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Structural analysis of the cell walls regenerated by carrot protoplasts*

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Abstract. A procedure was developed to isolate protoplasts rapidly from carrot (*Daucus carota* L. cv. Danvers) cells in liquid culture. High purity of cell-wall-degrading enzymes and ease of isolation each contributed to maintenance of viability and initiation of regeneration of the cell wall by a great majority of the protoplasts. We used this system to re-evaluate the chemical structure and physical properties of the incipient cell wall. Contrary to other reports, callose, a $(1 \rightarrow 3)\beta$-D-glucan whose synthesis is associated with wounding, was not a component of the incipient wall of carrot protoplasts. Intentional wounding by rapid shaking or treatment with dimethyl sulfoxide initiated synthesis of callose, detected both by Aniline blue and Cellufluor fluorescence of dying cells and by an increase in $(1 \rightarrow 3)$-linked glucan quantified in methylation analyses. Linkage analyses by gas-liquid chromatography of partially methylated alditol-acetate derivatives of polysaccharides of the incipient wall of protoplasts and various fractions of the cell walls of parent cells showed that protoplasts quickly initiated synthesis of the same pectic and hemicellulosic polymers as normal cells, but acid-resistant cellulose was formed slowly. Complete formation of the wall required 3 d in culture, and at least 5 d were required before the wall could withstand turgor. Pectic substances synthesized by protoplasts were less anionic than those of parent cells, and became more highly charged during wall regeneration. We propose that de-esterification of the carboxyl groups of pectin uronic-acid units permits formation of a gel that envelops the protoplast, and the rigid cellulose-hemicellulose framework forms along with this gel matrix.

Key words: Callose – Cell culture (cell-wall regeneration) – Cell wall (composition, regeneration) – *Daucus* (cell-wall regeneration) – Pectic substances – Protoplast (wall formation)

Introduction

According to several reports, the incipient walls of plant protoplasts are very different from the cell walls of the source tissues (Asamizu and Nishi 1980; Blaschek et al. 1981; Pilet et al. 1984; Gould et al. 1986). The three most important differences reported were: 1) $(1 \rightarrow 3)\beta$-D-glucan (callose) is synthesized by wall-regenerating protoplasts, rather than $(1 \rightarrow 4)\beta$-D-glucan (cellulose), 2) half of the polymers synthesized by protoplasts are found in the extracellular medium rather than tightly associated with the protoplast surface, and 3) protoplasts synthesized polysaccharides that were different from those found in normal cells. Most of these earlier studies were plagued by the unavailability of commercial enzyme preparations pure enough to digest the parent wall without exposing the plasma membrane to proteolytic damage or contributing toxic substances which could adversely affect metabolism or even viability. Although callose is a structural component of a few specialized walls, such as the cell-plate, pollen tubes and cotton fibers (Bacic et al. 1988; Maltby et al. 1979), it is typically synthesized in response to wounding (Bacic et al. 1988). Further, extracellular polysac-

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Abbreviations: DEAE = Diethylaminoethyl; DMSO = dimethyl sulfoxide; ECP = extracellular polymers; EDTA = ethylenediaminetetraacetic acid; HGA = homogalacturonan; RG = rhamnogalacturonan; Tes = *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TFA = trifluoroacetic acid

charides (ECPs) may result from deliberate secretion, or they may be integral wall polymers that fail to polymerize or assemble because of loss of complementary polymers or enzymes required for crosslinking. Investigation of the processes of renewed synthesis and assembly of the cell wall requires protoplasts unwounded by mechanical or enzymic damage so that the chemical and physical factors directly responsible for that assembly can be deciphered. We first optimized the procedure for isolation and culture of healthy carrot protoplasts and characterized the composition of the walls and ECPs of carrot cells during culture to establish both physical and chemical alterations during normal culture. We then compared the physical and chemical properties of walls regenerated by carrot protoplasts with those of the carrot cells. By using procedures that maintained 75% viability of the protoplasts for over 7 d, we found that very little callose was made and the incipient wall comprised non-cellulosic polymers found in the walls of the parent culture. Protoplasts secreted ECPs in proportions similar to those of cells in culture, and rapid re-formation of the cell wall by protoplasts was accompanied by conversion of weakly anionic pectins to stronger anionic polymers.

Material and methods

Plant material. Suspension cultures of carrot cells (*Daucus carota* L. cv. Danvers) were grown in medium containing MS salts, pH 5.0 (Murashige and Skoog 1962; commercial preparation, Grand Island Biological Co., New York, N.Y., USA) supplemented with 0.1 g/l *myo*-inositol, 2.0 mg/l glycine, 0.4 mg/l 2,4-dichlorophenoxyacetic acid, and either 30 g/l sucrose or 31.5 g/l glucose as carbon source (carrot medium). The medium was autoclaved, and pyridoxine, thiamine-HCl, and nicotinic acid were filter-sterilized and added to the autoclaved medium to a final concentration of 0.5 mg/l each. Glucose was filter-sterilized to avoid decomposition. Cells were subcultured weekly and inoculated at 8 mg fresh weight/ml in 25 ml of medium in 125-ml Erlenmeyer flasks. To scale up yields in some experiments, carrot cells were inoculated at the same density in 100-ml of medium in 500-ml Erlenmeyer flasks.

Isolation and fractionation of cell walls and other material. All experiments contained at least duplicate samples, and each experiment was done twice or as noted. Whole cells 4 (duplicates), 7 (triplicates), or 10 d (four replicates) after subculture were filtered on sintered-glass funnels, and samples of about 1 g were homogenized in 5 ml of ice-cold 0.05 M *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (Tes) buffer containing 0.01 M ascorbate, pH 7.2, then centrifuged for 5 min at $1000 \cdot g$. The pellets were washed twice with ice-cold 0.5 M potassium-phosphate buffer, pH 6.8, four times with water, twice with chloroform:methanol (1:1; v/v) at 40° C for 30 min each, once with acetone, twice methanol, and three times with water. The isoluble material remaining was resuspended in water, frozen and lyophilized.

The medium filtrates containing the ECPs were dialyzed against running deionized water overnight, frozen, and lyophilized. Material from the Tes supernatant was precipitated by addition of four volumes of ethanol and incubation at −20° C. The precipitate was collected by centrifugation and washed four times with ice-cold 80% ethanol. The pellet was suspended in water, frozen, and lyophilized.

The washed wall material was stirred in dimethylsulfoxide (DMSO) at ambient temperature for 24 h. The DMSO-soluble material was dialyzed overnight against running deionized water and lyophilized. The insoluble wall material was collected by centrifugation, washed once with water, and extracted twice with 0.5% ammonium oxalate in a boiling-water bath for 60 min each to remove the pectic substances. The extract was dialyzed overnight against running deionized water and lyophilized. The remaining insoluble wall material was washed once with water and lyophilized.

The dried wall material was extracted sequentially with 0.1 M, 1 M and 4 M KOH, each containing 3 mg/ml $NaBH_4$ to prevent end "peeling" (Aspinall et al. 1962). Each extraction was carried out under N_2 for 55 min at ambient temperature. The pellet was extracted a second time with 4 M KOH at ambient temperature overnight. The KOH supernatants were filtered through Whatman GF/F glass fiber mats (from Fisher Scientific, Itasca, Ill., USA) and neutralized with glacial acetic acid. Two milliliters were reserved for total-sugar and uronic-acid assays, and the remainder was dialyzed against deionized water and lyophilized. Cellulose content in the material remaining after KOH extractions was determined according to Updegraff (1969).

Chemical composition. Protein was assayed according to Bradford (1976) (commercial reagents from Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as standard, total sugar by the phenol-sulfuric method (Dubois et al. 1956) with glucose as standard, and uronic acid by a carbazole method (Dische 1947), modified by addition of sulfamate (Galambos 1967), with galacturonic acid as standard. Starch was quantified by release of glucose with glucoamylase and determination by enzymic assay (Carpita and Kanabus 1987). Samples were suspended in 1 ml of DMSO and sonicated 1 h. The DMSO solution was diluted to 20% with water, and glucose released by digestion of the starch with glucoamylase [(1 → 4)α-D-glucohydrolase, exo-(1 → 4)α-D-glucosidase (EC 3.2.1.3) from *Rhizopus niveus;* Miles Laboratories from Seikagaku Kogyo Co., Tokyo, Japan] was measured by a coupled hexokinase and glucose-6-phosphate dehydrogenase assay. The efficiency of the starch hydrolysis was determined by digestion of amylopectin (Sigma Chemical Co., St. Louis, Mo., USA), and the glucose analysis was quantified by comparison with D-glucose standards.

Sugar composition. Duplicate samples from each fraction (approx. 5 mg each) were hydrolyzed with 2 M trifluoroacetic acid (TFA) containing 1 μmol *myo*-inositol (internal standard) for 90 min at 120° C in a heating block. The TFA was evaporated under a stream of N_2, and the sugars were converted to their corresponding alditol acetates (Blakeney et al. 1983) as modified by Carpita and Whittern (1986). Sugars were separated by gas-liquid chromatography on a 2 mm-diameter, 3 m-long packed column of 3% SP-2330 (Supelco, Bellafonte, Penn., USA) temperature programmed from 170° to 240° C at 5° C/min and then held at 240° C for 12 min. Temperatures of the injection port and flame-ionization detector were 250° C.

