*Phytochemistry*, 1980. Vol. 19, pp. 1943–1947. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0901-1943 $02.00/0

# WALL LOSS AND ORIGIN OF SOLUBLE CARBOHYDRATES DURING MATING OF *CHLAMYDOMONAS REINHARDI*

KENNETH M. SOLTER, HSIAN-CHIA CHOW, DARRYL A. RAY and AHARON GIBOR*

Department of Biological Sciences, University of California, Santa Barbara, Santa Barbara, CA 93106, U.S.A.

(*Received* 12 *November* 1979)

**Key Word Index**—*Chlamydomonas reinhardi*; Chlorophyta; cell wall; lytic-factor; mating; carbohydrates; cell wall sugars; hydroxyproline.

**Abstract**—In the mating reaction between gametes of the green alga *Chlamydomonas reinhardi*, a lytic factor which solubilizes the cell wall is released. It has been shown that carbohydrates accumulate in the supernatant of mating gametes. We present here data which support the notion that the released carbohydrates arise from solubilized gametic cell walls. The evidence is based, in part, on the comparison of the carbohydrates and amino acids in the acid hydrolysates of cell-free supernatants to the reported composition of isolated cell walls. In both cases the three predominant sugars are mannose, arabinose and galactose, and also, in both cases, large amounts of the amino acid hydroxyproline are present. In addition, it is shown that if gametic cell walls are removed prior to mating interactions by treatment with a preparation of lytic factor, much less carbohydrate is subsequently released into the supernatant, when such 'naked' gametes are mated.

## INTRODUCTION

The mating reaction that occurs between plus and minus gametes of the unicellular biflagellate green alga *Chlamydomonas reinhardi* involves a series of discrete steps. An initial massive agglutination is followed by pairing, cell wall loss, fertilization tubule formation and finally cell fusion [1]. The agglutination between the gametes is mediated by the flagellar tips [2, 3]. Flagellar interactions also appear necessary for cell wall loss [4] and fertilization tubule formation by the plus gamete [1]. The mating reactions are being studied by morphological, biochemical [1, 4–11] and genetic techniques [3, 12–14].

The cell wall of *Chlamydomonas reinhardi* has been studied genetically and biochemically. Mutants with cell-wall defects, including some lacking walls, have been isolated [15]. Walls of normal cells contain no cellulose and are multilayered, with part of the wall composed of a highly ordered, complex glycoprotein lattice [16–19]. Mannose, arabinose and galactose account for 47% of the wall by mass and protein accounts for 30% [16, 20]. The predominant amino acid of the cell wall is hydroxyproline [20]. Using such agents as lithium chloride and sodium perchlorate, the wall can be dissociated into several components. Upon removal of these salts, the wall components reassemble, *in vitro*, by a process of self-assembly [19, 20]. Self-assembly requires available sugar residues (mannose in particular), has a pH optimum of 5 and a temperature optimum of 20° [21].

During the mating reaction, gametic wall loss results from secretion of a species-specific lytic factor [22–24]. Activity of the lytic factor has been observed both microscopically [24–26] and quantitatively [27]. Claes [25] reported that high levels of concanavalin-A interfere with lytic factor activity. Low amounts of concanavalin-A, or adhesion of the gametes to polystyrene, will induce lytic factor release in minus gametes [28]. Gametic lytic factor is also active on vegetative cell walls, and allows vegetative protoplasts to be formed. Protoplasts are observed to regenerate a new wall within 2–3 hr. The regeneration can be inhibited by cycloheximide and disturbed by concanavalin-A [29].

During mating, carbohydrates are found to accumulate in the supernatant of mixed gametes. The accumulation was found to be specific; it occurred only when active plus and minus gametes were mixed. Because of the time course of soluble carbohydrate appearance, we postulated that it was a result of the action of lytic factor on discarded cell walls [5]. In this paper we present further evidence that the carbohydrates and soluble protein accumulation is a result of lytic factor release and the subsequent solubilization of the cell walls.

