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Release of Small Polyuronides from *Nitella* Cell Walls during Ionic Exchange

Claude Gillet*, Pierre Cambier, and Françoise Liners
Facultés Universitaires de Namur, B-5000 Namur, Belgium

ABSTRACT

Mono-divalent ion exchange in isolated cell walls of *Nitella flexilis* (L.) Ag. induces a marked loss of wall polymers and a decrease in the wall cationic exchange capacity. These data correlate with the replacement in the walls of adsorbed Mn^{2+} by Na^{+} ions. Boiling wall samples in methanol for 1 h or keeping the ionic solutions chilled to 4°C does not inhibit the cell wall polymer leakage but modifies the kinetics both of the ionic exchange and of the released polymers. These data are more compatible with physical rather than enzymic induced processes. The extracted polymers in the successively renewed NaCl solutions initially belong to the wall protein and pectin fractions and mainly to pectic fractions subsequently. Determination of the average degree of polymerization shows that the average molecular size of the lost acidic polysaccharides increases with extraction time up an average polymerization degree of 25. Enzyme-linked immunosorbent assay inhibition tests show the presence of homopolymer blocks equal to or higher than 10 in the released polymer fragments. Compositional analysis of released polysaccharides suggests that the pectin lost by action of monovalent ions was largely composed of rhamnogalacturonans whose acidic residue fraction is approximately 60% in association with galactose chains. Small quantities of glucuronylated xylans are also found.

Small quantities of cell wall polysaccharides may be secreted into the free space of the wall from where they diffuse into the growth medium of suspension cultures of many higher plant cells (19). This polysaccharide release may be increased when the wall-loosening enzymes are activated during growth (11) or when the walls are treated with solutions of chelating agents or with solutions of monovalent ions (17). These solutions remove from the wall divalent ions that are implicated through ionic bridges in the cross-linking and the holding of pectin molecules in the wall. These modifications in the pectic content may be investigated in situ by measuring the variations in the CEC[1] of the wall because the pectic fraction is mainly responsible for the density of negatively fixed charges in the wall. In an isolated cell wall of Characeae, a group of freshwater algae whose unmethylated pectin content is high (3) and whose carbohydrates are clearly similar to those of the primary wall of higher plants (3, 20), Gillet et al. (7) observed a significant loss of CEC with a lowering of the concentration of divalent ions in the wall. This loss depends on the ionic strength within the wall. Homblé et al. (8), using Fourier transform IR spectroscopy, also showed in Characeae a loss of negatively charged carboxylic groups in the same external ionic conditions. The purpose of this research was to understand the nature of the charged polymers progressively released by the characean cell walls when incubated in the presence of NaCl solutions with a view to correlating the leaching of these different molecules with the modifications observed in the CEC and the additional possible effects on the ionic selectivity of the wall.

[1] Abbreviations: CEC, cationic exchange capacity; $\overline{DP}$, average polymerization degree; DP, polymerization degree.

MATERIALS AND METHODS

General Procedure

After the cell walls were isolated, the pieces of walls were all suspended in renewed 5 mM $MnCl_2$ solutions at 25°C. The Mn^{2+} content of the walls was measured. This amount is expressed on a dry weight basis and is assumed to be equal to the maximum initial amount of ionizable groups of the walls (initial CEC in equivalents per kilogram dry weight). The wall suspension was then divided into four approximately equal batches referred to as batches A, B, C, and D. Two batches, A and B, were dipped in boiling methanol to neutralize wall autolytic enzymes. After this treatment, the walls of these two batches were again suspended in 5 mM $MnCl_2$ solutions, and after equilibration, their Mn^{2+} wall content was measured. It was assumed to be equal to the remaining CEC after methanol pretreatment. The two other batches (C and D) were not pretreated and left in 5 mM $MnCl_2$ solutions.