Linkage analysis. Samples were methylated by a modification of the method of Kvernheim (1987). Duplicate samples (approx. 3 mg each) were placed in 15-ml Corex (Corning Glass from

Fisher Scientific, Chicago, Ill.) tubes and stored in a vacuum desiccator over P_2O_5 overnight. The tubes were sealed with serum sleeve stoppers, and 1 ml of anhydrous DMSO was added by syringe. The tubes were evacuated and sonicated 4 h in a water bath that warmed to 50° C. Ultra-high purity Ar (Matheson Gas, Chicago) was introduced by means of a syringe needle with a second needle inserted for escape flow, and 250 µl of 2.5 M *n*-butyllithium in hexanes (Aldrich Chemical Co., Milwaukee, Wis., USA) was added to each tube. After the solution had stirred for 4 h under continuous Ar flow, 500 µl of CH_3I was added to each tube. The Ar flow was stopped, and the samples in sealed tubes were left stirring overnight.

The per-*O*-methylated polymers were recovered as described by Carpita and Whittern (1986) and hydrolyzed in 2 M TFA for 90 min at 120° C. The mixture was cooled to 30° C, and the TFA was evaporated under a stream of N_2. The per-*O*-methylated sugars were reduced with $NaBD_4$ and acetylated according to the method of Blakeney et al. (1983).

The per-*O*-methylated alditol acetates were separated on a 0.25-mm-diameter, 30-m-long vitreous silica wall-coated open-tubular capillary column of SP-2330 (Supelco; Shea and Carpita 1988). Separations were carried out with a Finnigan/MAT 9610 gas chromatograph coupled to a Finnigan/MAT 4021 quadrupole mass spectrometer interfaced to a Finnigan/MAT 2100C INCOS data system (Finnigan Instruments, Cincinnati, Oh., USA). Samples in dichloromethane were introduced by means of a Varian split/splitless capillary injector operated in the split mode (split ratio 50:1). The injector temperature was 225° C, and the gas chromatograph oven was temperature programmed from 160° to 210° C at 2° C/min, then from 210° to 240° C at 5° C/min. Helium was used as the carrier gas at 180 kPa. The interface oven temperature was 240° C, and the ion source temperature was 300° C. Mass spectra were recorded at 70 eV with an emission current of −0.4 mA and A/V sensitivity of 10^{-7}. The scan time was 0.95 s (0.05 s reset) over the *m/z* range of 41-350. The derivatives were identified by comparison with spectra of standards prepared from *p*-nitrophenylglycosides (Carpita and Shea 1988).

Separation and identification of radioactive derivatives of polysaccharides obtained in the radiolabelling experiments with protoplasts (see below) required modification of the chromatographic conditions. Alditol acetates were separated on a 0.75-mm-diameter, 30-m-long wide-bore glass capillary column of SP-2330, 0.2 mm film thickness (Supelco). Separations were carried out with a gas chromatograph coupled to a Packard Model 894 gas proportional counter (United Technologies, Downer's Grove, Ill.). Samples in ethyl acetate were introduced by direct injection. The injector temperature was 250° C, and the oven was temperature programmed from 160° to 240° C at 5° C/min. Helium carrier flow was 2 ml/min, with 112 ml/min make-up added directly to the column effluent; the effluent was split 2% to the flame-ionization detector and 98% to the gas proportional counter. The signal was calibrated by gas-chromatographic separation of known amounts of radioactive alditol-acetate standards. The values for arabinose and xylose were corrected for the lack of the C-6, and then the molar distribution of the radioactivity was determined from the Bq counts in each sugar. Partially methylated alditol-acetate derivatives were separated with the wide-bore SP-2330 column temperature programmed at 160° C with a 5-min hold then to 240° C at 1.5° C/min. Derivatives were quantified according to the effective carbon response factors of Sweet et al. (1975).

Quantitation of individual polysaccharides. Each fraction of the cell wall contained a mixture of polysaccharides. Although it is difficult to deduce the structure of individual polysaccharides based solely on linkage analysis, a considerable body of literature on characteristic linkage structures of purified polymers from many dicotyledenous species has made estimates reasonably accurate (Bacic et al. 1988). These estimations were used in order to simplify observation of changes in distribution of these polysaccharides through a normal cell-culture cycle. Ammonium oxalate extracts pectic substances, which, typical of cell walls of carrot and other dicotyledons, comprise homogalacturonan (HGA), rhamnogalacturonan (RG), and neutral arabinans, galactans and arabinogalactans as side-chains of RG (Jarvis 1984; Konno et al. 1986). The dilute KOH then extracts additional pectic substances that likely are crosslinked through ester linkages with phenolic compounds (Fry 1986; Jarvis 1984). The hemicelluloses are polysaccharides extracted with strong alkali, and in dicotyledons these polymers are typically xyloglucan and smaller amounts of arabinoxylan (Darvill et al. 1980; Bacic et al. 1988). Arabinogalactans were either Type I, comprising 4- and 3,4-galactosyl units and the stoichiometric amount of *t*-arabinosyl units, or Type II, comprising 3-, 6- and 3,6-galactosyl units and a stoichiometric amount of *t*-arabinosyl units (Fincher et al. 1983; Jarvis 1984). The remaining *t*-arabinosyl units and the linked and branched residue arabinosyl units constituted arabinan. Xyloglucan was the sum of the 4,6-glucosyl units and stoichiometric terminal xylosyl units as well as the 2-xylosyl, 2-galactosyl, *t*-fucosyl, and a fraction of the 4-linked glucan one-third that of the 4,6-glucosyl based on the hepta- and nonasaccharide unit structure (Bacic et al. 1988). Arabinoxylan was the sum of the 4-, 2,4- and 3,4-xylosyl units and stoichiometric amounts of *t*-arabinosyl units (Darvill et al. 1980; Bacic et al. 1988).

Protoplast isolation. Onozuka cellulase (Kinki Yakult Mfg. Co., Nishinomiya, Japan; Type R-10) and Macerase (Calbiochem, San Diego, Cal., USA; Cat. No. 441201) were partially purified before use by a modification of the method of Gamborg (1975). Each enzyme was dissolved in water (2 g/10 ml), and any insoluble material was removed by centrifugation. The supernatant was applied to a 15-mm-diameter, 300-mm-long column of Sephadex G-25 (Pharmacia, Uppsala, Sweden; purchased from Sigma Chemical Co.) and eluted with water. Fractions (2 ml) were collected and assayed for protein. Fractions containing protein were pooled, frozen, and lyophilized. Worthington cellulase (Worthington Biomedicals Corp., Freehold, N.J., USA; Type CELF) and Worthington pectinase (Type PASE) were used directly as supplied. The enzymes were filter-sterilized before use, and the final concentrations of the enzymes were 0.5% (w/v) Worthington cellulase and 0.1% (w/v) Worthington pectinase or 0.65% (w/v) Onozuka cellulase and 0.2% (w/v) Macerase. Protoplasts were isolated by a modification of the method of Klein et al. (1981), and procedures were carried out under aseptic conditions. Cells at various stages of culture were collected by centrifugation at 120·*g* for 2 min. The cells were washed once with carrot medium and once with carrot medium containing 400 mM sorbitol. Ten milliliters of protoplast medium (carrot medium containing 100 mM glucose and 400 mM sorbitol) containing wall-digesting enzymes was added to 10 ml packed volume of cells. The suspension was transferred to a Petri dish and incubated for 1 h on a gyratory shaker at 30 rpm at room temperature in the light.

The suspension was filtered through Nitex cloth (Tetko, Elmsford, N.Y., USA; linear pore size, 48 µm) to remove undigested cells, and the filtrate was centrifuged at 120·*g* for 8 min for gentle pelleting of the protoplasts. The supernatant was discarded, and the protoplasts and debris were suspended in 24% (w/v) Ficoll (Sigma; type 400-DL) in protoplast medium. The top layer of a Ficoll step gradient was formed by pipetting 10 ml of protoplast medium into a conical centrifuge tube. A second layer, 9% (w/v) Ficoll in protoplast medium, was in-

jected underneath the first with a glass syringe fitted with a No. 19 gauge (0.7 mm internal diameter), 10-cm-long blunt-end needle. The suspension containing the protoplasts was injected under the first two layers. The gradient was centrifuged for 20 min at 170·*g*. The protoplasts were collected from the interface between the top two layers, washed with protoplast medium three times, and collected by centrifugation after each wash (3 min at 120·*g*). The protoplasts were resuspended in protoplast medium to a final concentration of $2 \cdot 10^6$ protoplasts/ml; 2 ml of the protoplast suspension were transferred to each of three Petri dishes (6 cm diameter, 1.5 cm high) and incubated without shaking at room temperature in the light. The sorbitol in the medium was gradually diluted by the addition of fresh carrot medium, 0.4 ml at 4 d, 0.4 ml at 8 d, 0.5 ml at 13 d, and 1 ml at 17 d. At 21 d, the suspensions from the three dishes were combined in a 50-ml Erlenmeyer flask and 5 ml of fresh medium added, and at 28 d, the suspension was transferred to a 125-ml Erlenmeyer flask and 9 ml of medium were added. The cell density was assayed every 7 d, and the suspension was subcultured routinely afterwards.