## RESULTS AND DISCUSSION

Table 1 summarizes the results of sugar analysis by GLC of the concentrated supernatant from mating gametes and zygotic cell walls. Mannose, arabinose and galactose are the three major sugars found in the supernatant from mixed gametes and are also the predominant sugars found in vegetative cell walls [20]. The supernatant from mixed gametes contained xylose, a sugar only rarely found in vegetative and zygotic walls. In addition, we can detect a small amount of glucose, the predominant sugar of zygotic walls which is absent from vegetative walls.

The cell walls of both vegetative and zygotic cells of *Chlamydomonas reinhardi* contain protein [20, 33]. Therefore, if it is assumed that the carbohydrates found to accumulate in the cell free supernatant are of cell-wall origin, the supernatant would also be expected to contain a protein or amino acids. Figure 1 clearly shows that protein is released into the supernatant of mixed plus and minus gametes. Protein does not appear in the supernatant of unmixed gametes, gametes mixed with vegetative cells or with plus and minus mixed vegetative

* To whom correspondence should be addressed.

Table 1. Comparison of the composition of the soluble sugars released into the cell-free supernatant of mating gametes of *Chlamydomonas reinhardi* to the sugar composition of normal cell walls

| Sugar | % of total for vegetative cell-wall relics* | % of total for supernatant of mixed gametes† | % of total for zygotic walls‡ |
|---|---|---|---|
| Mannose | 22.5 | 32.0 | 22 |
| Arabinose | 30.0 | 21.5 | 6.9 |
| Galactose | 47.5 | 23.3 | 22 |
| Xylose | rare | 18.5 | rare |
| Glucose | — | 4.7 | 49.1 |
| Total | 100.0 | 100.0 | 100.0 |

* Data taken from Roberts [20].

† Crude supernatant was isolated from plus and minus gametes 3 hr after mixing. Hydrolysis of crude concentrated supernatant was done with 1.0 M $H_2SO_4$ for 18 hr at 110°. The hydrolysed samples were analysed by GLC.

‡ Data taken from Catt [33] and converted to per cent of total.

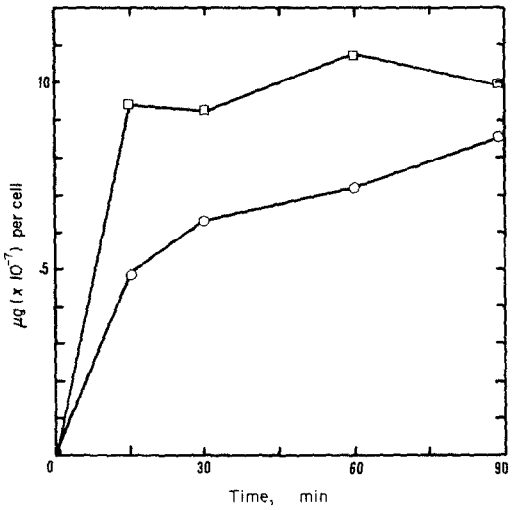


Fig. 1. Carbohydrate and protein release from mixed plus and minus gametes. Gametes of both mating types were washed and resuspended in N-free medium to a concentration of $1 \times 10^8$ cells/ml. At time 0, equal numbers of plus and minus gametes were mixed. Samples of the cellular suspension were taken at timed intervals for determination of levels of carbohydrate and protein in the cell free supernatant (see Experimental). □——□, Carbohydrate; ○——○, protein.