With a view to inducing a loss of their pectic fraction by progressive replacement of their Mn^{2+} content by a monovalent ion, cell walls of the four batches were removed from the $MnCl_2$ solutions and were allowed to reequilibrate in 0.1 M NaCl solutions, which were renewed every 20 min. These ionic exchanges were carried out at 4°C for the walls of batches A and C and at 25°C for those of batches B and D. For each batch, every 20 min, the remaining Mn^{2+} and the remaining CEC (after reequilibration in 5 mM $MnCl_2$) were measured in successive samples of the walls. The carbohydrates released by the walls were analyzed in the successive equilibrium solutions. In total, the walls were soaked 12 times in renewed NaCl solutions. Except where specified, all experiments were carried out in triplicate.

Materials

The plants used were specimens of *Nitella flexilis* (L.) Ag. which were grown in pond water in laboratory tanks. Cell walls were prepared by simply cutting off the apex of the axis to avoid possible contamination by epiphytic microorganisms in older parts and were isolated by the fragmentation of entire cells in a mortar. The wall pieces were washed several times, initially with a mixture of ethanol and diethyl ether, and subsequently in distilled water to ensure that the cell membranes and cytoplasmic content were completely removed.

Equilibration with $MnCl_2$ Solutions

The wall pieces (about 4 g fresh weight) were put in Mn^{2+} form. This form was chosen because extracellular calcification occurs mainly in mature internodal cells of Characeae. This amount of nonexchangeable Ca^{2+} is hard to distinguish from the mobile Ca^{2+} by means of the methods of analysis that we used (6) and makes it difficult to interpret the results of an ionic exchange in which Ca^{2+} is used to monitor the replacement of bivalent by monovalent ions. Thus, the wall pieces were equilibrated with 5 mM $MnCl_2$ solutions, renewed 10 times in 24 h with continuous stirring, at 25°C and pH 5.35 before the exchange with solutions of monovalent ions. Following this pretreatment, a sample of the walls was removed to measure the total Mn^{2+} content (initial CEC). To this end, the sample was blotted between two Whatman No. 3 filter papers. Its cationic content was extracted overnight with 2 mL of a 1 N HCl solution and analyzed with a Philips PU 9200 X atomic absorption spectrophotometer.

Mn^{2+}-Na^+ Exchanges

As soon as the pretreatment in Mn^{2+} solutions was completed, the wall pieces were soaked in 15 mL of a 0.1 M NaCl solution. After 20 min the suspension was filtered using a Bellco Cellector Merck, Belgium. A sample of the residue, which consisted of cell walls eluted for the first time, was taken and divided into two parts, referred to as part I and part II. In part I, we measured the amount of Mn^{2+} remaining in the walls after the first 20 min of the exchange Mn^{2+}-Na^+. The walls of part II were then soaked in 5 mM $MnCl_2$ solutions, and their Mn^{2+} content was measured after equilibration. This amount is assumed to be equal to the remaining CEC in the walls after the first step of the exchange with monovalent ions. The remaining residue was soaked again for 20 min in a renewed 0.1 M NaCl solution until the next filtration. The filtrate of the first filtration was air concentrated under a vacuum, redissolved in 1.5 mL of distilled water, and was sterile filtered with a 0.22-μm Minisart syringe. It was then extensively dialyzed against distilled water and kept in a freezer for sugar analysis. This procedure was repeated for the different steps of wall incubation in the NaCl solutions.

Inactivation of Wall Enzymes

Because the wall autolysis and release of pectic polysaccharides may be the result of an enzyme-mediated mechanism, two samples of wall were boiled in 70% methanol, for 1 h, at 100°C before treatment with monovalent ions. Because this pretreatment might not be sufficiently drastic to rule out the involvement of wall enzymes, the Mn^{2+}-Na^+ exchanges for two batches were achieved in solutions that were kept chilled at 4°C throughout the ionic exchange.

Sugar Analysis

Total carbohydrate content of the released polysaccharide fractions was determined by colorimetry using the phenol-H_2SO_4 method (490 nm) (5). Uronic acid concentrations were estimated after hydrolysis with H_2SO_4 by specific colorimetric assays based on reaction with the *m*-hydroxydiphenyl method (520 nm) (4). The reducing sugar content was determined by means of the A_{276} of their condensation products with 2-cyanoacetamide (9). The $\overline{DP}$ for the polysaccharides released by the walls in each fraction was then obtained by the total uronic acids to reducing sugar ratio. Sugar composition of the different fractions was obtained after hydrolysis (2 h in 2 M TFA, 120°C) by GC of the trimethylsilylated derivatives of neutral and acidic sugars (22).

