Ci) or [<sup>3</sup>H]proline (500  $\mu$ Ci) were added to a 500 ml culture of synchronized cells (cell density  $1 \times 10^6$  cells/ml) 6 h after beginning of the light period. Samples (100 ml) were taken 15 min (a), 30 min (b), 60 min (c) and 120 min (d) after addition of the radioactive label. The cells were collected by centrifugation, washed, extracted with aqueous LiCl and the LiCl-extracts processed for SDS/polyacrylamide-gel electrophoresis and fluorography as described in the Materials and Methods section. Labelled protein components extracted from  $10^8$  cells were analysed per slot. The fluorographs B and C show different results of the same type of experiment. e = protein molecular weight standards.



studied to clarify the question whether or not there is an educt-product relationship between some of the LiCl-extractable macromolecules. As already reported [26], different labelling patterns were observed after labelling with [ $^{35}\text{S}$ ]methionine (Fig. 7A) and [ $^3\text{H}$ ]proline (Fig. 7B, C). Furthermore, the methionine-rich macromolecules are labelled faster than the macromolecules predominantly labelled with [ $^3\text{H}$ ]proline. Since the relative amount of incorporated [ $^3\text{H}$ ]proline to incorporated [ $^{35}\text{S}$ ]methionine is much higher in the case of the insoluble wall layer than in the case of total LiCl-extract (Fig. 6B, C), this finding indicates that the methionine-rich polypeptides are not related to the cell wall. It is reasonable to suppose that these polypeptides are constituents of the plasmalemma and/or the flagellar membrane. It has to be mentioned that the labelling patterns obtained from the different pulse-labelling experiments with [ $^3\text{H}$ ]proline varied considerably whereas the labelling patterns with [ $^{35}\text{S}$ ]methionine were found to be much more reproducible. Two types of labelling patterns were obtained from the pulse-labelling experiments with [ $^3\text{H}$ ]proline: In some experiments, the high-molecular-weight cell wall subunits were found to be predominantly labelled (Fig. 7B), whereas in other experiments performed with the same laboratory strain under the same growth conditions macromolecules with considerably lower  $M_r$ -values were more intensely labelled (Fig. 7C). In some experiments, macromolecules with  $M_r$ -values of 36000 and 26000 were found to be predominantly labelled with [ $^3\text{H}$ ]proline [26]. In every case, however, all the components were labelled with the same time-course. No educt-product relationship could be observed.

The fact that the labelling patterns with [ $^3\text{H}$ ]proline are much less reproducible than the labelling patterns obtained with [ $^{35}\text{S}$ ]methionine could be explained by variations of the pool size or variations in the metabolism of the added [ $^3\text{H}$ ]proline. A careful analysis of the [ $^{35}\text{S}$ ]methionine labelling patterns, which were obtained from labelling experiments performed in parallel to the labelling experiments with [ $^3\text{H}$ ]proline, did not confirm this interpretation: The variations in the labellings of those protein components which are predominantly labelled with [ $^3\text{H}$ ]proline could be observed also after labelling with [ $^{35}\text{S}$ ]methionine. Therefore, I suppose that these variations of the [ $^3\text{H}$ ]proline labelling patterns have to be mainly referred to differ-

ences with respect to the posttranslational modification of some cell-wall polypeptides.

