Plant Physiol. (1986) 82, 301–306
0032-0889/86/82/0301/06/$01.00/0

Pea Xyloglucan and Cellulose[1]

IV. ASSEMBLY OF β-GLUCANS BY PEA PROTOPLASTS

Received for publication October 2, 1985 and in revised form May 2, 1986

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ABSTRACT

The synthesis and assembly of xyloglucan were examined during early stages of wall regeneration by protoplasts isolated from growing regions of etiolated peas. During early stages of cultivation, fluorescence microscopy showed that the protoplast surface bound Calcofluor and ammonium salt of 8-anilino-1-naphthalene sulfonic acid and, in time, it also bound fluorescent fucose-binding lectin. Based on chemical analysis, 1,3-β-glucan was the main polysaccharide formed by protoplasts and xyloglucan and cellulose were minor wall components. Binding between cellulose and xyloglucan was not as strong as that in tissues of intact pea plants, *i.e.* mild alkali could dissolve most xyloglucan from the protoplast. However, the addition of exogenous pea xyloglucan into the culture medium stimulated the deposition of new polysaccharides into the protoplast wall and enhanced the close association of newly formed xyloglucan with cellulose.

Protoplasts isolated from a variety of plant tissues are capable of regenerating new walls which are sufficiently normal in structure and composition to permit differentiation into specific cellular types, tissues and even whole plants (5). In early stages of this regeneration, cellulose microfibrils can be seen to arise apparently *de novo* on protoplast surfaces with a 'matted' random orientation (5), relatively low crystallinity (17), and low mol wt (2). This differs markedly from the more ordered wall framework commonly observed in cells grown *in situ*. In intact growing pea stems, a xyloglucan:cellulose complex appears to constitute an essential wall framework that maintains cell size and shape (12, 13, 16). It is not known whether newly formed protoplast cellulose is also tightly associated with xyloglucan in an interconnected macromolecular complex (12), since xyloglucan has not been identified among the components of regenerating walls to date. The main aim of the present study was to test for the biosynthesis of xyloglucan in regenerating cell walls of protoplasts from growing pea stems and to examine its association with cellulose.

In many studies on cultured protoplasts, the first and major polysaccharide which is generated is a glucan composed of 1,3-β-linkages (callose) (3, 18, 27). Wounded or stressed plants often secrete masses of this glucan into periplasmic spaces (8, 9) and it is commonly encountered in products formed by plasma membranes from UDP-glucose *in vitro* (1, 21). Biosynthesis of xyloglucan probably occurs in Golgi by concurrent transfer of both glucose and xylose from UDP-glucose and UDP-xylose (14, 15), in which event, this polysaccharide must be secreted through the plasma membrane where cellulose microfibrils are organized (3–6, 18, 24). Since the macromolecular complex of xyloglucan and cellulose in young cells appears to be responsible for the strength and extensibility, the deposition of xyloglucan as well as cellulose around young protoplasts may be critical to their survival and growth potential.

Nothing is known of xyloglucan synthesis in regenerating cell walls of protoplasts. The present study describes 1,3-β-glucan, cellulose, and xyloglucan deposition by pea protoplasts, and the influence of the addition of exogenous xyloglucan into the culture medium on cellulose-xyloglucan interaction in the new cell wall.

MATERIALS AND METHODS

Materials. The following products were obtained from Sigma: crude digestion enzymes, hemicellulase, and pectinase (*Aspergillus niger*), β-glucuronidase (*Helix pomatia*), aprotinin, bovine serum albumin, and glucose oxidase (method kit). Cellulysin (*Trichoderma viride*) was from Calbiochem-Behring. Ficoll 400 was from Pharmacia, Calcofluor from Ciba-Geigy, Aniline blue from Harleco and ANS[4] from Eastman Kodak. [U-^{14}C]Xyloglucan (0.1 μCi/mg) was prepared (12) from cell wall preparations of pea epicotyl tissues (2 g fresh weight) preincubated with 1 mCi of [U-^{14}C]glucose for 2 h.

Preparation and Culture of Protoplasts. Protoplasts were prepared from the apical sections of etiolated pea seedlings (120 g fresh weight) (24). Digestion was facilitated by shaking slices of the sections for 3 h with 33 mM Mes/Tris (pH 5.5) containing 125 mM $CaCl_2$, 125 mM KCl, 0.25% (w/v) aprotinin, 0.67% (w/v) BSA, 2.67% (w/v) cellulysin, 0.67% (w/v) hemicellulase, 1.33% (w/v) β-glucuronidase, and 5.0% (v/v) pectinase. After the protoplasts were released from the digested tissue, they were collected by centrifugation at 170*g* for 10 min and washed three times with 33 mM Mes/Tris (pH 5.5) buffer containing 125 mM $CaCl_2$, 125 mM KCl, and 0.25% aprotinin. The protoplasts and debris were resuspended in the same buffer containing 8% Ficoll, and the mixture was overlaid with buffer containing 6% Ficoll and buffer only. This discontinuous gradient was centrifuged at 200*g* for 20 min. Purified protoplasts (1×10^8 protoplasts, approximately 2.5 g fresh weight) were collected from the interface between 6% and 0% Ficoll. They were washed three times with buffer by centrifugation, resuspended in R-2 culture medium (22) containing 2% glucose and incubated at 25°C in the dark without shaking. The osmotic potential of culture medium had previously been adjusted between −1275 and −1325 kPa with sorbitol using a cryoscopic osmometer (Precision Systems

[1] Supported by grants from the Natural Sciences and Engineering Research Council of Canada and le Programme de Formation des Chercheurs et d'Action Concertée du Québec.