HPLC Determination of a Uronic Acid Pools

Samples (1 mL) of eluted fractions were injected onto a Pharmacia HPLC and separated using a 15-cm × 21.5-mm i.d. TSK DEAE-5PW column eluted with a linear gradient for 150 min from 0 to 0.25 M NaCl buffered with 25 mM sodium acetate buffer (pH 5.2) at a flow rate of 3 mL/min. Fractions of 3 mL were collected, and aliquots of 0.2 mL were assayed for uronic acid content (A_{520}).

The DP of the collected oligosaccharides were determined by comparison of elution times of peaks with that of a mixture of oligogalacturonic acids obtained by hydrolyzing a sample (10 gr/L) of a polygalacturonic acid (Sigma P1879). This hydrolysis was done in HCl by heating at reflux at 100°C for 45 h at pH 3.2.

Protein Measurement

The total protein content released into each NaCl solution was estimated using a colorimetric method after reaction with the Folin phenol and alkaline copper treatment (14). A_{660} was read against a BSA standard.

ELISA Test

The ELISA inhibition test was performed as follows: one volume of each polysaccharide fraction released (±80 μg/mL) was added to one volume of anti-pectin monoclonal antibody (2F4 ascites purified on proteins G, 450 μg/mL, diluted 1:40 in a 1-mN $CaCl_2$ and 150-mN NaCl solution). These mixtures were left overnight at 4°C. They were centrifuged for 10 min at 10,000 rpm before being used as primary antibody solutions in the conventional ELISA test as described by Liners et al. (12).

RESULTS AND DISCUSSION

Initial CEC

The mean initial CEC (as measured by bound Mn^{2+}) of the wall samples equilibrated in $MnCl_2$ solutions was 1.340 and 1.308 eq/kg dry weight for those treated with methanol before equilibration. No significant leakage of pectic fragments is thus induced by boiling methanol. These values can be compared with approximately 1.300 eq/kg dry weight obtained by Homblé et al. (8) for the apparent CEC of *Chara corallina* and by Morikawa et al. (16) for that of *Nitella axilliformis* in the presence of calcium ions and underline the fact that Mn^{2+} at the concentration used replaces Ca^{2+} in the walls.

Kinetics of Na^{2+} Exchange and of CEC Loss

The loss of CEC and the elimination of large quantities of Mn^{2+} from the walls occurred rapidly during the first nine treatments with renewed NaCl solutions (Fig. 1). The rate of Mn^{2+} exchange and CEC elimination was faster at 25°C for methanol-treated walls, but whatever the treatment, there were eventually no divalent ions left in the walls and the maximum CEC loss was similar for all the wall samples. We also observed that the degree of ionic conversion and the subsequent CEC loss depended, for the same volumes of Na^+ exchange solutions, on the initial quantity of the treated walls.