### *Pulse-chase experiments*

Pulse-chase experiments have been performed to study the fate of the radioactively labelled components present in the different subcellular fractions and to investigate the possibility that the LiCl-extractable cell wall components are precursors of the insoluble cell wall layer. Synchronized cell cultures were labelled for 1 h with [ $^3\text{H}$ ]proline or [ $^{35}\text{S}$ ]methionine during the cell enlargement period. After labelling, the cells were collected by centrifugation, washed, resuspended in fresh culture medium containing unlabelled amino acids, and the disappearance of the radioactive label from the various subcellular fractions was measured. The disappearance of the radioactive label from the intracellular macromolecules labelled with [ $^3\text{H}$ ]proline was considerably faster than the decrease of [ $^{35}\text{S}$ ]methionine-labelled intracellular proteins (Fig. 8A). The specific radioactivity of the LiCl-extract of cells labelled with [ $^3\text{H}$ ]proline was significantly increased during the first hour of the chase (Fig. 8B). Thereafter, the specific radioactivity of the LiCl-extractable protein components decreased. Increase of the radioactive label during the first hour of the chase as well as the decrease after this time period were much less striking in the case of cells labelled with [ $^{35}\text{S}$ ]methionine (Fig. 8B). Radioactive labelling of the insoluble cell wall layer also increased during the first hour of the chase period (Fig. 8C). The increase of the specific radioactivity of the insoluble cell wall component was much more striking than in the case of the LiCl-extracted protein components and highly significant even in the case of the [ $^{35}\text{S}$ ]methionine-labelled cells (Fig. 8C). After the first hour of the chase period, the radioactive label started to disappear from the insoluble cell wall layer – especially in the case of cells labelled with [ $^3\text{H}$ ]proline. This finding indicates that a turnover of the insoluble cell wall component takes place also during the phase of cell enlargement. Radioactively labelled macromolecules are accumulated in the culture medium (Fig. 8D) when the radioactive label disappears from the insoluble cell wall layer (Fig. 8C). As shown in Fig. 9, the specific radioactivity of all the macromolecules present in the LiCl-extract of intact cells decreased with about the same velocity under chase conditions. As in the



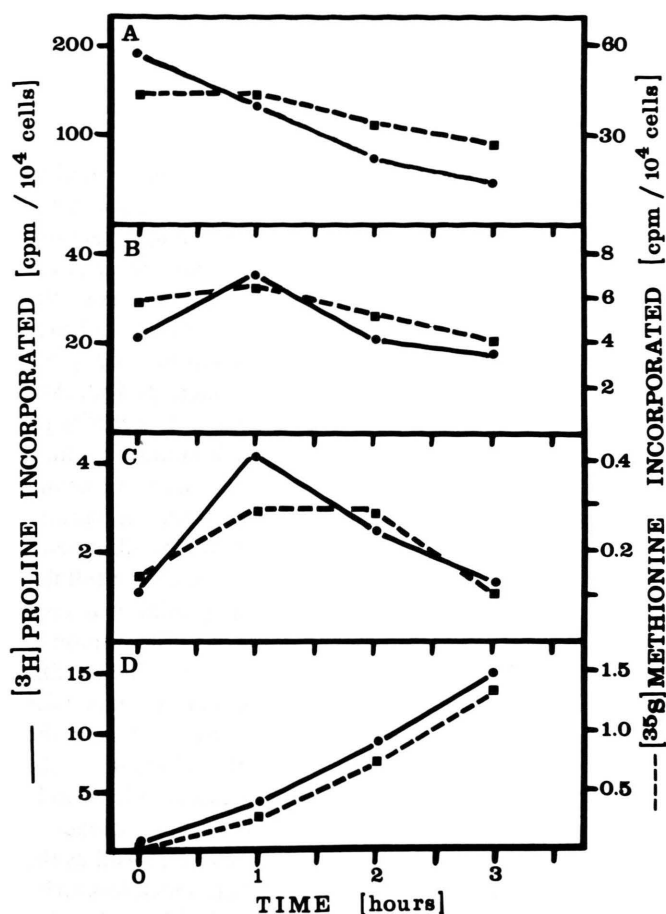


Fig. 8. Disappearance of radioactive label from intracellular macromolecules (A), the LiCl-extractable protein components (B) and the insoluble cell wall layer (C) of *Chlamydomonas reinhardtii* cells pulse-labelled for 1 h with  $[^3\text{H}]$ proline (—) or  $[^3\text{S}]$ methionine (---) after addition of unlabelled amino acids and accumulation of radioactively labelled macromolecules in the culture medium (D). A portion of  $[^3\text{H}]$ proline (1 mCi) or  $[^3\text{S}]$ methionine (250  $\mu\text{Ci}$ ) was added to a 1000 ml culture of synchronized cells (cell density  $1 \times 10^6$  cells/ml) 6 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 or 10 mM unlabelled methionine. Samples (200 ml) were taken at the indicated time points. Cell fractionation was performed as described in the Materials and Methods section.