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[4] Abbreviation: ANS, ammonium salt of 8-anilino-1-naphthalene sulfonic acid.

Osmette). All buffers, dyes, and media were sterilized by filtration or autoclaving before use. During the isolation and incubation procedures, appropriate precautions were taken to maintain sterility. No visible contamination by bacterial or fungal contaminants was observed using light (Nomarski) or fluorescence microscopy throughout these experiments.

Osmotic Stabilization. The protoplasts prepared in this manner were stable in culture, able to withstand manipulations such as shaking, low speed centrifugation, transfer by pipette to new solutions, etc, without lysing, provided the osmotic potential was maintained below −1275 kPa (24). However, if the protoplast cultures were diluted 10-fold with distilled H_2O, the cells burst immediately unless they had been cultured so as to develop a firm cell-wall able to withstand endogenous turgor. Cell number in the suspensions was determined microscopically with a haemacytometer (American Optical). Calcofluor and Aniline blue were used at 0.01 and 0.05%, respectively, with R-2 culture medium adjusted to an osmotic potential of 1280 kPa.

Fractionation of Polysaccharides from Regenerated Cell Walls. Cultured protoplasts (approximately 8×10^7, 2.0 g fresh weight) were centrifuged at 310*g* for 10 min. The supernatant (medium) was poured into ethanol to make a 70% ethanol solution, and the precipitated polysaccharides were washed six times with ice-cold 70% ethanol to obtain the extracellular (medium) fraction. The pelleted protoplasts were washed four times with fresh culture medium, boiled with 70% ethanol for 5 min, washed three times with ice-cold 70% ethanol, and twice with acetone. The pellet was air-dried to obtain a total insoluble preparation which was extracted successively three times with 0.1 M EDTA (pH 7.0) at 85°C for 2 h, 4% KOH/0.1% $NaBH_4$ in an ultrasonic bath for 2 h, and 24% KOH/0.1% $NaBH_4$ in an ultrasonic bath for 2 h (12).

Carbohydrate Analysis. Total polysaccharide was assayed by the phenol-sulfuric acid method (10) calibrated with glucose. This method yields a value for pure pea xyloglucan which is 1.72 times the actual weight of xyloglucan because of high O.D. of xylose (12). Xyloglucan was determined by the iodine-sodium sulfate method (19), and cellulose was assayed as glucose in the 24% KOH-insoluble fraction after dissolving in 72% (w/w) H_2SO_4. For measurements of 1,3-β-glucan content, a new assay method was developed which depended on removing other polysaccharides by extended periodate consumption. A sample was incubated at 4°C for 14 d in the dark with 30 μmol sodium metaperiodate in a total volume of 1 ml. The reaction mixture was then reduced in 100 μmol $NaBH_4$ for 12 h. Trifluoroacetic acid was added to a final concentration of 2 M, and the sample was hydrolyzed by heating at 100°C for 2 h. The mixture was concentrated to dryness to remove the acid and deionized with Rexyn 101 (H^+). Borate was removed by repeated addition of methanol and evaporation (total of 4 additions). 1,3-β-Glucan content was determined as glucose by the glucose oxidase method (26). This method yielded values for 1,3-β-glucan which corresponded closely to the amount of 3-linked glucose based on methylation analysis of the original sample.

Methylation of polysaccharides was performed as described earlier (12). The partially methylated alditol acetates were ana-