Microscopy. Viability was determined each day with Evans blue (Taylor and West 1980). For fluorescence microscopy, cells were stained with either 0.05% Aniline blue (Matheson, Coleman & Bell, Cincinnati, Oh.) in protoplast culture medium adjusted to pH 9, or simultaneously with 0.01% fluorescein diacetate (Sigma) and 0.01% Cellufluor (Polysciences) in protoplast culture medium (pH 5). The Aniline-blue-stained protoplasts were observed and photographed with an inverted Nikon (Nippon Kogaku K.K., Tokyo, Japan) microscope with epifluorescence and Diaphot attachments. A 420–485-nm interference filter was used for excitation, and a 510-nm dichroic mirror and 520–560-nm eyepiece absorption filter were used for observation. Protoplasts stained with fluorescein diacetate and Cellufluor were observed and photographed with a Nikon research microscope with epifluorescence and Optiphot attachments. A 330–380-nm interference filter was used for excitation, and a 400-nm dichroic mirror and 420-nm eyepiece absorption filter were used for observation. Microscopic observations were recorded on Ektachrome ASA 160 Tungsten film (Eastman-Kodak, Rochester, N.Y.).

Analysis of regenerated walls. All experiments were done three times, each with duplicate samples. In some experiments, protoplasts were deliberately damaged by incubation on gyratory shakers at low speed (30 rpm) or high speed (110 rpm) or by incubation in 0.2% DMSO without shaking. The protoplasts were separated from the medium by gentle centrifugation (3 min at 170·*g*), and polymeric material in the supernatant and pelleted fractions was precipitated with four volumes of ethanol and heated 5 min in a boiling-water bath, cooled to −20° C for 30 min, and washed four times with ice-cold 80% ethanol. The pelleted material was washed twice with chloroform:methanol (1:1; v/v) and resuspended in methanol. Samples were assayed for total sugar and uronic acid, and polysaccharides in samples of freshly isolated protoplasts as well as 4-d- and 8-d-old protoplasts were per-*O*-methylated, and alditol-acetate derivatives were prepared, separated, and identified as described above.

Radiolabeling of regenerating walls of protoplasts. Freshly isolated protoplasts were incubated for 3 h to recover from shock of manipulation before addition of radioactive glucose. Experiments were done twice, each in duplicate. For chemical analysis of newly synthesized material, protoplasts which were to be labeled 3, 48, and 72 h after isolation were incubated in medium containing 10 mM glucose. Each day the medium was assayed for reducing sugar (Somogyi 1952), and the concentration of glucose was readjusted to 10 mM. Protoplasts which were to be labeled after 3 d from time of isolation were incubated in medium containing 100 mM D-glucose. By 5 d, the protoplasts had consumed enough glucose from the medium to reduce the concentration to just below 10 mM; before pulse labeling, the concentration was readjusted to 10 mM with fresh, sterile D-glucose solution. Duplicate dishes without radioactivity were sampled for determination of viability with Evans blue. For each experiment, 3.7 MBq of [U-^{14}C]-D-glucose (0.19 MBq/μmol; ICN Radiochemicals, Irvine, Cal.) were added to each of four dishes of protoplasts. After incubation for 3 h, protoplasts were pelleted by centrifugation 120·*g* for 3 min to separate the polymers associated with the protoplasts from the ECPs. The protoplast pellet was washed three times with 5 ml of protoplast medium to remove any extracellular material, and the ECPs were precipitated with four volumes of ice-cold ethanol. Insoluble materials from both were washed sequentially with 80% ethanol and chloroform:methanol (1:1; v/v) as described above. Samples of each were taken for assay of radioactivity in cellulose and non-cellulosic material. The material precipitated with 80% ethanol was washed with ice-cold 80% ethanol and chloroform:methanol as described, and resuspended in 2 ml methanol. The insoluble material was more finely suspended in methanol than water so aliquots of the suspension were easier to pipet. Radioactivity was determined in 50 μl of each sample by liquid scintillation spectroscopy. Alditol acetate and partially methylated alditol-acetate derivatives were each prepared as described with half of the sample, and the remainder was fractionated by ion-exchange chromatography.

Diethyl amino ethyl-Sephadex chromatography of ECP and cell-wall material. The radioactive ECP and cell walls from protoplasts (approx. 1 mg each), and the lyophilized material from the ammonium-oxalate extract and ECP of 4-d cells (3 mg each) were suspended in 1 ml of 20 mM phosphate buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA; brought to pH 5.0 with 10 mM citric acid) and warmed in a sonicated water bath for 1 h. Standards of polygalacturonic acid (Polysciences) and citrus pectin (Sunkist Growers Inc., Corona, Cal.) were washed with 80% ethanol to remove monosaccharides and oligomers and then dissolved in the phosphate-citrate-EDTA buffer. The insoluble material was removed by centrifugation, and the supernatant was applied to a 10-mm-diameter, 100-mm-long column of diethylaminoethyl (DEAE)-Sephadex A-50 (Pharmacia; repackaged by Sigma) equilibrated in the phosphate-citrate buffer. The column was washed with 25 ml of phosphate-citrate buffer followed by a 100-ml linear gradient of NaCl to 0.8 M in phosphate-citrate buffer. Fractions (2.6 ml) were collected, and samples were taken for determination of radioactivity, total sugar, and uronic acid.

Results

Protoplast isolation. The density-gradient-flotation technique efficiently isolates viable protoplasts from hydrolytic enzymes and the few remaining cells. The highest yield of protoplasts after 2 h of wall digestion was from 4-d cell cultures, regardless of enzyme source (Fig. 1). The combination of Worthington cellulase and pectinase gave consistently higher yields of protoplasts than did Onozuka cellulase R-10 with Macerase. Maximum yields were reached with Worthington enzymes in

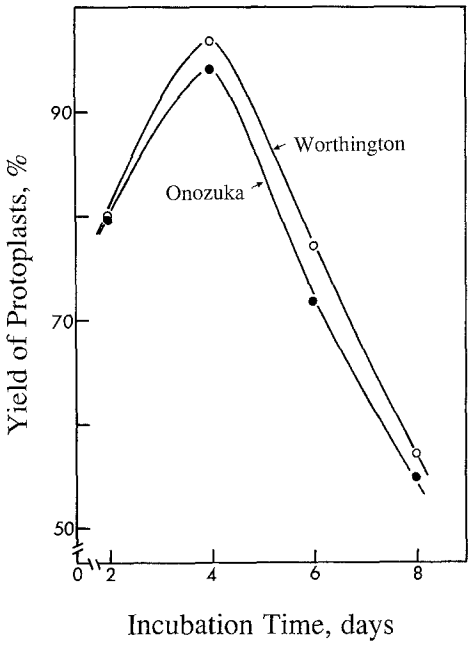


Fig. 1. Yield of protoplasts from carrot cells at various stages of the culture cycle. Cells were exposed to the Worthington enzyme preparation for 2 h or the Onozuka preparation for 4 h. Values are the mean of duplicate experiments with variance ranging from ±1.5 to ±5.2%