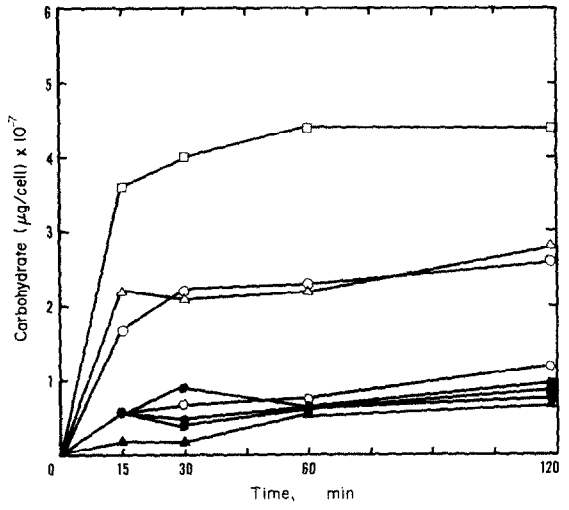


Fig. 2. Effect of pretreatment with lytic factor on subsequent carbohydrates released by mixed plus and minus gametes. Gametes of both mating types were washed and resuspended to a concentration of $2 \times 10^7$ cells/ml in N-free medium. Aliquots of each mating type were treated separately with lytic factor in the following manner. The cells were centrifuged and resuspended in N-free medium at a concentration of $5 \times 10^7$ cells/ml. An equal volume of crude lytic factor was added. After 30 min the cells were washed once with N-free medium and resuspended to their original volume. At time 0 wall-less gametes of opposite mating type were either mixed together, or each separately mixed with untreated (walled) gametes of the opposite mating type. At timed intervals samples were removed and assayed for carbohydrate as in Fig. 1. □——□, Control (both mating types walled); ○——○, plus wall-less mixed with minus walled; △——△, minus wall-less mixed with plus walled; ◇——◇, mixed plus and minus (both mating types wall-less); ■——■, plus alone; ●——●, minus alone; ▲——▲, plus wall-less alone; ⬬——⬬, minus wall-less alone.

cells. The time course of accumulation for protein is almost identical to that of carbohydrates, although, per cell, less protein is released.

As can be seen in Table 2, the amino acid composition of the protein from the supernatant of mating gametes more closely resembles that of vegetative cell walls [20] than that of zygotic cell walls [33]. In both vegetative cell walls and the supernatant from mixed gametes the predominant amino acid is hydroxyproline. In a related species *Chlamydomonas gymnogama* over 87% of a cell's hydroxyproline is found in the wall [34]. The large amounts of hydroxyproline in the supernatant suggests the dissolution and accumulation of cell wall materials.

The similarities between amounts of other amino acids in the supernatant and vegetative walls (Table 2) provide further evidence for a cell-wall origin of the proteins.

If the carbohydrate released into the supernatant originates from lysed cell-wall material, then when gametes whose walls have been previously removed with lytic factor are mated, less carbohydrate should appear. This, as Fig. 2 shows, is clearly the case. The amounts of carbohydrate released by mating gametes without walls is barely above background levels. When gametes of one mating type without walls are mated with gametes of the opposite mating type with intact walls, about one half the control levels of carbohydrate appear. Wall removal did not alter the mating efficiency which was 90%, or greater, in all cases.

The ratios between the various sugars as found in the supernatant of mating gametes, differ from that seen for vegetative and zygotic walls (Table 1). These differences can be explained in several ways. The cell-wall chemistry of *Chlamydomonas reinhardi* seems to vary with different stages of the life cycle. Catt [33] has shown that the predominant sugar of zygotic walls is glucose (see Table 1), a sugar absent from vegetative cell walls. In addition, although gametic lytic factor is active on vegetative and gametic cells, it is inactive on zygotic cells [23].

Another reason that there are differences between the reported composition of vegetative cells walls and the supernatant of mixed gametes might be due to the way Roberts [20] obtained material for the analysis of cell walls. The material was obtained by isolating cell-wall relics that collected in old vegetative culture medium. These relics are left after vegetative mother cells have undergone cell division and the daughter cells released. Such cell-wall relics might not be identical to the walls of living cells.