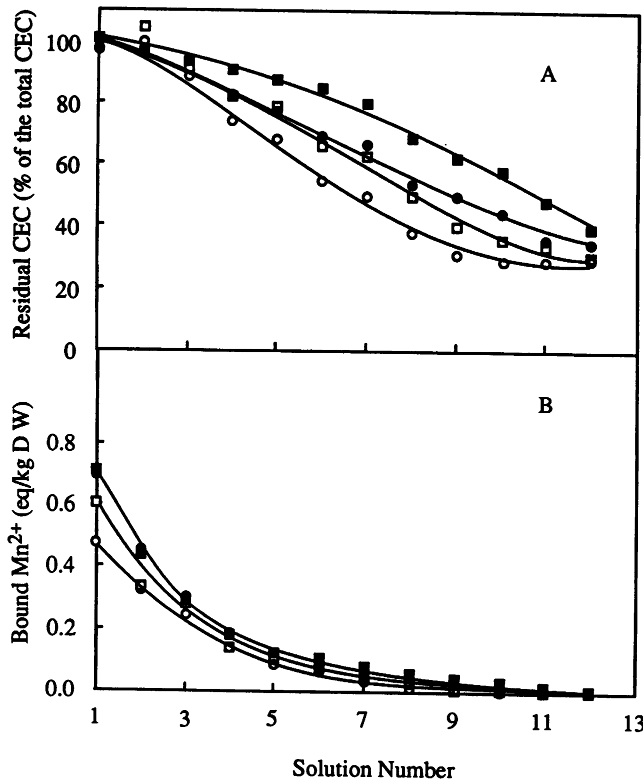

Figure 1. A, Time course of the CEC loss of *Nitella* cell walls first put in Mn^{2+} form and then soaked in renewed 0.1 M NaCl solutions. B, Time course of the decrease in the Mn^{2+} as the Mn^{2+}-Na^+ exchange proceeds. ●, Walls boiled in methanol before an ionic exchange was achieved in solutions kept chilled at 4°C; ○, boiled but ionic exchange at 25°C; ■, not boiled, ionic exchange at 4°C; □, not boiled, ionic exchange at 25°C. DW, Dry weight.

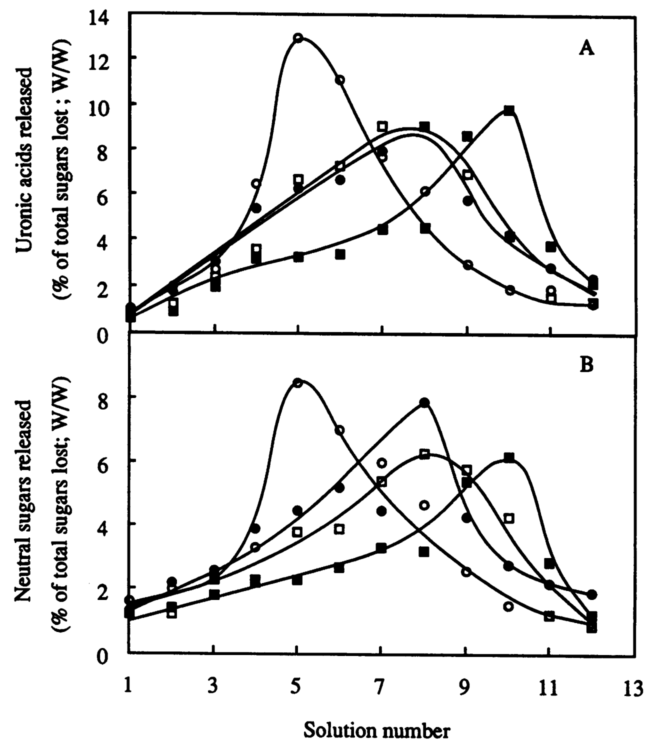

Figure 2. Time course of the amounts of acidic (A) or neutral (B) sugar residues found after hydrolysis of polymers released from *Nitella* cell walls soaked in renewed 0.1 M NaCl solutions. Same symbols as in Figure 1.

Release of Sugars by NaCl Treatments

When NaCl was substituted for $MnCl_2$, carbohydrates started to be released from the cell walls whether pretreated

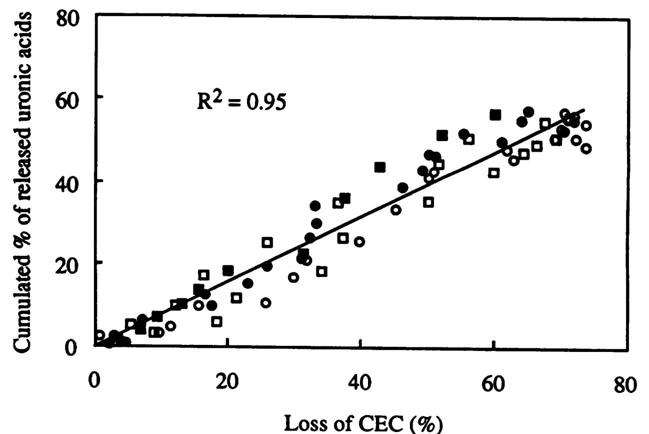

Figure 3. Linear relationship between the wall CEC loss and the cumulated amounts (percentage) of acidic residues found after hydrolysis of polymers released from *Nitella* cell walls in 0.1 M NaCl solutions. Same symbols as in Figure 1.