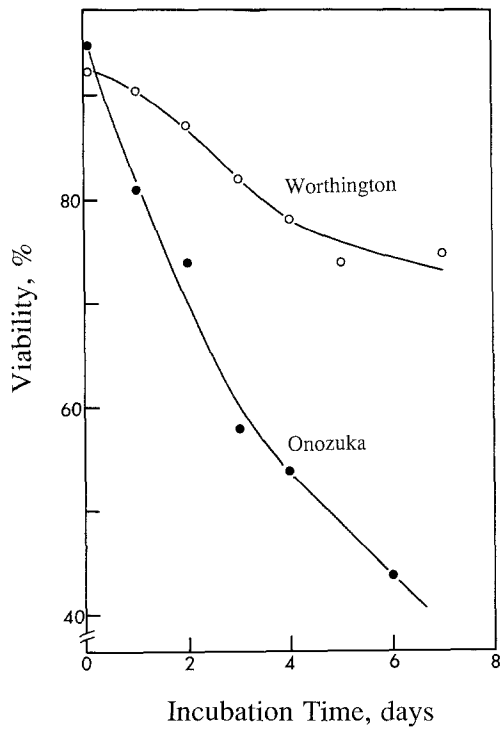


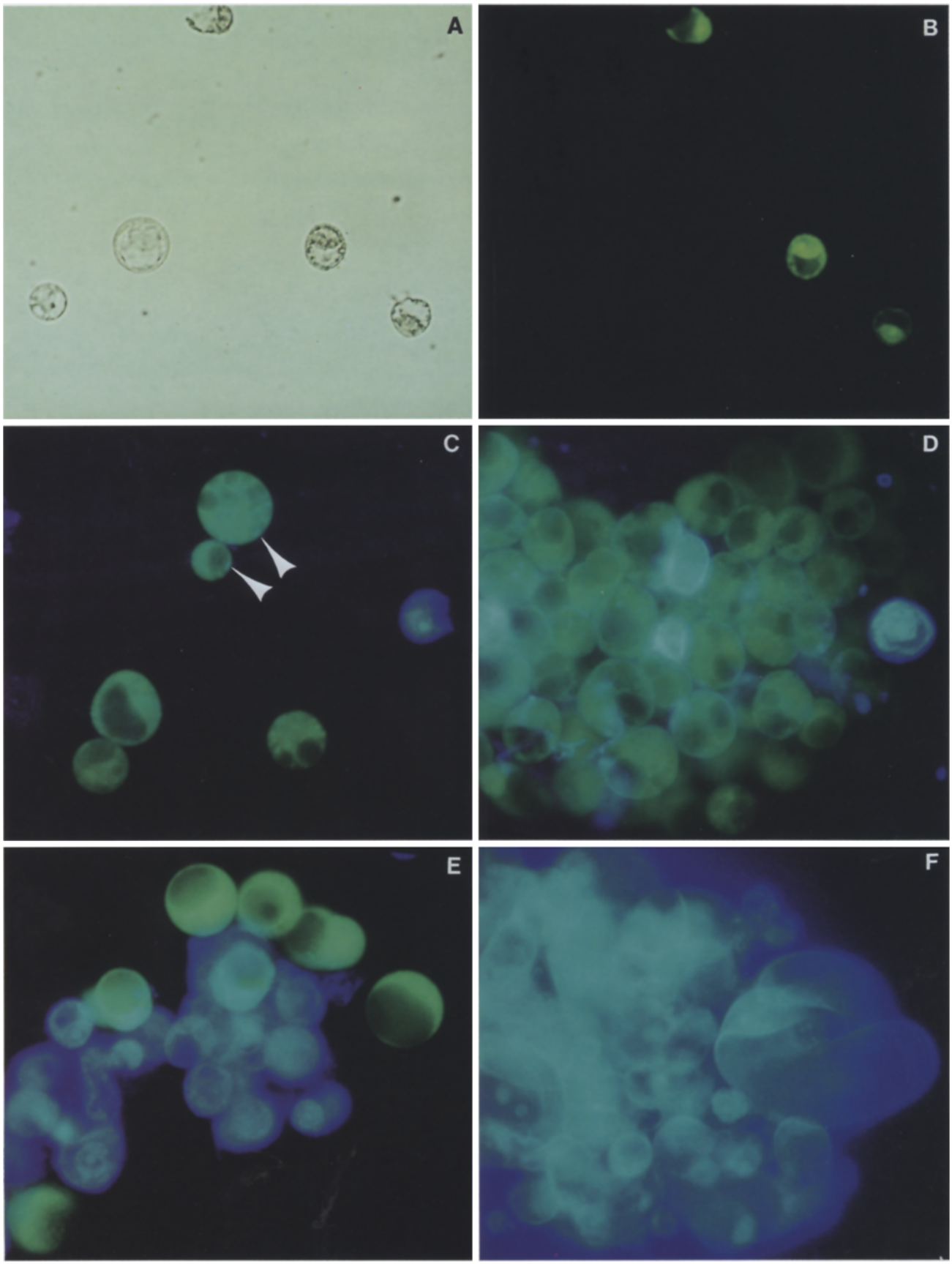
Fig. 2. Viability of carrot protoplasts determined by exclusion of Evans blue during subsequent incubation after digestion of the cell walls with the Worthington and Onozuka enzyme preparations and purification by the Ficoll flotation gradient. Values are the mean of four (Onozuka) and seven (Worthington) experiments with standard deviation ranging from ±0.6 to ±7.9%

less than 2 h, but the combination of Onozuka cellulase and Macerase required at least 4 h. When protoplasts were isolated with Worthington enzymes, over 75% of the protoplasts were still alive after 8 d of culture, whereas viability of protoplasts obtained with the Onozuka-Macerase mixture had dropped to 40% in 6 d (Fig. 2).

From observations not shown, the incipient cell wall completely enveloped the protoplasts by 3 d and had sufficient independent structure so that plasmolysis was observed when the protoplasts were transferred to higher concentrations of osmoticum. However, the incipient walls of 3-d protoplasts had not developed sufficient tensile strength to withstand increases in turgor, so most cells burst when the medium was diluted with water. After 5 d, the protoplasts could withstand fivefold dilution of the medium but still burst in the absence of osmoticum. Two to three weeks after protoplast isolation, an actively growing culture was re-established and could be maintained in normal liquid culture.

Fluorescence microscopy of the regenerating cell wall. Wall material deposited at the surface of protoplasts was visualized by fluorescence microscopy. Protoplasts were stained with Aniline blue (pH 9.0) for (1 → 3)β-D-glucan or with Cellufluor as a stain for both callose and cellulose (Hayashi et al. 1986). Neither of these stains reacted with the surfaces of freshly isolated protoplasts. Both stains reacted intensely with protoplasts which had died by 4 h after isolation but not with live protoplasts (Fig. 3A, B, C). Four hours after isolation, live cells did not have a complete wall, but patches of material sticking to the plasma membrane were stained by Cellufluor (Fig. 3C). Two-day protoplasts had a very thin wall which stained with Cellufluor (Fig. 3D), and by 6 d, a wall completely enveloped most of the viable cells (Fig. 3F).

When incubated on a rapidly moving shaker, 60% of the protoplasts were unable to exclude Evans blue after 24 h, and none could exclude the dye after 72 h (Fig. 4). Almost half of the protoplasts were damaged when they were cultured in 0.2% DMSO, but 10% of the damaged protoplasts recovered their ability to exclude Evans blue (Fig. 4). After 24 h, 30% of the protoplasts in unshaken dishes were unable to exclude the dye, but 15% of the cells recovered by 48 h. Protoplasts on the slow-moving shaker were not seriously affected at first, but 30% were unable to exclude Evans blue after 72 h. When protoplasts were stained for the presence of (1 → 3)β-D-glucan with Aniline blue, the strongly fluorescent material was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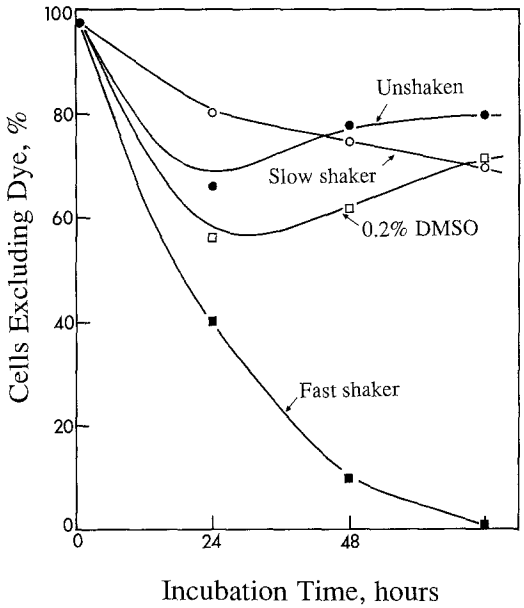

Fig. 4. Staining of carrot protoplasts with Evans blue during treatments designed to damage them. Protoplasts were incubated without shaking, on a slow gyratory shaker (30 rpm; 2.5 cm displacement), on a fast gyratory shaker (110 rpm; 2.5 cm displacement), or in presence of 0.2% DMSO without shaking

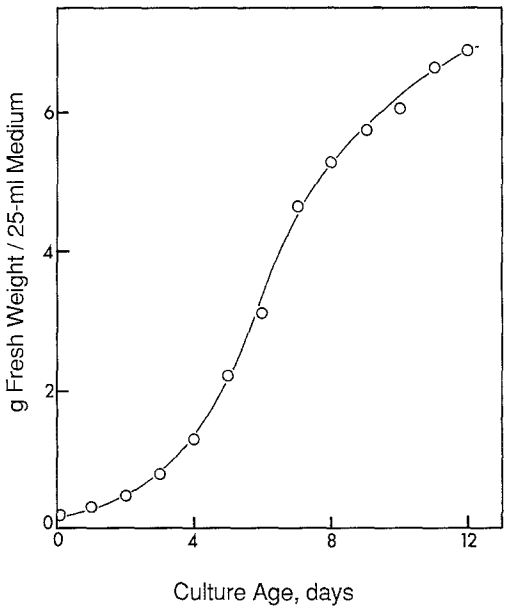

Fig. 5. Growth of carrot cells in liquid culture. Cells were inoculated at 0.2 g FW into 25 ml of carrot medium in 125-ml Erlenmeyer flasks and incubated by gyratory shaking. Values are the means of thirteen experiments over four years. Standard deviations from 4 d on, when substantial growth had occurred, ranged from ± 0.12 to ± 0.58 g

associated only with dead protoplasts (Fig. 3A, B). Two days after isolation, protoplasts damaged by slow shaking or 0.2% DMSO had produced strongly Cellufluor-positive material, whereas viable cells, discerned by accumulation of fluorescein, had not (Fig. 3D, E).

Fig. 3A–F. Fluorescence microscopy of carrot protoplasts after staining with fluorescein diacetate, Aniline blue, and Cellufluor. **A** Bright-field illumination of protoplasts stained for (1 → 3)-glucan with Aniline blue 4 h after isolation. Two protoplasts were viable, but three had died. **B** Same as **A** but viewed by fluorescence microscopy. Only the protoplasts which had died fluoresced. **C** Protoplasts stained for viability with fluorescein diacetate and for carbohydrate with Cellufluor 4 h after isolation. The viable protoplasts appear green and have only small patches of material stained with Cellufluor (*arrows*). In contrast, protoplasts which have died by 4 h stain intensely with Cellufluor and appear blue. **D** Two-d-old protoplasts cultured with slow shaking also doubly-stained with fluorescein diacetate and Cellufluor. Most of the cells had formed walls and give even intensity of staining with the Cellufluor. When shaken, protoplasts clump immediately; even unshaken protoplasts eventually clump after about 5 d. **E** Two-d-old protoplasts incubated in the presence of 0.2% DMSO and doubly-stained with Cellufluor and fluorescein diacetate. About half of the protoplasts had died and stain intensely with Cellufluor; the surviving protoplasts, green from accumulation of fluorescein, formed thin walls barely detectable by Cellufluor. **F** Cell walls formed by protoplasts after 6 d in culture. Complete walls stain well with Cellufluor, and their asymmetric, elliptical and kidney shapes were similar to those of the parent culture

Analysis of walls of carrot cells. The sugar-linkage composition of material in cell-wall fractions of the carrot cells was determined for comparison with walls regenerated by protoplasts. Further, walls were examined from cells at several stages throughout the culture cycle to assess changes that occur normally as cells transit from early stages, where cell division predominated (0–4 d), to later stages where cells mostly expanded and elongated. Increase in cell number began within 1 d of transfer to fresh medium, and mitotic index was highest at 4 d (Shea 1988). At 4 d, when cells exited lag phase (Fig. 5), the density of cytoplasmic material was greatest. Cell expansion predominated through 7 d, and amounts of Tes-soluble, starch, ECP, and cell wall decreased markedly in proportion to the fresh weight of the cells (Table 1). After 7 d, cell expansion continued and FW increased slightly, but the amount of cell wall more than doubled (Table 1).