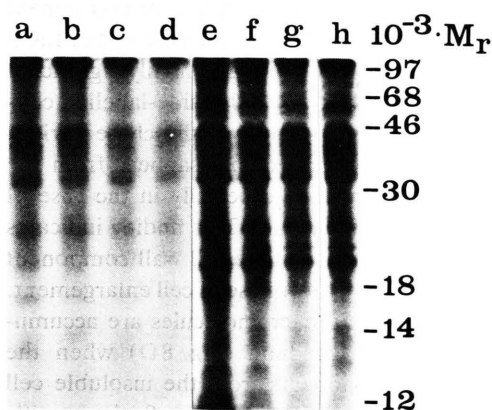


Fig. 9. Disappearance of radioactively labelled components from the LiCl-extractable protein fraction of *Chlamydomonas reinhardtii* cells pulse-labelled for 1 h with  $[^3\text{S}]$ methionine (a) or  $[^3\text{H}]$ proline (e) after addition of un-

labelled amino acids. A portion of  $[^3\text{S}]$ methionine (250  $\mu\text{Ci}$ ) or  $[^3\text{H}]$ proline (1 mCi) was added to a 1000 ml culture of synchronized cells (cell density  $1 \times 10^6$  cells/ml) 6 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 unlabelled methionine, respectively. Samples (200 ml) were taken immediately (a, e), 1 h (b, f), 2 h (c, g) and 3 h (d, h) after addition of unlabelled amino acids. Cells were extracted with aqueous LiCl and the LiCl-extracts processed as described in the Materials and Methods section. LiCl-extracted proteins corresponding to  $1 \times 10^8$  cells were submitted to SDS-PAGE on slab gels containing 20% acrylamide. a–d = fluorograph of a gel slab with LiCl-extracts from cells labelled with  $[^3\text{S}]$ methionine, e–h = fluorograph of a gel slab with LiCl-extracts from cells labelled with  $[^3\text{H}]$ proline.

pulse-labelling experiments (Fig. 7), no educt-product relationship could be observed. The radioactive label was lost very slowly from the LiCl-extractable macromolecules (Fig. 8 and 9).

## Discussion

During the vegetative cell cycle of *Chlamydomonas reinhardtii*, two phases of cell wall biosynthesis can be distinguished:

1. *de novo* synthesis of the daughter cell walls during cytokinesis and
2. cell wall enlargement during cell growth.

Formation of the daughter cell walls includes the *de novo* synthesis of the insoluble wall components. Our pulse-labelling experiments revealed that during cytokinesis, maximal incorporation of radioactive precursors into the insoluble cell wall material precedes the increase of LiCl-soluble wall glycoproteins. This finding indicates that the insoluble wall layer of the daughter cell walls is formed prior to the LiCl-soluble wall layers.

Robinson and Schlösser [45] have studied the cell wall regeneration by protoplasts of *Chlamydomonas smithii* and the *de novo* formation of the daughter cell walls. In both cases, they observed a fringe of globular material emanating from the plasmalemma, which resembles the innermost wall layer W1, prior to the formation of a clear and definite wall layer (W2). Together with our results that the insoluble wall component of the daughter cell walls is formed prior to the LiCl-soluble wall layers, this finding apparently agrees with the cell wall model of Roberts and coworkers [21]. The assumption of Roberts and coworkers [21] that the insoluble cell wall component of *Chlamydomonas reinhardtii* corresponds to the innermost wall layer W1, however, is contradicted by the results of Goodenough and Heuser [27] and our own data (Voigt, Wachholz, Manshard and Mix, unpublished results), which indicate that the insoluble cell wall material is located within the central triplet.

Pulse-labelling experiments performed during cell wall enlargement revealed that radioactive precursors are incorporated into the LiCl-soluble cell wall glycoproteins after a considerably shorter lag phase than into the insoluble cell wall material. The conclusion that the LiCl-soluble cell wall fraction must contain high proportions of precursors of the insoluble wall layer is supported by the results of our pulse-chase experiments.

