

THE CHEMICAL COMPOSITION OF THE CELL WALL OF *CHLAMYDOMONAS GYMNOGAMA* AND THE CONCEPT OF A PLANT CELL WALL PROTEIN

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ABSTRACT

Cell walls of *Chlamydomonas gymnogama*, shed during sexual mating, were collected and analyzed. Ultrastructural examination indicates that the walls are free of cytoplasmic contamination and that they exhibit a regular lamellate structure. The walls are composed of glycoprotein rich in hydroxyproline. The hydroxyproline is linked glycosidically to a mixture of heterooligosaccharides composed of arabinose and galactose. Altogether, the glycoprotein complex accounts for at least 32% of the wall. The amino acid composition of the walls is extraordinarily similar in widely different plant species. The implications of these similarities as well as the widespread occurrence of these glycoproteins are discussed.

The cell walls of all green plants so far examined, with the exception of the Charales (10), possess hydroxyproline-rich glycoproteins (10, 15, 19, 35), indicating that these glycoproteins are of fundamental importance.

In most of the plant kingdom, hydroxyproline arabinoside is the only hydroxyproline oligosaccharide released by alkaline hydrolysis (19); in a recent study of *Chlamydomonas reinhardtii*, hydroxyproline was found linked to a variety of oligosaccharides unlike those in any other plant cell wall (24). Unfortunately, the cell wall preparations obtained from *C. reinhardtii* were relatively crude, because the fragility of the cell walls made their isolation by mechanical means extremely difficult. Thus, an accurate measure of the contribution of these heterooligosaccharides to the wall was not possible; particularly bothersome was the possibility that the location of certain of these

oligosaccharides might be cytoplasmic only. Nevertheless, their uniqueness and the likelihood that they were genuine wall components led us to believe that there might be other important differences between *Chlamydomonas* cell walls and the walls of more advanced green plants.

Also, persistent reports have been made expressing doubt about the location of hydroxyproline-rich protein in the cell walls of both algae (33) and higher plants (34).

For these reasons we initiated the present study involving a more complete chemical analysis of cell walls of *Chlamydomonas*. To overcome problems of cell wall purity in the present study, we used *Chlamydomonas gymnogama*, which sheds its cell walls *intact* during the mating process. Differential centrifugation allowed the separation of these cell walls from whole cells, and their analysis is reported here.

MATERIALS AND METHODS

Cultures of *C. gymnogama* (IUCC no. 1638) were obtained from Temd Deason (7). They were grown axenically in 6 liters of Bristol's medium in 12-liter flasks, bubbled with air, and maintained in synchronous culture (4). After 5-days' growth, when packed cell volumes of 6.0 ml/liter had been attained, the cultures were centrifuged, the old medium was decanted, and cells were resuspended in nitrate-free Bristol's medium, and inoculated into another 12-liter flask of 6 liters of minus nitrate Bristol's. These cultures were bubbled with air and maintained in continuous yellow orange light (orange Cinemoid plastic filter). After about 5 days under these conditions some 20–50% of cells had mated and shed their walls. Low-speed (250 g) centrifugation pelleted whole cells and higher speeds (3,000 g) pelleted cell walls. Cell walls were washed twice with distilled water, resuspended in distilled water, freeze-dried, weighed, and analyzed. Cultures were assayed for sterility by using both nutrient broth and microscope examination.

Microscopy

Cell wall preparations were examined using both phase-contrast and electron microscopy. For the latter, walls were fixed by the method of Franke et al. (9), and poststained in uranyl acetate followed by lead citrate. Sections were cut with diamond knife and examined with a Zeiss EM 9 electron microscope.

Preparation of Old Culture Medium

The medium in which vegetative cells had been grown was concentrated at 35°C under vacuum from 6 liters to 100 ml and again centrifuged. It was then dialyzed overnight and was made up to 80% ethanol. The resultant flocculent precipitate was washed with 95% ethanol, dried, and analyzed in the same manner as the cell walls.