Although Minami and Goodenough [35] reported the secretion of glycopolypeptides into the medium by young zygotes, we believe that the carbohydrates accumulated and measured in our assay are not of zygotic origin. The carbohydrates we measure are very quickly released into the medium, maximal amounts being reached within 15 min after mixing of the gametes. The zygotic polypeptides begin to appear after *ca* 1 hr [30]. Also, carbohydrate accumulation can occur in the absence of zygote formation [4, 8].

We thus conclude that the carbohydrates found to accumulate in the supernatant of mixed plus and minus gametes arise from the loss and dissolution of the gametic cell wall as a result of lytic factor action. This conclusion is based on several facts, including chemical similarities of the concentrated supernatant to vegetative cell walls, and the fact that no carbohydrates accumulate when gametic walls are removed prior to mating. Thus, measurement of supernatant carbohydrates is a valid assay for detecting cell-wall loss and dissolution in cells of *Chlamydomonas*.

The amount of cell-wall material released from gametes upon mating varies greatly from one preparation to the next (compare Figs. 1 and 2). We obtained values for solubilized carbohydrates from 12 to $4 \times 10^{-7}$ $\mu$g per cell in different preparations. These variations are probably due to differences in the age of the gametes and in conditions during gametogenesis.

Table 2. Comparison of the composition of the soluble amino acids released into the cell-free supernatant of mating gametes of *Chlamydomonas reinhardi* to the amino acid composition of normal cell walls (per cent by mass)

| Amino acid | Vegetative wall relics* | Supernatant of mixed gametes† | Zygotic walls‡ |
|---|---|---|---|
| Lys | 4.36 | 4.8 | 5.6 |
| His | 0.74 | 1.0 | 0 |
| Arg | 5.77 | 4.4 | 7.1 |
| Asp | 9.77 | 8.8 | 8.5 |
| Thr | 6.17 | 6.9 | 2.2 |
| Ser | 6.91 | 7.2 | 4.8 |
| Glu | 7.90 | 8.4 | 9.4 |
| Pro | 5.91 | 6.3 | 3.1 |
| Gly | 4.30 | 5.8 | 11.4 |
| Ala | 6.80 | 7.9 | 7.3 |
| Val | 5.65 | 7.0 | 5.6 |
| Met | 1.83 | 1.3 | 5.4 |
| Ile | 3.02 | 3.6 | 2.2 |
| Leu | 7.52 | 7.3 | 12.4 |
| Tyr | 4.16 | 4.0 | 5.0 |
| Phe | 5.81 | 4.1 | 4.5 |
| Hypro | 13.42 | 11.1 | 5.6 |

* Data taken from Roberts [20].

† Hydrolysis of the concentrated crude supernatant was carried out by incubation with mercaptoethane-sulfonic acid for 24 hr. Almost total hydrolysis of peptide bonds resulted, since samples showed very low amounts of polypeptides upon analysis. Norleucine (a synthetic amino acid) was used to measure losses during the analytical process and over 90% of the norleucine was detected on the amino acid analyser.

‡ Data taken from Catt [33] and converted to per cent by mass.

## EXPERIMENTAL

*Cultures and production of gametes.* *Chlamydomonas reinhardi* wild type strain 137C (MT plus and MT minus) were obtained from Dr. M. Margulies of the Radiation Biology Laboratory, Smithsonian Institute. The cells were grown in phosphate buffered medium as described previously [5], except they were exposed to a 12:12 light–dark cycle and thus were in synchronous condition. Gametes were induced in liquid culture using growth medium without a N source ('N-free medium') as described by Kates and Jones [30], with the following changes. During the overnight conversion period, cultures were bubbled with a mixture of 95% air and 5% $CO_2$ and continuously illuminated. The mating efficiency was measured as previously described [7].