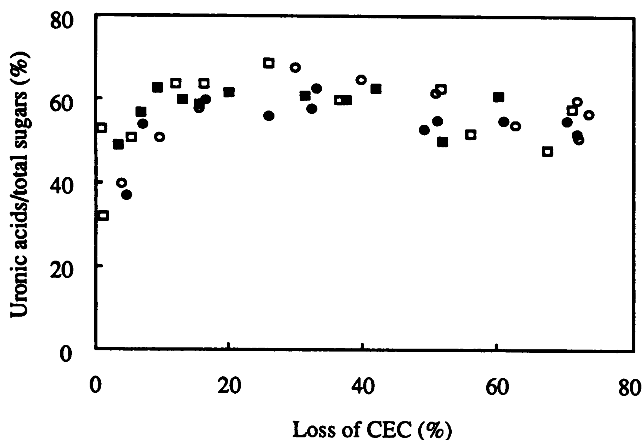

Figure 4. Variations in ratios of acidic residues to the amount of total sugars found after hydrolysis of polymers released from *Nitella* cell walls in accordance with the CEC lost by action of 0.1 M NaCl solutions. Same symbols as in Figure 1.

or not by boiling methanol, with maxima occurring at various times depending on the pretreatment and the temperature at which the assays were carried out. Figure 2 shows the different profiles of released quantities of neutral and acidic sugar residues. The temperature dependence and the shapes of these curves suggest that the rate of carbohydrate loss is mainly a wall matrix diffusion-controlled phenomenon. These data are compatible with a diffusion of low mol wt carbohydrates in the first NaCl solutions and of higher mol wt carbohydrates in the following ones, from an intact wall matrix or from a matrix whose degree of porosity has been increased by hot methanol polymer cleavage.

Boiling methanol pretreatment of the walls was realized with a view to neutralizing the enzyme activity. Because the pretreated walls exhibit a carbohydrate loss that may be even greater than the one occurring with the nonpretreated walls, it is unlikely that wall enzymes participate in the reorganization of the walls of *Nitella* when they are soaked in monovalent ion solutions. This apparent lack of correspondence may be similar to the failure of Thompson and Preston

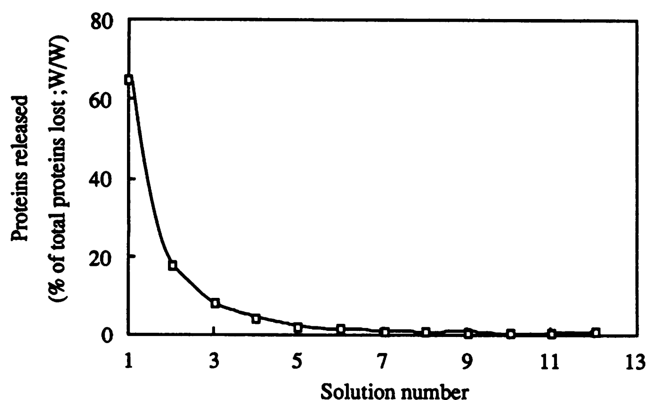

Figure 5. Time course of the amount of proteins released from *Nitella* cell walls soaked in 0.1 M NaCl renewed solutions. Same symbols as in Figure 1.