Walls were assayed for their polysaccharide content as described earlier (24). Uronic acid content was determined by the modified carbazole method of Bitter and Muir (2). Amino acid analyses were made using a modified Technicon Autoanalyser (16). Lipid content was determined by extracting the walls twice in boiling chloroform:methanol (2:1) for 5 h (32). Ash content was determined by heating a sample of walls on a platinum boat in an oven at 800°C for 4 h. Water content was estimated by repeated drying over P_2O_5 *in vacuo*.

The preparation, separation, and analysis of hydroxyproline oligosaccharides were performed as detailed elsewhere (24). Because sequence analysis of the heterooligosaccharide required much larger amounts of material than were obtainable with pure cell walls, a crude cell wall preparation similar to one described previously (24) was used for sequencing. The assumption that the heterooligosaccharides were the same in either of these

two different cell wall preparations is supported by findings reported in Results.

To assess the contribution of hydroxyproline to the weight of cell wall as well as whole cells, a fraction of cell wall and whole cell preparations were diluted and counted in a hemacytometer. These preparations were dried in the frozen state and weighed, then assayed for their hydroxyproline content (16).

RESULTS

A typical sample of cell walls as seen by phase-contrast microscopy is shown in Fig. 1. Fig. 2 shows a similar sample examined in the electron microscope. This preparation and others like it showed the complete absence of membranous material, indicating, as we had surmised, that the gametes carry their plasmamembrane with them when they shed their walls. In addition, the shed gamete walls (Fig. 3) resemble attached cell walls of both vegetative cells (Fig. 4) and gametes (Fig. 5), as well as exhibiting a lamellar pattern similar to that observed in the cell walls of *C. reinhardtii* (29). In Fig. 5, an osmiophilic globule can be seen. These

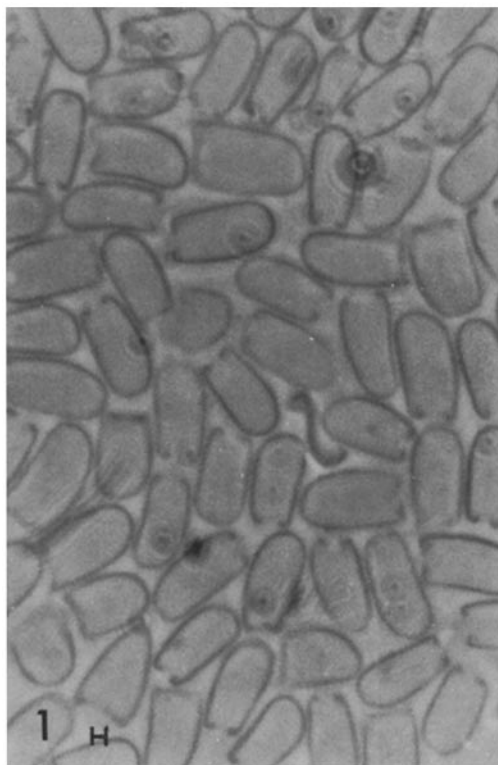


FIGURE 1 A typical cell wall preparation viewed by phase-contrast microscopy. $\times 1,300$.