*Production and isolation of lytic factor.* Large quantities of vegetative cells were grown in 5 l. diphtheria toxin bottles and bubbled with a mixture of 95% air and 5% $CO_2$. Cells were converted to gametes as described above. For lytic factor production the gametes were washed once with N-free medium and resuspended in fresh N-free medium to a concn of $2 \times 10^8$ cells/ml. Equal numbers of opposite mating type gametes were mixed and mating allowed to occur, in the light and at room temp. (21°). After 90 min the cell suspension was centrifuged to remove a majority of the cells. The few remaining cells and cellular debris were removed by a high speed centrifugation (29 000 *g* for 10 min at 4°) in a Sorvall RC-2B. The crude lytic factor present in the supernatant was kept on ice until further use.

*Sample preparation and methods for analysis of lytic products.* Vegetative cells were grown and converted to gametes as described for production of lytic factor. Gametes were harvested by a low speed centrifugation and resuspended to a concn of $1 \times 10^8$ cells/ml. Equal numbers of gametes of each mating type were mixed and allowed to mate for 3 hr. After the mating period, the cell suspension was centrifuged at low speed (to remove the cells) and the supernatant then centrifuged at 12 000 *g* for 10 min to remove any cells still remaining. The cell-free supernatant was transferred to a 500 ml flask and concd by flash evapn at 80°.

*Monosaccharide analysis by GLC.* The concd crude soluble material from the mating mixture was dissolved in 10 ml 1.0 M $H_2SO_4$ and heated in a sealed, air-free Pyrex tube at 100° for 18 hr. After cooling the tube was opened and the soln neutralized with $BaCO_3$. The ppt. which formed upon neutralization was removed by centrifugation. Excess of barium ions was pptd by addition of an equivalent amount of dil. $H_2SO_4$ and removed by filtration. The hydrolysate was then lyophilized. Tri-Sil 'Z' (Pierce Chemical Co.) was added to the powdered sample to produce the trimethylsilated derivatives.

GLC separation of the sugar derivatives was carried out on a Becker Gas Chromatograph (Packard Model 417) equipped with a $H_2$ flame ionization detector, a mini laboratory integrator and a chart recorder. The column (4 mm i.d. × 1.5 m) contained SE-30 ultraphase 3% on chromosorb W (HP) 80/100. $N_2$ flow was 36 psi, hydrogen 20 psi, and air 30 psi. The injector block temp. was maintained at 240° and the detector at 220°. Column temp. was initially set at 150° for 15 min and programmed to rise linearly at 1°/min to a final temp. of 220°.

*Amino acid analysis.* The method used for protein and polypeptide hydrolysis was described in ref. [31]. Hydrolysis was performed in a sealed air-free Pyrex tube. A portion of the crude sample (see above) was first hydrolyzed with 3 ml 3 N mercaptoethane-sulfonic acid at 110° for 24 hr. After hydrolysis the tube was cooled and the hydrolysate neutralized with 1 N NaOH. The neutralized hydrolysate was then lyophilized. The amino acid content of the hydrolysate was determined with a Beckman Model 120° Amino Acid Analyzer.

*Carbohydrate release assay.* A suspension of gametes at $2 \times 10^7$ cells/ml of one mating type was mixed with a suspension of equal vol. (and concn) containing opposite mating type gametes. The mixed cell suspension was sampled at timed intervals by centrifuging out the cells and assaying the cell-free supernatant for released carbohydrate using the anthrone reaction as previously described [5].

*Protein release assay.* The procedure for this assay was identical to that for carbohydrates, except the cell concn used was $1 \times 10^8$ cell/ml, and the protein concn of the cell free supernatants was determined by the method of ref. [32]. Bovine serum albumin was used as a standard for these determinations.

*Acknowledgements* — We would like to thank Ms. K. McCollum for technical assistance with the amino acid analysis, Dr. H. Nakada for assistance with GLC and Dr. N. Sharon for helpful suggestions. This work was supported by NSF Grant PCM 77-07380.