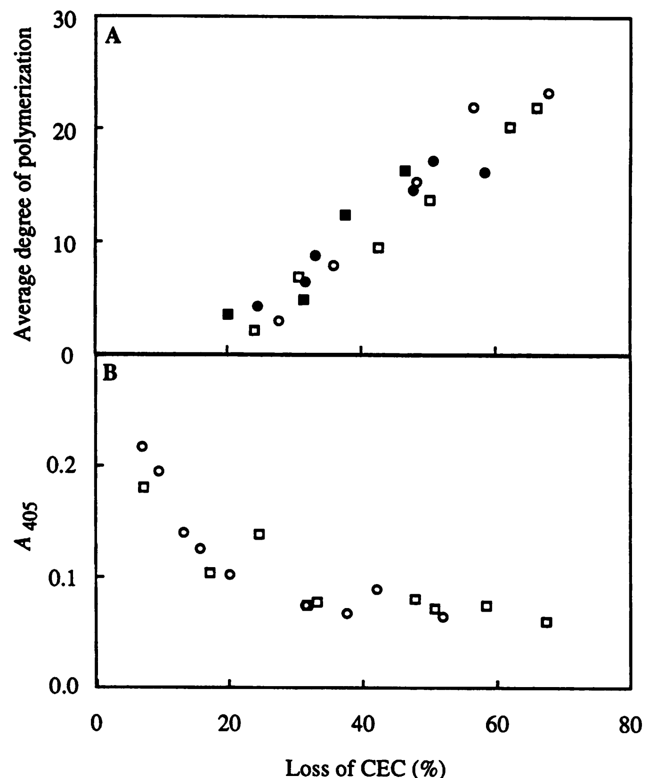

Figure 6. Variation in $\overline{DP}$ of polysaccharides released from *Nitella* cell walls as a function of the CEC lost by action of 0.1 M NaCl solutions. A, The $\overline{DP}$ was estimated from the ratio of the total uronic acids to reducing sugars in each eluted fraction after determination of their content in these two respective kinds of sugars by means of specific colorimetric methods (4, 9). Same symbols as in Figure 1. B, The $\overline{DP}$ was monitored by means of inhibition ELISA tests on an aliquot of each eluted fraction. Inhibition ELISA test: effect of the $\overline{DP}$ of polysaccharide samples on the recognition of polygalacturonic acids by 2F4 monoclonal antibodies. The antibodies were incubated with the tested polymer fractions arising from walls treated with (O) or without (□) methanol at 25°C. The resulting mixtures were centrifuged, and the supernatants were dispensed in polygalacturonic acid-coated microwells. In these inhibition ELISA tests, the absorbance decreased with increasing $\overline{DP}$ until there was a minimum corresponding to the presence of homogalacturonan blocks of at least nine residues in the collected sugars. Values are the means of duplicates.

(21) or Métraux and Taiz (15) to detect a relation between enzyme reactions and wall loosening during extension in *Nitella*. Our data and the previous conclusions substantiate the hypothesis that, in the ion-mediated structural changes in the wall of *Nitella*, direct physical reactions are involved rather than enzymic ones.

Figure 3 shows a linear correlation between the quantity of CEC lost by the walls and the accumulated amounts of acidic residues found after hydrolysis of the polymers that diffused out into the NaCl solutions from walls whether pretreated or not. Because the CEC is a measure of the nondiffusible charges of the pectin, this direct relationship confirms that the released acidic polymers arise from the pectin fraction. Thus, there is up to 60% of the pectin fraction