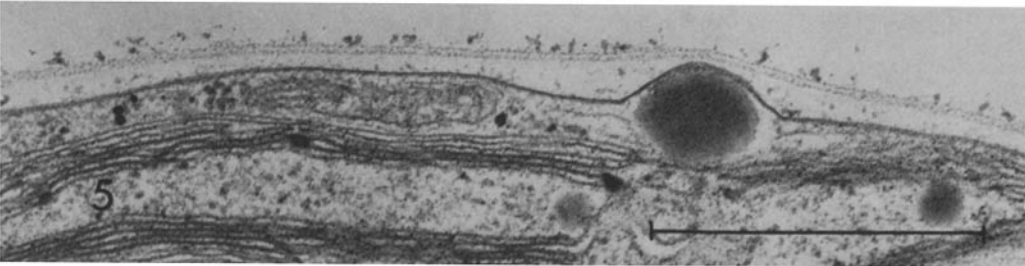
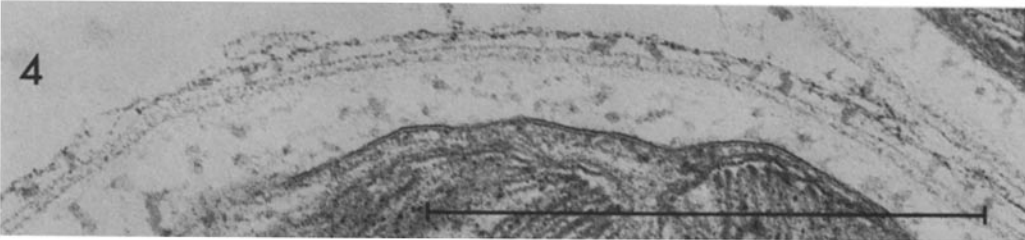
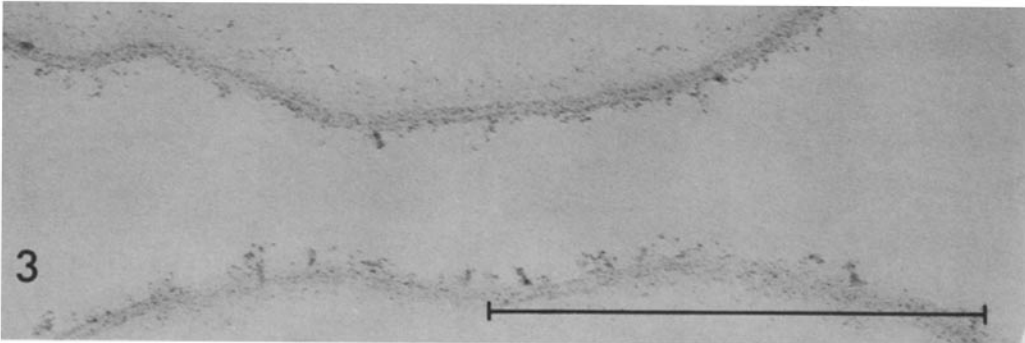
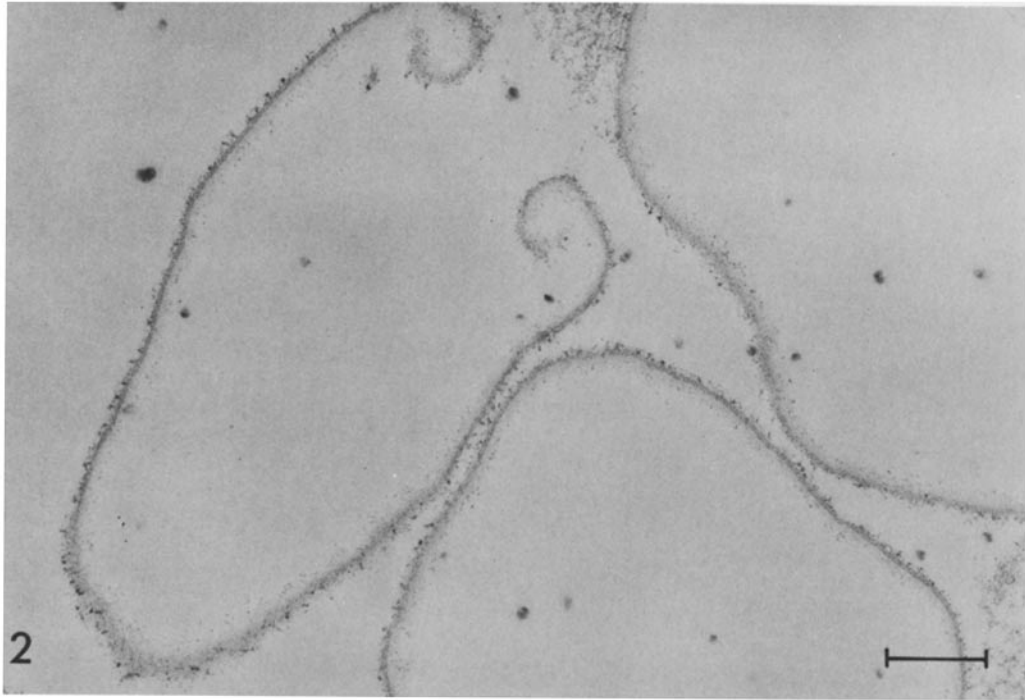