The ability to extract polymers from the wall also changed during the culture cycle. Although the proportion of pectic substances extracted by ammonium oxalate were nearly constant, amounts of material extracted by 0.1 M and 1 M KOH decreased from about 10% and 13% to about 3% and 7%, respectively (Table 1). The material solubilized by acetic-nitric acid nearly tripled from 9% to 24%, whereas the amount of crystalline cellulose

Table 1. Distribution of material in wall fractions of carrot cells. Cells were harvested on the days indicated, and FW was determined. The ECPs were dialyzed against running deionized water and lyophilized. Cells were homogenized in Tes buffer, and the cell walls and starch were pelleted by centrifugation and washed extensively with 0.5 M potassium phosphate, pH 6.8, water, chloroform:methanol, acetone, methanol, and water. The Tes-soluble material was precipitated with 80% ethanol. Starch and small amounts of other material were extracted from the cell walls with DMSO, and the walls were washed with water and lyophilized. Cell-wall materials were then extracted sequentially with ammonium oxalate and increasing concentrations of KOH, and the α-cellulose was washed with water and lyophilized. Crystalline cellulose in the α-cellulose was then determined after hydrolysis of non-cellulosic polysaccharides in acetic-nitric acid (*A-N*) reagent (Updegraff 1969). Values are the means of two (4-d cells), three (7-d cells), or four (10-d cells) experiments, each in duplicate. To scale up yields, cells were inoculated in 100 ml of carrot medium in 500-ml Erlenmeyer flasks. As a result, yields of cells (g FW/ml medium) were about 70% that of comparable cultures grown in smaller flasks (Fig. 5)

Cell fractions	Culture age (d)		
	4	7	10
mg/100-ml culture			
Fresh weight	3350	14520	17720
ECP	18.1	34.2	35.9
DMSO-soluble (starch)	6.8	20.1	21.9
Tes-soluble	47.9	95.6	107.5
Wall material	42.4	98.3	216.3
wt% of wall material			
Ammonium oxalate	36.8	38.3	32.1
0.1 M KOH	9.6	4.4	3.4
1 M KOH	12.7	6.1	6.9
4 M KOH	9.3	6.1	6.2
4 M KOH overnight	11.1	11.1	11.0
A-N-soluble	8.7	16.3	23.8
Cellulose	11.9	17.7	16.6

rose slightly from 12% to 17%. Amounts of total KOH-extractable material decreased from 43% to 28% of the wall between 4 and 10 d of culture, largely a result of the decreases in the amount of material extracted by 0.1 M and 1 M KOH (Table 1).

The material precipitated from the Tes extract was mostly arabinose and galactose with lesser amounts of xylose and glucose, and the material was enriched in mannose compared with the other fractions (Table 2). The Tes fraction comprises soluble proteins and newly synthesized and unincorporated pectins and hemicelluloses, whereas DMSO extracts mainly starch.

Uronic acids constituted about 45% of the ammonium-oxalate-extractable material of the carrot cells (Table 2), and both 2- and 2,4-linked rhamnosyl units were detected (Table 3). Most of the neutral-sugar fraction was arabinose and galactose, and the arabinose was mostly non-reducing *t*-, 5- and 3,5-linked (Table 3). When the amounts of the material in linkages attributed to arabinan, arabinogalactan I, and arabinogalactan II were summed, they accounted for over 70% of the neutral-sugar portion of the ammonium-oxalate fraction from 4-d cells; this proportion decreased during culture, but these polymers still constituted 56% of the material by the stationary phase (Table 4). Polymers in the 0.1 M KOH extract resembled those in the ammonium-oxalate extract (Tables 3, 4).

Much of the material extracted by higher concentrations of KOH was xyloglucan, deduced from the abundant 4- and 4,6-glucosyl units and corresponding *t*-xylosyl units (Tables 3, 4). Units of 2-xylosyl, 2-galactosyl and *t*-fucosyl units were also observed. Some 4-, 2,4- and 3,4-linked xylosyl units

Table 2. Distribution of neutral sugars and uronic acids in soluble and cell-wall polymers of fractions of 4-d-old carrot cells. Cells were collected from 4-d-old cell cultures and homogenized in Tes buffer. After removal of starch and cytoplasmic contaminants from the walls, the pectic substances were extracted with hot ammonium oxalate and the hemicelluloses with increasing concentrations of KOH. Polymers soluble in Tes buffer were precipitated with 80% ethanol, washed in additional 80% ethanol, suspended in water and lyophilized. The ECPs in the filtered culture medium, and the ammonium-oxalate and glacial-acetic-acid-neutralized KOH extracts were dialyzed against running deionized water and lyophilized. Duplicate samples of each material (approx. 5 mg each) were hydrolyzed in 2 M TFA, and alditol acetates prepared, separated, and quantified as reviewed (Carpita and Shea 1988). Values are in mol% and are the means of duplicate samples, with variance always less than ±5%

Sample	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic acids
Tes-soluble	5	2	27	8	8	27	12	11
DMSO-soluble	tr[a]	tr	2	3	1	13	81	nd
Ammonium oxalate	6	tr	22	7	tr	13	7	45
0.1 M KOH	6	1	26	10	4	18	22	13
1 M KOH	4	3	16	28	1	15	20	13
4 M KOH	3	3	15	19	3	12	34	11

[a] tr = trace amounts less than 0.5%; nd = not determined

Table 3. Distribution of neutral sugar linkages in soluble and cell-wall polymers of fractions of 4-d-old carrot cells. Fractions were obtained as described in Table 2. Aliquots of 3 mg from each sample in duplicate were permethylated, and partially methylated alditol-acetate derivatives were prepared. Derivatives were separated by gas-liquid chromatography and identified by electron-impact mass spectrometry (Carpita and Shea 1988). Values are in mol% and are the mean of duplicates, with variance always less than ±7%

Sugar linkage	Tes-soluble	Cell-wall fractions				
		Ammonium oxalate	0.1 M KOH	1 M KOH	4 M KOH	ECP
t-rhm[a]	1	tr[b]	1	tr	tr	1
2-rhm	3	2	1	1	tr	1
2,4-rhm	3	2	3	1	1	2
Total	*7*	*4*	*5*	*2*	*1*	*4*
t-fuc	tr	tr	tr	1	2	2
t-ara*f*	15	21	22	9	8	9
t-ara*p*	4	2	3	1	1	7
2-ara*f*	1	tr	tr	tr	tr	tr
3-ara*f*	tr	1	1	tr	tr	tr
5-ara*f*	11	24	16	8	6	13
3,5-ara	3	10	7	4	3	5
2,5-ara	3	8	5	3	2	2
Total	*37*	*66*	*54*	*25*	*20*	*36*
t-xyl*p*	3	2	3	14	16	7
2-xyl*p*	tr	tr	1	5	7	3
3-xyl*p*	nd	nd	nd	nd	tr	nd
4-xyl*p*	3	1	3	11	4	5
2,3-xyl	tr	tr	tr	1	1	tr
2,4-xyl	tr	tr	tr	4	2	1
3,4-xyl	tr	tr	tr	1	1	1
Total	*7*	*3*	*8*	*37*	*31*	*17*
t-man	1	nd	1	tr	nd	nd
4-man	2	1	nd	tr	4	tr
Total	*3*	*1*	*1*		*4*	
t-glc	11	1	1	1	1	1
2-glc	4	nd	tr	tr	nd	nd
4-glc	5	4	5	6	11	5
2,4-glc	nd	tr	tr	tr	1	nd
4,6-glc	1	1	1	15	16	13
Total	*21*	*6*	*7*	*22*	*29*	*19*
t-gal*f*	1	2	2	2	2	2
t-gal*p*	2	3	2	2	2	3
2-gal	nd	2	nd	2	1	2
3-gal	2	1	1	tr	nd	3
4-gal	2	2	5	3	5	3
6-gal	2	3	5	3	2	6
4,6-gal	8	3	1	tr	tr	nd
3,6-gal	5	2	8	1	1	5
3,4-gal	nd	2	1	tr	nd	nd
Total	*22*	*20*	*25*	*13*	*13*	*24*

Table 4. Distribution of neutral polymers deduced from linkage analyses in fractions of walls of carrot cells during culture. Rationale for assignments of each polymer are given in the *Material and methods*. Values are mol%

Cell-wall fraction	Age of culture (d)		
	4	7	10
Ammonium-oxalate-soluble:			
Arabinogalactan I[a]	2	5	5
Arabinogalactan II	6	5	1
Arabinan	65	47	50
Arabinoxylan	1	2	2
Xyloglucan	6	7	14
4-linked Glucan	4	13	12
Other	15	21	16
0.1 M KOH-soluble:			
Arabinogalactan	6	4	14
Arabinogalactan II	21	6	7
Arabinan	46	43	37
Arabinoxylan	4	5	2
Xyloglucan	6	17	13
4-linked Glucan	4	16	15
Other	13	8	12
1 M KOH-soluble:			
Arabinogalactan I	4	1	2
Arabinogalactan II	5	2	4
Arabinan	24	29	25
Arabinoxylan	21	22	28
Xyloglucan	41	32	38
4-linked Glucan	1	3	2
Other	3	12	2
4 M KOH-soluble:			
Arabinogalactan I	4	4	3
Arabinogalactan II	3	2	3
Arabinan	19	16	17
Arabinoxylan	10	6	17
Xyloglucan	48	63	45
4-linked Glucan	6	2	8
Other	9	7	7

[a] Proportions of individual polymers were deduced from linkage analysis and based on criteria described in text

from arabinoxylan were also observed, and arabinans made up much of the remainder (Tables 3, 4).