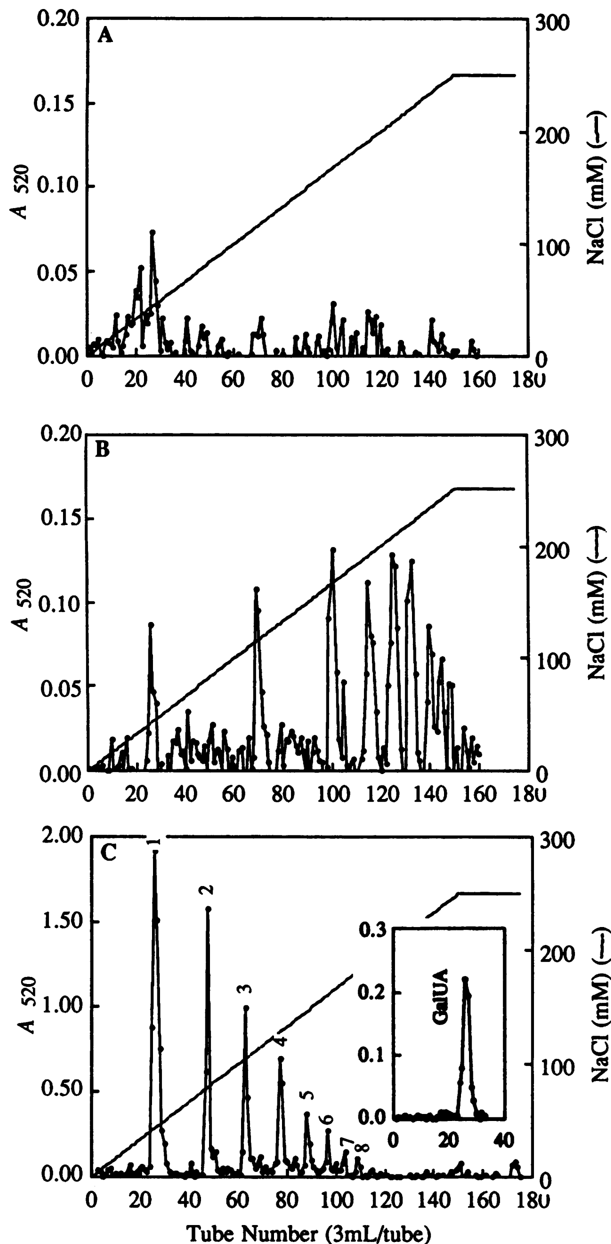

Figure 7. A, Elution profile of the oligosaccharides released by *Nitella* cell walls in the fourth 0.1 M NaCl solution (25°C, not methanol pretreated). The oligosaccharides were applied to a TSK DEAE-5PW column in 25 mM sodium acetate buffer and eluted with a linear gradient of NaCl (diagonal line) as shown. B, Elution profile of the oligosaccharides released by *Nitella* cell walls in the eighth 0.1 M NaCl solution (same procedure). C, Elution profile in the same conditions of oligogalacturonides obtained after acid hydrolysis (pH 3.2, 100°C, 45 h) of a polygalacturonic acid sample purchased from Sigma (note the difference in scale). The inset displays as reference the elution of glacturonic acid monomers purchased from Aldrich.

that is extracted by the presence of monovalent ions and that diffuses out under the electrostatic force of repulsion that appears between polymerized chains charged in the same manner (7).

Electrical Charges of Released Sugars

For each of the successive solutions of NaCl treatment, we calculated the ratio of acidic residues to the total amount of (acidic + neutral) sugars released. These results are plotted in Figure 4 as a function of the percentage of the CEC loss to eliminate the time diffusion parameter peculiar to each condition of the ionic exchange. It appears that, in the first pectic fractions released from the walls, there were more neutral residues than acidic ones and that this ratio was reversed afterward. The acidic residue fraction was then about 0.6. Because the repulsive forces between the pectic polymers are proportional to the number of electrical charges of the same sign carried by these molecules, these results suggest that the carbohydrates that first leave the wall, in spite of their lesser electrical charge, should be shorter macromolecules and, therefore, more diffusible.

$\overline{DP}$ of Released Sugars

Insufficient amounts of sugars were released in the first elution solutions (and in the last one) to enable accurate quantitative measurements of the $\overline{DP}$ to be made with the colorimetric methods usually recommended to evaluate the quantity of reducing sugars. In addition, a rapid loss of proteins, which may be in part glycoproteins (1), occurs as the Mn^{2+}-Na^{+} exchange begins (Fig. 5) and may interfere with the method, making the $\overline{DP}$ values appear weaker in the first fractions. Nevertheless, as the CEC loss becomes large enough, the results obtained with these methods show that the $\overline{DP}$ of released sugars increased with time and reached a maximum value of 25 (Fig. 6).

Detection of a range of oligomers released at two different times of elution (corresponding to solution Nos. 4 and 8 from walls not methanol pretreated) by means of HPLC analysis confirmed that there were relatively large amounts of longer chain length oligomers in the late fraction (Fig. 7, A and B). A comparison with an HPLC profile of oligomers obtained by hydrolysis of a sample of commercial polygalacturonic acid (Fig. 7C) reveals that most of the eluted peaks from *Nitella* were shifted in time, suggesting the presence of neutral sugars in the released wall oligogalacturonides. This comparison also shows the presence of oligomers with a $\overline{DP}$ higher than 8 in these two fractions from *Nitella* walls. This observation is in agreement with the results of an ELISA inhibition test. In this test, the 2F4 monoclonal antibodies (13) were first incubated with the various fractions collected, and the mixtures were then used as primary antibodies in a classical ELISA test against homopolygalacturonic acid.