TABLE I
Chemical Composition of Cell Walls of
C. gymnogama

Constituent	Percentage of dry weight
Arabinose	24.8
Galactose	19.6
Mannose	1.4
Xylose	2.2
Glucose	1.8
Uronic acid (calculated as galacturonic acid)	5.3
Unknown sugars	2.0
Hydroxyproline	3.0
Other amino acids	7.2
Lipid	6.9
Ash	10.2
Water	8.0
Total accounted for	92.4

Hydrolyses for sugars and amino acids were done with 2 N trifluoroacetic acid and 6 N HCl, respectively. Stronger acid hydrolysis (72% H₂SO₄) yielded no increase in the glucose content of the walls.

structures are numerous in gametes and appear to be extruding from the cell.

Table I shows the chemical composition of the cell walls. Arabinose and galactose are the predominant sugars, with lesser amounts of uronic acid, glucose, mannose, and two unknown monosaccharides. The sugars constitute about 50% of the wall. Amino acids account for another 10% of the wall, with hydroxyproline being the predominant one. When added together, the various wall constituents total only about 90% of the dry weight of the walls. This probably represents inaccuracies in measuring water of hydration as well as a certain amount of decomposition of sugars and especially amino acids during acid hydrolysis. In this connection, considerable darkening of the hydrolysate occurred during hydrolysis in 6 N HCl; some humin was visible after completion of the hydrolysis, and considerable ammonia was present in the amino acid analyses, indicating that

decomposition occurred. The TFA hydrolysis done for sugar analysis yielded a clear pale yellow solution with no visible particulate matter. Thus, the 90% yield probably reflects amino acid rather than sugar losses.

Table II shows the amino acid composition of *C. gymnogama* cell walls. Because the protein in these walls was synthesized under conditions of nitrogen starvation, we were concerned that this might result in an atypical amino acid composition of the walls. To test for this possibility, we analyzed an alcohol precipitate prepared from concentrated medium in which vegetative cells had grown and divided. After cell division, the mother cell wall gradually becomes diffuse and disappears into the medium. Thus old medium should contain quantities of cell wall glycoprotein typical of vegetative cell walls. The amino acid composition in the column marked *old medium* was from such and is very similar to that of the cell walls, as are those from the extracellular matrix of *Volvox carteri* and cell walls of tomato. The significance of these similarities will be discussed later.

Fig. 6 shows a profile of the hydroxyproline oligosaccharides released from the cell wall. An estimate of the average number of sugars attached to all the hydroxyproline in the wall indicates that the glycoprotein complex accounts for a total of at least 32% of the weight of the wall. *C. gymnogama* walls appear to release a much more complex mixture of oligosaccharide than do *C. reinhardtii*. This fact prevented a complete sequencing of the different species of oligosaccharide. We succeeded in determining a tentative sequence for seven of the heterooligosaccharides, listed in Table III. It appears that the sugars attached to the hydroxyproline are basically similar to those in *C. reinhardtii* walls, consisting of heterooligosaccharide composed of a mixture of arabinose and galactose. There are, however, several differences between the hydroxyproline oligosaccharides found in the two species of *Chlamydomonas*. For example, neither Hyp-Gal nor Hyp-Ara-Glc-Ara-Gal-Ara is

FIGURE 2 Cell walls as seen by electron microscopy. $\times 12,000$.

FIGURE 3 A higher magnification of two adjacent released cell walls. $\times 64,000$.

FIGURE 4 A cell wall and adjacent portion of a vegetative cell. Note the plasmamembrane and the similarity between the lamellate structure of this cell wall and that in Figs. 3 and 5. $\times 76,000$.

FIGURE 5 A wall and adjacent cell of a gamete. Note the osmiophilic globule pushing up against the wall. These structures are prevalent in gamete cells. $\times 45,000$.