## REFERENCES

1. Goodenough, U. W. (1977) *Microbial Interactions (Receptors and Recognition)* (Reissig, J. L., ed.) Ser. B, Vol. 3, p. 324. Chapman & Hall, London.
2. Wiese, L. (1965) *J. Phycol.* **1**, 46.
3. Forest, C. L., Goodenough, D. A. and Goodenough, U. W. (1978) *J. Cell Biol.* **79**, 74.
4. Solter, K. M. and Gibor, A. (1977) *Nature* **265**, 444.
5. Solter, K. M. and Gibor, A. (1977) *Plant Sci. Letters* **8**, 227.
6. Solter, K. M. and Gibor, A. (1978) *Exp. Cell Res.* **115**, 175.
7. Ray, D. A., Solter, K. M. and Gibor, A. (1978) *Exp. Cell Res.* **114**, 185.
8. Ray, D. A., Solter, K. M. and Gibor, A. (1978) *J. Cell. Biol.* **79**, 164a.
9. Bergman, K., Goodenough, U. W., Goodenough, D. A., Jawitz, J. and Martin, H. (1975) *J. Cell Biol.* **67**, 606.
10. Wiese, L. and Wiese, W. (1978) *Cell–Cell Recognition* (Society for Experimental Biol. Symposium No. 32) (Curtis, A. S. G., ed.) p. 83. Cambridge University Press, Cambridge.
11. Wiese, L. and Hayward, P. C. (1972) *Am. J. Botany* **59**, 530.
12. Goodenough, U. W., Hwang, C. and Martin, H. (1976) *Genetics* **82**, 169.
13. Goodenough, U. W., Hwang, C. and Warren, A. J. (1978) *Genetics* **89**, 235.
14. Forest, C. L. and Togasaki, R. K. (1975) *Proc. Natl. Acad. Sci., U.S.A.* **72**, 3652.
15. Hyams, J. and Davies, D. R. (1972) *Mutation Res.* **14**, 381.
16. Horne, R. W., Davies, D. R., Roberts, K. and Gurney-Smith, M. (1971) *Nature* **232**, 493.
17. Hills, G. J. (1973) *Planta* **115**, 17.
18. Hills, G. J., Gurney-Smith, M. and Roberts, K. (1973) *J. Ultrastruct. Res.* **43**, 179.
19. Hills, G. J., Philips, J. M., Gay, M. R. and Roberts, K. (1975) *J. Molec. Biol.* **96**, 431.
20. Roberts, K. (1974) *Phil. Trans. R. Soc. London* **268B**, 129.
21. Catt, J. W., Hills, G. J. and Roberts, K. (1978) *Planta* **138**, 91.
22. Schlosser, U. G. (1966) *Arch. Mikrobiol.* **54**, 129.
23. Schlosser, U. G., Sachs, H. and Robinson, D. G. (1976) *Protoplasma* **88**, 51.
24. Claes, H. (1971) *Arch. Mikrobiol.* **78**, 180.
25. Claes, H. (1975) *Arch. Mikrobiol.* **103**, 225.
26. Goodenough, U. W. and Weiss, R. L. (1975) *J. Cell. Biol.* **67**, 623.
27. Matsuda, Y., Tamaki, S. and Tsubo, Y. (1978) *Plant Cell Physiol.* **19**, 1253.
28. Claes, H. (1977) *Exp. Cell Res.* **108**, 221.
29. Robinson, D. G. and Schlosser, U. G. (1978) *Planta* **141**, 83.

30. Kates, J. R. and Jones, R. F. (1964) *J. Cell Comp. Physiol.* **63**, 157.
31. Penke, B., Gerenzi, R. and Kovacs, K. (1974) *Analyt. Biochem.* **60**, 45.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
33. Catt, J. W. (1979) *Plant Sci. Letters* **15**, 69.
34. Miller, D. H., Mellman, I. S., Lamport, D. T. A. and Miller, M. (1974) *J. Cell Biol.* **63**, 420.
35. Minami, S. A. and Goodenough, U. W. (1978) *J. Cell Biol.* **77**, 165.