It is reasonable to suppose that the extracellular precursors of the insoluble cell wall layer are localized between the plasmalemma and the insoluble wall layer. This assumption is supported by our finding that the innermost wall layer W1 is removed by treatment of intact cells with aqueous LiCl (Voigt, Wachholz, Manshard and Mix, unpublished results). The fringe of globular material emanating from the plasmalemma, which is observed prior to the formation of a clear and definite wall layer [45] must therefore largely consist of LiCl-soluble precursors of the insoluble wall layer. For these reasons, our observation that during cytokinesis labelling of the insoluble wall material peaked prior to the accumulation of labelled LiCl-soluble macromolecules (Fig. 4) merely indicates that cross-linking of the LiCl-soluble precursors is very fast as compared to their excretion or exocytosis. It has to be assumed that the velocity of the cross-linking process is not only dependent on the concentrations of the soluble precursors and the corresponding enzyme but also on the amount of cross-linking sites of the insoluble wall material. Based on this assumption, I conclude that the velocity of cross-linking decreases as soon as the insoluble wall layers of the daughter cells are almost completed. The decreased consumption of precursors must result in an accumulation of LiCl-soluble cell wall glycoproteins. This interpretation is supported by the observation that a turnover of the insoluble cell wall component occurs during cell wall enlargement. The formation of new cross-linking sites of the insoluble wall layer is a prerequisite for cell wall extension. The labelling kinetics of the different LiCl-extractable components, the disappearance of the radioactive label from these macromolecules under chase conditions and the differential labelling with [<sup>3</sup>H]proline and [<sup>35</sup>S]methionine of these polypeptides indicate that several components of the LiCl-extract of intact cells (with  $M_r$ -values between 20000 and 100000) are also precursors of the insoluble wall component – in addition to the high-molecular-weight cell wall glycoproteins [17–24, 46]. Investigating the cell surface polypeptides of *C. reinhardtii* by *in vivo* vectoral labelling by glucose oxidase-coupled lactoperoxidase-dependent [<sup>125</sup>I]iodination, Monk *et al.* [47] have also identified those macromolecules which are associated with the cell wall. 17 Cell-wall-associated polypeptides with  $M_r$ -values between 28000 and 280000 have been identified by these authors. Essentially the same macromolecules

were found in cell walls shed into the culture medium by the imp-1 mutant of *C. reinhardtii* [48] and cell walls mechanically isolated from gametes of *C. reinhardtii* [49]. All these polypeptides were also found in the LiCl-extracts from intact cells ([26], this paper). The main components of the cell-wall-associated macromolecules, described by Monk *et al.* [47], Matsuda *et al.* [48] and Imam *et al.* [49] were found to be more strongly labelled with [<sup>3</sup>H]proline than with [<sup>35</sup>S]methionine and might therefore be cell wall structural glycoproteins. The cell-wall-associated polypeptides which are more strongly labelled with [<sup>35</sup>S]methionine could be cell-wall-associated enzymes [50–52].

Neither the pulse-labelling nor the pulse-chase experiments revealed educt-product relationships between these protein and glycoprotein components. Therefore, a relative large number of different cell wall precursors are synthesized within the cell, released by secretion or exocytosis, incorporated into the soluble wall layers and then cross-linked with the insoluble cell wall layer – apparently without further modification. This interpretation of the results presented in this communication, is supported by an investigation of the polypeptide chains, which are released from the insoluble cell wall material by chemical deglycosylation (Vogeler, Voigt and König, unpublished results). Preliminary results with antibodies raised against purified cell wall glycoproteins indicate that the polypeptides released from the in-

soluble wall layer by chemical deglycosylation are immunologically related to the LiCl-soluble cell wall glycoproteins.

Although the LiCl-extract of intact cells is a very crude fraction, a comparative SDS-PAGE analysis of LiCl-extracted macromolecules from wild-type cells and the cell-wall deficient mutant CW-15 revealed that at least the high-molecular-weight cell wall glycoproteins can be unequivocally identified on SDS/polyacrylamide gels [26]. The results of our pulse-labelling experiments performed during the vegetative cell cycle show that the putative precursors of the insoluble cell wall material are synthesized during the time period between the end of mitosis and the end of the following cell enlargement period. It has to be clarified, whether the genes coding for the cell wall polypeptides are repressed during the time period between the end of cell growth and cytokinesis or whether other processes like processing of the primary transcripts, translation, post-translational modification or exocytosis/secretion of the cell wall precursors are decreased during this time period.

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