TABLE II
Amino Acid Compositions of Four Samples

	<i>C. gymnogama</i> cell walls	Old medium	<i>Volvox</i> sheath*	Tomato cell wall†
Cyst	3	0	0	5
Hyp	30	30	30	30
Asp	10	12	12	8
Thr	10	9	10	6
Ser	11	12	11	15
Glu	7	9	5	9
Pro	6	9	6	8
Gly	9	10	25	8
Ala	11	14	11	7
Val	7	11	8	8
Ilu	4	3	3	5
Leu	9	9	6	9
Tyr	2	3	3	3
Phe	4	4	2	3
Lys	4	5	4	11
His	0	1	1	2
Arg	4	5	4	4
Total residues	131	145	141	139

The analyses have been normalized to 30 residues of hydroxyproline in view of the calculations which indicate the fundamental wall protein subunit to be in the range of 125–250 residues (17).

* Lamport and Kochert (1971. Unpublished results). Analysis of *Volvox* matrix after release by proteolytic digestion and ethanol precipitation.

† Taken from Lamport (16).

present in *C. gymnogama*. One possible explanation for this is that these differences reflect the use of clean wall preparations in this report vs. the crude preparations used earlier (24). Thus, the missing heterooligosaccharides might be of cytoplasmic origin. We tested this possibility in two ways, and in both cases it appears unlikely.

First, the hydroxyproline analysis and weighings of cell wall and whole cell preparations indicate that (a) hydroxyproline accounts for 3% of the cell wall weight and 0.10% of whole cell weight; (b) the average weight of a gamete cell wall is 2.9 pg, while that of an intact gamete is 99 pg; thus, about 2.9% of a gamete's weight is due to its cell wall. This means that over 87% of the hydroxyproline in a cell must be located in the wall, making the contribution of cytoplasmic hydroxyproline-rich glycoprotein rather insignificant, except as it might contribute specific hydroxyproline heterooligosaccharides to the mixture released by alkaline hydrolysis. Even this possibility appears unlikely, however, because the elution pattern of both whole cell and cell wall heterooligosaccharide as well as a qualitative analysis of each major peak are identi-

cal in either fraction. This means that none of the oligosaccharides are located exclusively in the cytoplasm or the wall, because no noticeable enrichment of any of them was seen in these different elution patterns. Thus, the differences in hydroxyproline heterooligosaccharide seen between the two species of *Chlamydomonas* more likely reflect species differences rather than preparative differences.

In *C. reinhardtii*, also, the likelihood that certain hydroxyproline heterooligosaccharides are of strictly cytoplasmic origin appears remote. Thus, we analyzed a whole cell sample from the wall-less mutant, CW 15+, of *C. reinhardtii* (6) and found that it releases hydroxyproline heterooligosaccharides in the same profile and of the same qualitative analysis as are present in crude cell wall preparations of both + and – mating types of wild type *C. reinhardtii*.

DISCUSSION

It is important to stress that the cell walls collected for these analyses are biologically and biochemically pure, since, as the micrographs demonstrate,