During cell culture, proportions of linkages typical of xyloglucan and other glucans (Table 3) increased markedly in ammonium-oxalate and weak-alkali extracts (Table 4). A substantial amount of polysaccharide was also released into the incubation medium throughout cell culture

[a] *t*-rhm = non-reducing terminal rhamnosyl unit; 2-rhm = 2-linked rhamnosyl unit deduced from the 1,5-di-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methyl-deoxyhexitol derivative, and so forth

[b] tr = trace amounts less than 0.5%; nd = not detected

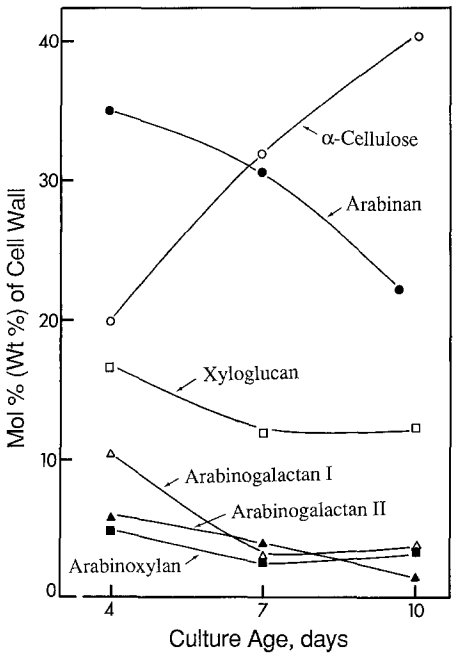

Fig. 6. Contribution of the major polysaccharides to the composition of the cell walls of carrot cells during the culture cycle. Individual polymers were from mol% contribution of sugar linkages deduced by methylation analysis, and α-cellulose was weight% of the material remaining after exhaustive extraction of the wall with 4 M KOH

(Table 1). A large proportion of this material was type II arabinogalactan (Tables 3, 5); the remainder represented a mixture of polymers of both pectin and hemicellulose of the cell wall.

The contribution of KOH-soluble xyloglucan, arabinoxylan and arabinogalactan II to the total wall was almost constant during the culture cycle (Fig. 6). The molar fraction of arabinan and arabinogalactan I decreased markedly, and only α-cellulose increased. Because cell number is nearly constant after 4 d, the values represent small changes in the proportion of the different polymers during cell expansion. After 4 d in culture, the mass of the wall doubled each 3 d (Table 1) so small decreases in mol% of the arabinan and arabinogalactan indicate that the polymers are still synthesized but rates of accumulation vary slightly from other polymers (Fig. 6; Table 1). Accounting for the increase in mass of a 100-ml culture (Table 1), the molar fraction of arabinan (Fig. 6) corresponds to an increase of about 16 mg between 4 and 7 d and 18 mg between 7 and 10 d. For comparison, about 5 and 15 mg of xyloglucan accumulated during these respective time periods. These values, however, do not account for any polymers lost to the extracellular medium or hydrolyzed and recycled during growth. α-Cellulose increased 25 mg between 4 and 7 d and over 50 mg between 7 and 10 d, concomitant with increased rates of xyloglucan accumulation.

Table 5. Neutral polymers accumulated in the walls and ECP regenerated by carrot protoplasts and intact cells. Values in mol%; nd = not detected

Linkage groups	Cell wall			ECP		
	Protoplasts		Cells	Protoplasts		Cells
	4 d	8 d	4 d	4 d	8 d	4 d
Arabinogalactan I and II	9	8	16	11	9	35
Arabinan	8	5	36	6	21	13
Xyloglucan	10	14	17	18	12	31
Arabinoxylan	1	1	5	4	3	9
4-linked glucan	55	63	16	50	41	5
3-linked glucan	3	1	nd	nd	1	nd
Other	14	8	10	11	13	7

Chemical analysis of the incipient wall of protoplasts. Over half of the material accumulated in the wall preparations of 4-d protoplasts was 4-linked glucan (Table 5). Starch constituted about 14% of the weight of the walls of 4-d cells (Table 1) and was undoubtedly a large proportion of the carbohydrate of the freshly isolated protoplasts. A substantial amount of the 4-linked glucan must be from starch remaining in the 4-d protoplasts, and because external sugar concentrations were initially 100 mM, additional starch was synthesized during the course of wall regeneration. The remainder of the polysaccharide was mostly xyloglucan or type I and II arabinogalactans and arabinans (Table 5). Very little arabinoxylan was present. Callose (3-linked glucan) was only 3% of the material, and this proportion decreased to only 1% after 8 d of incubation. By 8 d, arabinogalactans had also become a smaller proportion of material synthesized, and both xyloglucan and 4-linked glucan increased (Table 5). Surprisingly, a substantial amount of the extracellular material was also 4-linked glucan (Table 5). Arabinan also increased substantially in the extracellular fraction (Table 5).

The chemical analyses were useful for determinations of the amounts of specific polymers that had accumulated during wall regeneration (Table 5). To determine the changes in specific synthetic pathways during this development, incorporation of [^{14}C]-D-glucose into polysaccharide was examined with short 3-h pulses with either freshly isolated protoplasts, which begin synthesis of the incipient wall, or 5-d-old protoplasts, when the in-

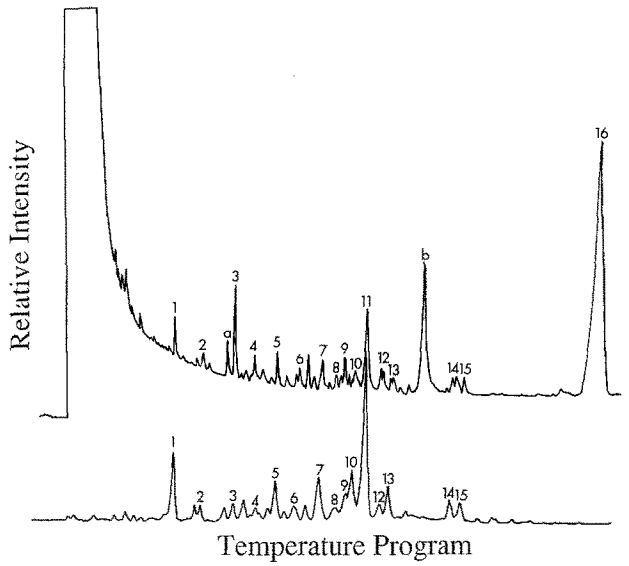

Fig. 7. Partially methylated alditol-acetate derivatives separated by gas-liquid chromatography. Relative mass was quantified by flame-ionization (*upper trace*) and radioactivity was determined by radiogas proportional counting detection (*lower trace*). Derivatives were from radioactive samples of the developing cell walls of 5-d protoplasts. Molar fractions of each derivative were deduced by integration and calculation by the effective carbon-response method (Sweet et al. 1975). A total of 833 Bq was injected; the smallest detectable peaks contain about 3 Bq. Some of the derivatives identified are: *1)* *t*-ara*f*, *2)* *t*-xyl*p*, *3)* 2-ara*f*, *4)* *t*-gal*p*, *5)* 5-ara*f*, *6)* 2-xyl*p* + 4-xyl*p*, *7)* 2,4-rhm, *8)* 3-gal*p*, *9)* 2,5-ara*f*, *10)* 3,5-ara*f*, *11)* 4-glc*p*, *12)* 2,4-xyl*p* + 3,4-xyl*p*, *13)* 6-gal*p*, *14)* 4,6-glc*p*, *15)* 3,6-gal*p*, *16)* *myo*-inositol (internal standard); *a* and *b* are non-carbohydrate contaminants of the derivatization procedure

Table 6. Distribution of neutral sugars in polymers of ECP and cell-wall material synthesized during a 3-h period immediately or 5 d after carrot protoplast isolation. Proportion of radioactivity in cell wall, extracellular polysaccharides and the acetic-nitric-insoluble cellulose were compared 0 d and 5 d after isolation of protoplasts with either the Onozuka or Worthington enzyme preparations. The distribution of radioactivity in various polysaccharides was determined with protoplasts isolated with the Worthington enzyme preparation. nd = not detected