In Figure 6, we report the absorbance of the ELISA test according to CEC loss. Because the absorbance decreased with the $\overline{DP}$ increase of the collected polyuronides until a minimum was reached in the presence of chains containing at least nine homogalacturonan blocks (13), these results support further the presence of larger proportions of polyu-

Table I. *Carbohydrate Composition of Polysaccharides Released in 0.1 M NaCl Solutions*
Mean for all fractions; in weight percentage of total released carbohydrates.

Gal Uronic Acid	Gal	Glc	Xyl	Rha	GlcUA	Fuc	Ara
60.0	9.4	8.3	7.2	6.2	6.1	1.5	1.3

ronides of chain lengths greater than nine residues in the late fractions, corresponding to a CEC loss of at least 40%.

Compositional Analysis of Released Carbohydrates

The results of the residue analysis of the soluble polymers collected in all fractions of the Mn^{2+}-Na^+ exchanges at 25°C are presented in Table I. Galacturonic acid was by far the most abundant residue: it accounted for 60% of the total weight of the released sugar residues. This percentage is similar to the 59% value obtained by Anderson and King (2) in the fractions of *Nitella* cell wall that can be considered as pectic.

Study of the time course of the predominant neutral sugars released in the different fractions reveals (Fig. 8) that the amounts of Rha and also of Gal residues reached a maximum at the moment when the amount of uronic acid lost was at its maximum (compare Figs. 2 and 8). Because we knew that minor quantities of a galactose resistant to partial hydrolysis were present in the pectic complex of *Nitella* (3), we believe that the polymers of the pectic fraction solubilized by NaCl action were rhamnogalacturonans, where the Rha residues act also as anchorage points for galactose side chains.

The other two most frequent residues, xylose and glucose, were released in a more regular pattern from the very beginning of the ionic exchange. We also found in the fractions in which the galacturonic acids were the most frequent small quantities of glucuronic acids. These residues are normally associated with xylose in the hemicellulose of *Nitella*, the fraction 17 reported in ref. 2. It appears, therefore, that glucuronylated xylans are probably leached from the walls in association with the soluble rhamnogalacturonans.

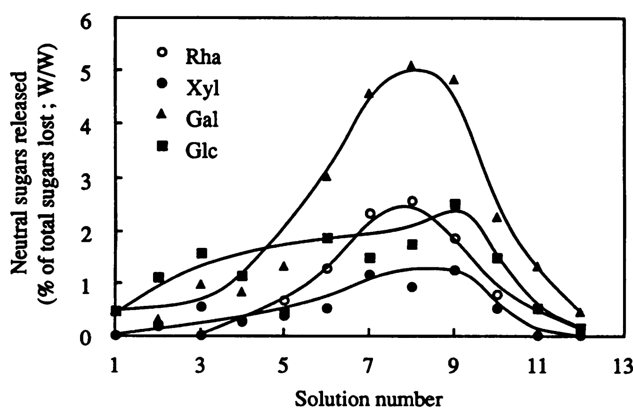

Figure 8. Time course of different neutral sugars found after hydrolysis of polymers released from *Nitella* cell walls soaked in renewed 0.1 M NaCl solutions (25°C, not methanol pretreated).

CONCLUSION

The replacement of divalent by monovalent ions induces the diffusion from the wall of small negatively charged polyuronides associated in the very first stages of the ionic exchange with proteins. These results emphasize the significant role of the noncovalent bonds in stabilizing the structure of the *Nitella* cell wall. As a consequence of this cell wall polymer leakage, a modification of the electrical charge of the wall occurs, and the further ionic exchange properties of this structure will depend only on its fraction of large nondiffusible polyuronides, the average molecular size of its homopolygaluctoran blocks being about 27 (10).

It is relevant to note that these results also suggest that local diffusion of oligouronides may spontaneously occur in the wall in response to the migration of the pH-banding pattern along the axis of the *Nitella* cell (18). The pH in the acid bands is low enough to remove divalent ions and, therefore, induce diffusibility of the short polymers. This phenomenon may contribute to the dynamic properties of the wall.

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