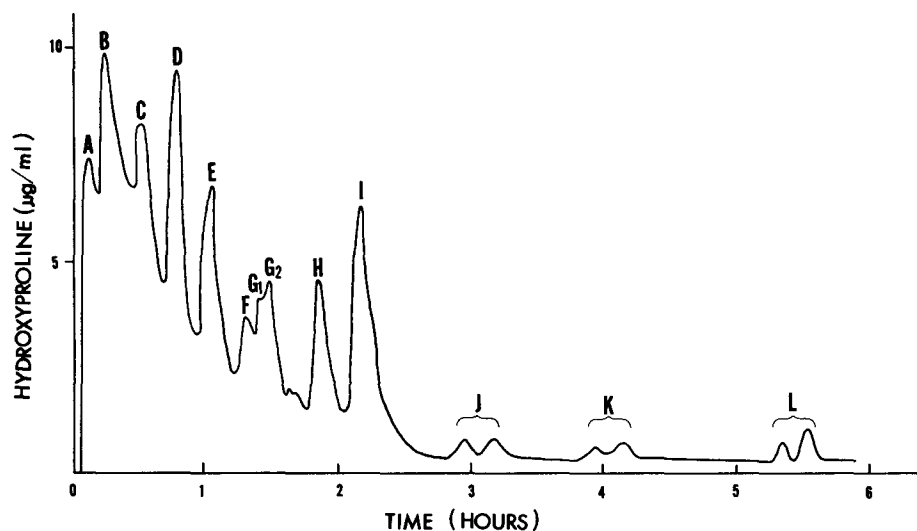


FIGURE 6 The hydroxyproline-oligosaccharide profile from cell walls. Several of the glycosides were further analyzed, and their tentative analysis is reported in Table III.

TABLE III
Composition and Partial Sequencing of Hydroxyproline-Glycosides Released from Cell Walls

Glycosides released	Hyp (% of total)	Estimated molar* ratios				Theoretical molar ratios	Tentative sequence of Hyp oligosaccharide
		Ara	Gal	Mann	Hyp		
Hyp A†	7.0	2.2	7.8	1.2	1	Hyp-Ara ₂ -Gal ₅ -Mann ₁	—
Hyp B†	21.0	3.8	5.7	0.3	1	Hyp-Ara ₄ Gal ₆	—
Hyp C	14.0	4.3	1.7	0	1	Hyp-Ara ₄ Gal ₂	—
Hyp D	16.5	3.9	1.5	0	1	Hyp-Ara ₃ -Gal ₂	<i>trans</i> Hyp-Ara-Ara-Ara-Gal-Gal§
Hyp E	9.0	3.1	1.7	0	1	Hyp-Ara ₃ -Gal ₂	<i>cis</i> Hyp-Ara-Ara-Ara-Gal-Gal
Hyp F	3.0	3.2	0.8	0	1	Hyp-Ara ₃ -Gal ₁	—
Hyp G ₁	5.7	2.8	0.85	0	1	Hyp-Ara ₃ -Gal ₁	—
Hyp G ₂	1.6	3.8	0.30	0	1	Hyp-Ara ₄	<i>cis</i> Hyp-Ara-Ara-Ara-Ara
Hyp H	6.7	1.8	0.9	0	1	Hyp-Ara ₂ -Gal ₁	<i>trans</i> Hyp-Ara-Ara-Gal
Hyp I	11.4	1.7	0.85	0	1	Hyp-Ara ₂ -Gal ₁	<i>cis</i> Hyp-Ara-Ara-Gal
Hyp J	1	1.8	0	0	1	Hyp-Ara ₂	<i>trans</i> and <i>cis</i> Hyp-Ara-Ara
Hyp K	1	0.9	0	0	1	Hyp-Ara	<i>trans</i> and <i>cis</i> Hyp-Ara
Hyp L	1.7	0	0	0	1	Hyp	<i>trans</i> and <i>cis</i> Hyp

* Except for Hyp-glycosides E, H, I, J, K, and L, each peak contained a mixture of *trans* and *cis* Hyp, indicating that they were composed of more than one glycoside. This made sequencing of the sugar attached to hydroxyproline impossible, and an accurate interpretation of the theoretical molar ratios tentative.

† The theoretical molar ratios of these glycosides can be only tentative because the glycoside eluted with the void volume of the column, and this fraction may contain free sugars released during alkaline hydrolysis. Hence, the exact sugar content is not yet known, but the elution position of these glycosides on a Sephadex G-25 column indicates a molecular weight consistent with this empirical formula.

§ This glycoside was not the only one present under peak D, as a mixture of *cis* and *trans* hydroxyproline was demonstrated. Another glycoside, probably richer in Ara than the one sequenced, was present, but in not enough quantity for analysis. The present data do not exclude a branched structure.

the organism retains its plasmamembrane intact while shedding its wall; thus, the wall should contain no or at most a minimum of cytoplasmic contamination. This eliminates the need for exten-

sive washing of walls with a variety of reagents, techniques which have been used previously (29, 35) and which must inevitably lead to the loss of some real wall components as well as the contami-

nants. It is possible that partial lysis of cell walls occurs before and during the wall-shedding process, as partial lysis of cell walls has been observed during mating in *C. reinhardtii* (5). This could lead to loss of some real wall components, and result in a different wall composition.