	Cell wall		ECP	
	0 d	5 d	0 d	5 d
Proportion (% radioactivity)				
Onozuka	54	80	46	20
Worthington	69	85	31	15
Acetic-nitric-insoluble material (% radioactivity)				
Onozuka	0.4	1.0	–	–
Worthington	1.8	2.1	–	–
Distribution (mol%)				
Arabinogalactan I	11	4	9	10
Arabinogalactan II	19	15	23	15
Arabinan	14	21	14	28
Arabinoxylan	17	9	20	14
Xyloglucan	1	6	9	2
4-linked Glucan	25	26	6	10
3-linked Glucan	1	nd	2	1
Other	12	19	24	20

cipient wall had completely enveloped the protoplasts. Because D-glucose is the actual carbon source for the wall-regenerating protoplasts, [U-^{14}C]-D-glucose is a marker for the relative synthesis of all polymers at any specific time of labeling. The cytoplasmic pool of UDP-glucose in carrot cells is saturated in as little as 10 min, and complete turnover of the vacuolar pool of glucose occurs in 40 min (Kanabus et al. 1986). Because labeling periods were 3 h in experiments described here, radioactivity from UDP-glucose to other nucleotide-sugar intermediates and, hence, into polymers, should be from well-saturated pools, and the values obtained should reflect the relative amounts of the different polymers made in the freshly isolated and 5-d-old protoplasts. Radioactivity incorporated into sugars exhibiting specific glycosidic linkages was determined by radiogas proportional counting of partially methylated alditol-acetate derivatives separated by gas-liquid chromatography. An example of one such analysis is given in Fig. 7. Freshly isolated protoplasts released substantial amounts of polysaccharide into the medium; 46% of the total synthesized was extracellular when Onozuka enzymes were used to generate protoplasts, but only 31% when the Worthington enzymes were used (Table 6). After 5 d, however, synthesis of ECP by protoplasts prepared from Worthington enzymes had fallen to just 15% of the total, a value essentially the same as the proportion secreted by cells between 4 and 7 d of culture (Table 1).

Protoplast type I and II arabinogalactans accounted for 32% of the label in ECP of freshly isolated protoplasts (Table 6). Linkages typical of xyloglucan made up 9% of the ECP, and arabinoxylan was 20% of the fraction. The differences in the distribution of radioactivity in sugar linkages between the ECPs of freshly isolated protoplasts and 5-d-old protoplasts were small. Amounts of radioactivity in arabinan doubled, whereas that in arabinoxylan and xyloglucan decreased (Table 6). Surprisingly, proportions of radioactivity doubled in secreted arabinan, whereas that in type II arabinogalactan decreased after 5 d. By comparison, the ECPs of cells were about 35% type II arabinogalactan (Table 5). Hence, in addition to the arabinogalactan normally released by cells, substantially more polymers typical of pectin and hemicellulosic fractions were in the medium of freshly isolated protoplasts. Release of these materials de-

creased during regeneration, but substantially more pectic substances were released into the medium than by normal cells (Tables 5, 6).

In the cell-wall material of freshly isolated protoplasts, the amount of type I and II arabinogalactan and 4-linked glucan accounted for about half of the total amount of material synthesized. The amount of arabinoxylan synthesized by 5-d protoplasts was only half the amount synthesized by freshly isolated protoplasts, and the difference was made up by an increase in the amount of radioactivity in xyloglucan and arabinan (Table 6). In contrast, incorporation of radioactivity into 4-linked glucan was only 25–26% of the total incorporation, and similar to the amounts expected from 4-linked glucan in cellulose and hemicellulosic glucans of 4-d intact cells (Tables 5, 6). The lower proportion of radioactivity in 4-linked glucan reaffirms that the large amounts of 4-linked glucan accumulated in protoplasts was likely from starch remaining after isolation of the protoplasts.

The principal difference between walls of intact cells and the new walls of protoplasts is the distribution of the 4-linked glucan resistant to acetic-nitric digestion. In the walls of cells, at least 12% of the cellulose is resistant to acetic-nitric hydrolysis (Table 1), whereas only 2% of the total wall material synthesized by protoplasts is resistant (Table 6).

The proportion of 3-linked glucan was very low, and synthesis could not be detected after incubation of unshaken protoplasts for 5 d (Table 6). To minimize distortion of observations by alterations in the rates of synthesis of the various polymers, we conveniently observed the increase in 3-linked glucan by comparing the ratio of 4-linked glucan to 3-linked glucan in damaged and undamaged protoplasts. After 4 d incubation, the ratio of 4-linked glucan to 3-linked glucan was lowered from 27 for unshaken, healthy protoplasts to only 10.8, 8.6, and 7.8, for rapidly shaken, DMSO-treated, and slowly shaken protoplasts, respectively. This increase in the relative amount of 3-linked glucan in the wounded protoplasts corroborates our observations by fluorescence microscopy (Fig. 3).

Fractionation of ECP and cell-wall material with DEAE-Sephadex. Extracellular and cell-wall polymers were separated into four fractions based on ion-exchange chromatography. Polygalacturonic acid and the RG and HGA of citrus pectin were all strongly bound and were eluted only with high concentrations of salt (not shown). More than half of the total sugar in the ECP of 4-d intact

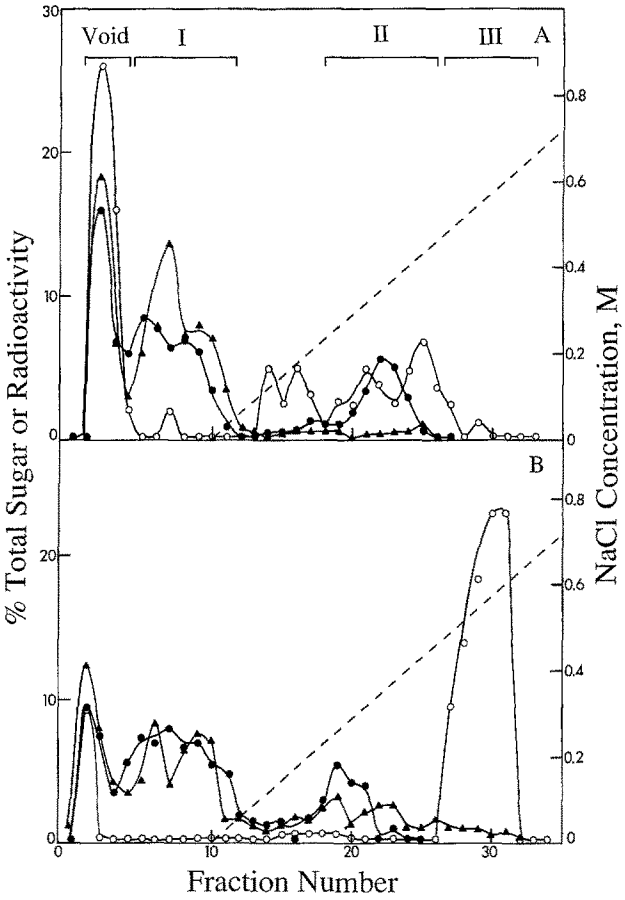

Fig. 8A, B. Anion-exchange chromatography of the ECP and ammonium-oxalate-soluble cell-wall material of 4-d cells from the parent culture, freshly isolated protoplasts, and 5-d protoplasts of carrot. Values represent the proportion of the total sugar from unlabelled material of cells or the proportion of radioactivity from protoplast material. Materials were loaded onto a 1-cm-diameter, 10-cm-long column of DEAE-Sepharose equilibrated with 10 mM phosphate-citrate-EDTA buffer, pH 5.0, washed with loading buffer, and then eluted with a 100-ml linear gradient to 0.8 M NaCl in phosphate-citrate buffer. **A** Comparison of distribution of extracellular polymers as radioactivity incorporated into material from freshly isolated protoplasts (●) and 5-d protoplasts (▲), and total sugar in material from 4-d parent cells (○). **B** Comparison of ammonium-oxalate-soluble pectic substances as radioactivity in material from walls of freshly isolated protoplasts (●) and 5-d protoplasts (▲), and total sugar, measured by the phenol-H_2SO_4 method (DuBois et al. 1956) in material from walls of 4-d parent cells (○)

cells was in the void fractions (Fig. 8A), but over 90% of the uronic acid in the ECP was retained and eluted in fractions II and III by NaCl. The ammonium-oxalate extract of 4-d cells also contained neutral-sugar-rich material that was in the void fraction, whereas a strongly acidic fraction required NaCl for elution (Fig. 8B). Sugar analyses indicated that arabinogalactans predominate in the void fractions, whereas HGA and RG were strongly bound (Shea 1988). In contrast, almost

70% of the labeled polymers synthesized by freshly isolated protoplasts were in the void or very weakly acidic fraction, and only about 20% were in a moderately acidic fraction eluted with NaCl (Fig. 8), even though uronic acids were a substantial proportion of the material. The proportion of strongly bound material increased markedly in 5-d protoplasts as over half was broadly distributed in the more strongly acidic fraction (Fig. 8 B). Even so, the pectins of protoplasts were far less anionic than those of the parent culture (Fig. 8 B).

Discussion

Yield of protoplasts depends on culture age. Highest yields of protoplasts were from 4-d cells (Fig. 1) which had the least amount of α-cellulose in the wall (Table 1). As amounts of α-cellulose increased (Fig. 6), the ability to generate protoplasts decreased, regardless of the enzyme preparation used (Fig. 1). The increase in α-cellulose was primarily a result of increased proportions of non-cellulosic polysaccharides resistant to extraction with KOH but solubilized by the acetic-nitric digestion (Table 1). Of course, cells from stationary phase transferred to fresh medium would also have walls resistant to hydrolysis, and only when sufficient new cells were produced during cell division would the walls have been more susceptible to digestion (Fig. 1). The acetic-nitric-soluble material from parent cultures probably represents pectic substances and hemicelluloses insolubilized by cross-linking by non-carbohydrate material. Increases in hydroxyproline content accompanied expansion of tobacco cells in culture (Iraki et al. 1989) and much of this was from incorporation of extensin into the expanding wall (Cooper et al. 1987; Lamport and Epstein 1983). Stevens and Selvendran (1984) also proposed that cross-linked polyphenolic materials contributed to the resistance to KOH extraction. Increases in both phenolic and extensin cross-linking might make the wall less accessible to the wall-digesting enzymes and, hence, explain the low yield of protoplasts from older cultures as well as from recently transferred cultures (Fig. 1).

Viable protoplasts make little callose. Maintenance of viability during wall regeneration is critical to differentiate the mechanism of regeneration from synthesis of wound polymers. Although the mixture of Onozuka cellulase and Macerase, used routinely in other laboratories to generate protoplasts, provided high yields (Fig. 1), impurities in the mixture resulted in sufficient damage to the protoplasts that more than half of them died by 6 d after isolation (Fig. 2). The superiority of the Worthington enzyme preparation permitted over 75% of the protoplasts to remain viable and resynthesize cell walls (Fig. 2). Our chemical data should thus reflect wall formation uncomplicated by wound phenomena associated with dying or damaged cells. Fluorescence microscopy with fluorescein diacetate as a vital stain and Cellufluor as a stain for both callose and cellulose showed that the intense fluorescence associated with callose was confined to protoplasts that had died some time after isolation (Fig. 3). Viable protoplasts showed no positive staining with Aniline blue, a stain specific for (1 → 3)β-D-glucan (Hayashi et al. 1986). Hayashi et al. (1986) observed very little fluorescence when pea protoplasts were stained with Aniline blue in spite of the large amount of (1 → 3)β-D-glucan synthesized by the protoplasts. Aniline blue fluoresces when illuminated with blue light of wavelength 390–430 nm only if the pH is greater than 8 (Clark 1973), and fluorescence may have been quenched at relatively low pH with the pea protoplasts (Hayashi et al. 1986). The high levels of callose synthesis in other studies probably indicates a wound response of damaged protoplasts in the cultures.

(1 → 3)β-D-Glucan is rarely a constituent of the dicotyledonous cell wall and is likely a wound polymer in protoplasts (Bacic et al. 1988). The enzymes previously used for protoplast isolation are heavily contaminated with proteolytic enzymes (Van der Walk 1984) which could damage the plasma membrane. Very few protoplasts in our study were damaged, and the very small amounts of callose that were synthesized were associated with dying protoplasts (Fig. 3). There was no detectable callose synthesis by older cultures. The proportion of (1 → 3)-glucan in the wall increased when cells in the culture were intentionally damaged. Delmer (1987 review) suggested that cellulose and callose are synthesized by the same enzyme system located on the plasma membrane, and any damage to the membrane disrupts cellulose synthesis so that callose is synthesized instead. The synthesis of callose in place of cellulose may be responsible for the low amount of cellulose or (1 → 4)-linked glucan others have observed in regenerated walls.

Protoplasts synthesize the same polymers as cells in culture but cellulose is largely not resistant to acetic-nitric digestion. Pectic substances constituted much of the material synthesized by protoplasts, and considerable amounts of pectin were released into the medium. The galactosyluronic acids of homogalacturonan (HGA) and galactosyluronic

acid-rich regions on rhamnogalacturonans (RG) can condense with Ca^{2+} forming ionic cross-links that constitute the gel matrix in which the cellulosic framework is embedded (Jarvis 1984; Rees 1977). However, a high proportion of the galactosyluronic acid groups may be secreted as their methyl esters (Jarvis 1984; Kauss and Hassid 1967; Moustacas et al. 1986), and cleavage of these esters is required for formation of the Ca^{2+}-cross-linked gel (Moustacas et al. 1986). Based on ion-exchange chromatography, the pectins initially secreted by protoplasts were largely esterified, and hence would lack the ability to form a proper gel matrix. Increase in the relative charge was correlated with retention of pectin by the protoplasts (Table 6; Fig. 8B).

Quantitation of cellulose is sometimes based on its insolubility in KOH (Takeuchi and Komamine 1978), but resistance to degradation by acetic-nitric acid (Updegraff 1969) is a better indication of its crystallinity. In these experiments only 1–2% of the material synthesized during a 3-h period was resistant to acetic-nitric acid (Table 6) compared to 12% of the wall of 4-d cells (Table 1); 26% of the radioactivity incorporated into polysaccharide by freshly isolated and 5-d-old protoplasts was in $(1 \rightarrow 4)$-linked glucan (Table 6). The degree of polymerization (DP) of cellulose synthesized by freshly isolated protoplasts may be very low and the crystallization may be disrupted. About 70% of the cellulose of carrot protoplasts had a DP of less than 200 units (Asamizu et al. 1977), and protoplast cellulose visually is not as crystalline as the cellulose microfibrils of the primary wall (Herth and Meyer 1977). The individual molecules that make up the microfibril may be discontinuous and, hence, more susceptible to acid hydrolysis (Table 6).

An alternative model for wall regeneration. Based on their microscopic observations, Prat and Roland (1971) proposed that without a cellulosic network, the newly synthesized cell wall is not captured and floats away from the protoplast. Wall development proceeds in a patchwork manner, presumably dependent on the presence of cellulose, until the protoplast is enveloped (Hanke and Northcote 1974). We propose an alternative, two-stage process in which synthesis and assembly of the cell-wall polymers begins soon after protoplast isolation, and then cross-linkage of the assembled polymers ensues to form a strong matrix capable of developing turgor. In contrast to the model of Prat and Roland (1971), we propose that formation of a pectin gel matrix proceeds independent of cellulose synthesis during regeneration of the wall. Jarvis (1984) and Moustacas et al. (1986) suggest that pectins are synthesized as methyl esters, and the uncharged polymers are deposited into the wall space where they are de-esterified by pectin methylesterase. De-esterification permits cross-linking with Ca^{2+} to form the gel. Because pectin methylesterase would be lost when the wall is removed, this cross-linking may occur more slowly in protoplasts; "patches" of these gels eventually coalesce to form the complete matrix (Fig. 3C). Correlated with this visualization is an increase in uronic acid-rich material binding to DEAE-Sephadex (Fig. 8B). Peroxidases and other functional enzymes of the wall lost upon generation of the protoplast may now be retained by this matrix and participate in cross-linking of the pectic substances. This matrix may facilitate organization of cellulose microfibrils and the hemicellulosic polymers that form the fundamental structure, but a cellulosic matrix may not be necessary to "entrap" the pectic substances.

The first phase of wall regeneration is renewed synthesis of pectic and hemicellulosic matrix polymers along with cellulose. Although the wall is complete by 5 d and polymers constituting the matrix are similar to those of the normal cell wall, the protoplasts still burst in the absence of osmoticum. Hayashi et al. (1986) demonstrated that the α-cellulose network still retained its integrity after pectins and hemicelluloses were extracted from walls of intact cells. In 6-d-old protoplasts, however, wall structure was lost and the cell-wall ghost collapsed when pectins and hemicelluloses were removed from walls (Hayashi et al. 1986). Formation of the enveloping gel around the protoplasts occurs within a few days, whereas formation of high-DP, crystalline cellulose takes considerably longer (Asamizu et al. 1977; Herth and Meyer 1977; Hayashi et al. 1986). These observations are consistent with our finding that very little of the 4-linked glucan synthesized was resistant to acetic-nitric hydrolysis (Table 6).

The second phase of wall regeneration needed for normal cell development is the acquisition of tensile strength. Polymers and cross-linking substances other than polysaccharides may be involved in acquisition of this strength, and such interactions should be investigated. The hydroxyproline-rich glycoprotein, extensin, is a major component of the primary cell walls of dicotyledons and is thought to cross-link the cellulose microfibrils into a strong matrix (Cooper et al. 1987; Fry 1986; Lamport and Epstein 1983). Esterified and etherified phenolic substances can impart additional

structural integrity (for a review see Fry 1986). Recent work from our laboratory also has demonstrated that cells in liquid culture adapted to severe osmotic stress fail to polymerize a substantial proportion of the soluble extensin precursors and, consequently, have substantially weaker walls than unadapted cells (Iraki et al. 1989). To our knowledge, synthesis of the extensin-cellulose matrix has not been investigated in protoplasts that are regenerating cell walls and the role of extensin polymerization in cross-linking the matrix into a strong wall clearly needs further examination.

Our results demonstrate that the carbohydrate polymers in the incipient wall of protoplasts are not particularly unique, and as predicted several years ago (Willison and Klein 1982), protoplasts may serve as a useful model to study the assembly of the cell-wall polysaccharides and proteins at the cell surface. Now that suitable enzyme preparations are available and damage to the plasma membrane is limited, these studies are technically feasible. When the wall is removed from the cell, the functional wall enzymes, such as pectin methylesterase and cross-linking peroxidases, are removed as well. Their secretion and activity in relation to the functional re-organization of the wall should be of special interest in future studies.

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