In discussing how clean the walls are, it is important not to lose sight of the fact that some degree of interaction between the wall and the cytoplasmic interior takes place. Thus, as gametes develop toward sexual maturity, they produce large numbers of osmiophilic globules which appear to extrude through the membrane. The composition of these bodies (perhaps lipid or mucilage) and a determination of how much they contribute to the chemical composition of the wall remain to be investigated. Similar densely staining material, presumed to be wall precursor, was observed in *C. reinhardtii* (6). Evidence is also beginning to accumulate that carbohydrate from the plasmamembrane is released as part of a continual turnover process (14). This plus the presence of enzymes on cell walls (31) emphasize the fact that the wall is in a state of flux with the cytoplasm and the external environment. Thus, cell wall chemistry may vary considerably at different stages of development of the cell itself.

Some cell wall preparations contained quantities of mucilage, which is produced by young zygotes and accumulates in nitrate-free cultures after sexual fusion has occurred. Since separation of walls and mucilage by centrifugation was impossible, we obtained walls only from cultures which had just mated, and hence had a minimum of mucilage.¹ However, chemical analyses indicated that wall preparations with or without mucilage were very similar, both consisting of hydroxyproline-rich glycoprotein. Slime and cell walls are also similar chemically in other species of algae (21, 23). Thus, it would seem that the organization of the glycoprotein into highly lamellate cell walls or less organized mucilage is less dependent on its empirical chemical composition than it is on the organization and self assembly of the glycoprotein components. Preliminary analyses of the mucilaginous sheath surrounding colonies of *V. carteri* (18) indicate it to have a composition similar to that of *C. gymnogama* walls. In addition, the isolation from old vegetative culture medium of a glycopro-

¹Sex and slime seem to be related throughout the entire plant and animal kingdoms.

tein similar chemically to that of cell walls reinforces the idea that what are at present undetectable or relatively subtle differences in chemical linkages make a great deal of difference in the final organization of the components. We have observed the gradual dissolution of the old mother wall which occurs concomitant with the deposition of new walls around daughter cells during cell division. Such a simultaneous deposition of new wall and decomposition of old wall raises interesting questions about the extracellular control of wall organization. Evidence for continual cell wall turnover (13) as well as nucleating agents controlling wall assembly in *C. reinhardtii* (11) has been noted.

Another interesting finding of the present study is the fact that the glycoprotein complex accounts for at least 32% of the weight of the cell wall. The alkaline hydrolysis treatment which releases the hydroxyproline oligosaccharides shown in Fig. 6 almost certainly hydrolyzes some glycosidic linkages and strips some of the sugars from the carbohydrate part of the glycoprotein. Thus, it is quite reasonable to assume that the bulk of the cell wall in *C. gymnogama* is composed of hydroxyproline-rich glycoprotein.

The low levels of glucose found in the walls indicates an absence of cellulose. Several reports on *C. reinhardtii* (1, 13, 30) indicate a lack of cellulose in its walls. Our results corroborate and extend these findings to *C. gymnogama*. In addition, many other species of green algae (8, 20), particularly marine members (22, 27, 28), have noncellulosic walls.

Hydroxyproline-rich glycoprotein now appears to be a more nearly ubiquitous cell wall component than is cellulose, being present in cellulosic as well as noncellulosic walls (10, 15, 19, 25, 26, 28, 35). In fact, in all chemical examinations of green plant cell walls, except for the Charophyta (10, 35), the presence of hydroxyproline-containing protein has been demonstrated. There have been past instances when no hydroxyproline was found (26, 29), but more exhaustive efforts have resulted in a successful demonstration of its presence (10). In fact, we recently re-examined the amino acid content of *Platymonas* cell walls and found that they contained 0.1% hydroxyproline, which had been previously (22) overlooked. One possible exception may be *Pleurochrysis* (3). The relative amounts of hydroxyproline among the other amino acids observed in the wall vary considerably, however,

from 0.06% (10, 25) in *Chlorella pyrenoidosa* up to 30% in *C. gymnogama*. This variation may be due to the presence of cytoplasmic protein contaminants or hydroxyproline-poor protein in the wall. At any rate, what is clear is that hydroxyproline-containing protein is very nearly ubiquitous in green plant cell walls.

Because this hydroxyproline-rich glycoprotein is so widespread in the plant kingdom, some comparative biochemical analysis will be of interest. There is some variation between different plant species in the oligosaccharide attached to hydroxyproline in these glycoproteins. For example, *C. gymnogama* walls contain more hydroxyproline arabinoside than *C. reinhardtii*, and neither Hyp-Ara-Glc-Ara-Gal-Ara nor Hyp-Gal was present, though they were found in *C. reinhardtii*. Basically, however, a mixture of arabinose and galactose heterooligosaccharides predominates in both species. No differences among hydroxyproline oligosaccharides released from cell walls, whole cells, or cytoplasmic preparations were found within either species of *Chlamydomonas*; this fact makes it unlikely that certain hydroxyproline oligosaccharides are localized in cytoplasm or cell wall fractions exclusively. We examined a third species of *Chlamydomonas*, *C. ulvaensis*, because it was found to be unique among the chlamydomonads surveyed in that its extracellular polysaccharide was composed of glucose and xylose, rather than arabinose and galactose (21). To see if any of this glucose was hydroxyproline bound, we released hydroxyproline oligosaccharide from *C. ulvaensis* and found that, as in the other two species of *Chlamydomonas*, arabinose and galactose were the only sugars present, indicating that the glucose and xylose were probably not part of the glycoprotein complex. *Codium*, another noncellulosic green alga containing hydroxyproline in its walls (28), was also examined. Alkaline hydrolysis of its cell walls released more than 95% of the hydroxyproline free of sugar, while the remainder consisted of hydroxyproline galactoside. All this seems to indicate that there is considerable variation in the sugars and their linkages to hydroxyproline in the glycoprotein complexes of green algal cell walls, just as there is variation in the polysaccharide content of different cell walls.

Thus, it seems that if a common structural unit exists in the glycoprotein, it will most likely reside in its amino acid sequence. The strongest evidence for this comes from the striking similarity between

the amino acid compositions of the cell wall preparations shown in Table II. Perhaps most telling is the comparison between the cell walls of *C. gymnogama* and tomato, two very distantly related green plants. It will be of great interest to determine the amino acid sequence of the protein in these two plants in order to verify what we suspect from the amino acid compositions. If the pentapeptide sequence, Ser-Hyp-Hyp-Hyp-Hyp, previously found in tomato and sycamore (17), were also to be found in *Chlamydomonas* walls, this would indicate that the amino acid sequence has been retained throughout a long evolutionary span and would emphasize the structural and functional importance of this particular pentapeptide sequence. Clearly, the glycoprotein already appears to have considerable similarities, at least in amino acid content and the possession of a hydroxyproline-sugar linkage.

Despite the mounting evidence of continued new reports of these glycoproteins in plant cell walls, persistent reports doubting their wall location in both green algae (34) and higher plants (33) have appeared. We believe that the present analysis demonstrating large quantities (at least 32%) of hydroxyproline-rich glycoprotein in what are indisputably pure cell wall preparations as well as the striking similarities of amino acid composition shown in Table II greatly strengthens the hypothesis that these proteins are of fundamental importance to plant cell walls.

We are, however, left in somewhat of a dilemma when it comes to making an accurate assessment of the importance and functional significance of hydroxyproline-rich glycoproteins. They are very nearly universally present in green plant cell walls and there are indications of considerable similarities in their amino acid content among widely disparate green plants. Yet, some groups of green plants, notably the Charophyta (10, 35) as well as other major divisions of plants, the brown and red algae (10), are completely without such glycoprotein. A resolution to this seemingly paradoxical situation awaits further research.

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