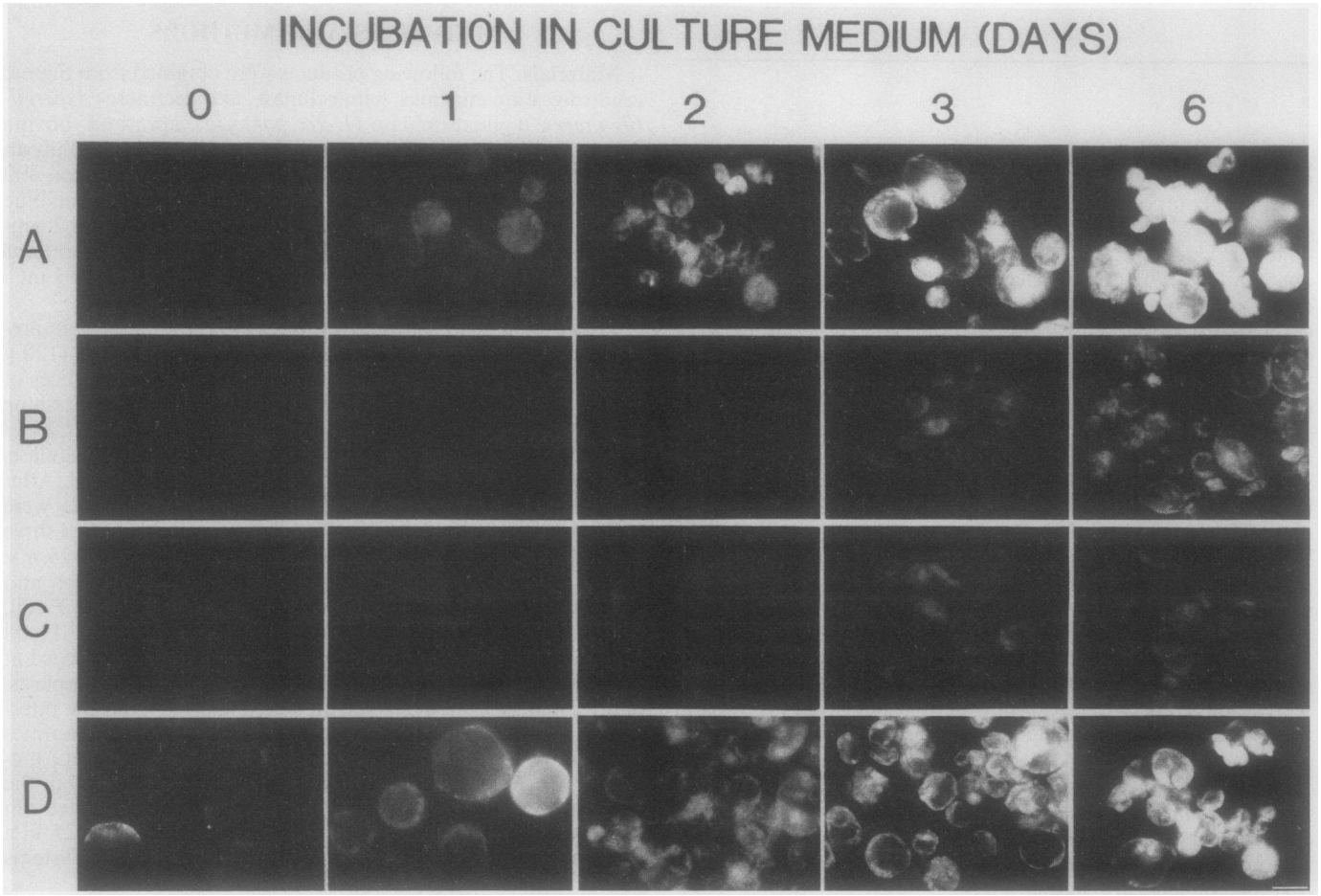

FIG. 1. Fluorescence microscopy of cultured protoplasts during early stages of wall regeneration. Protoplasts were stained with Calcofluor (A), Aniline blue (B), fucose-binding fluorescein lectin (C), and ANS (D). Magnification was the same in all photographs (bar represents 10 μm).

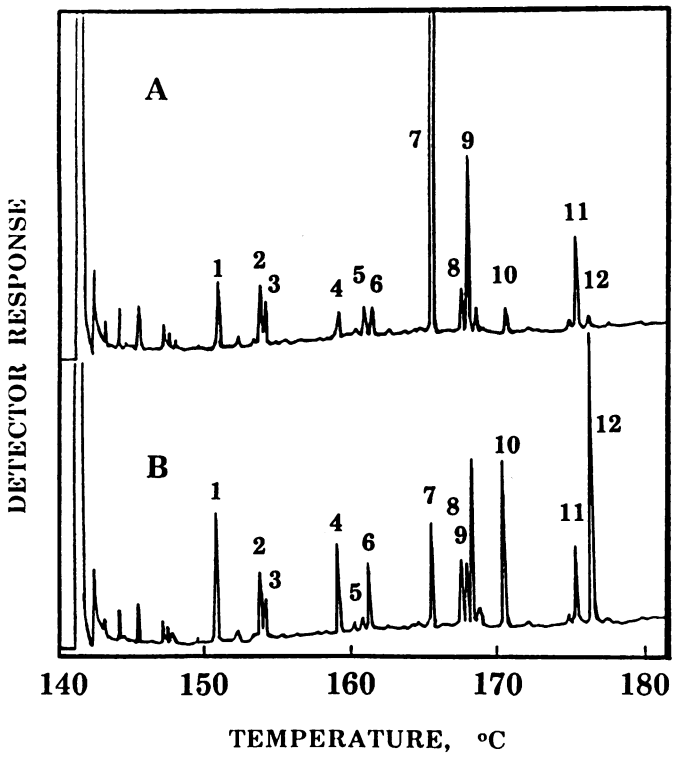

FIG. 2. Gas chromatogram of the methylated alditol acetates of protoplast cell walls (A) and polymers secreted into the culture medium (B) of pea protoplast suspensions. The materials were isolated from 6-d-old suspension culture and derivatized as described in "Materials and Methods." Peak numbers (identified with standards) refer to the permethylated derivatives of 1, T-arabinose; 2, T-xylose; 3, T-fucose; 4, 5-arabinose; 5, 2-xylose; 6, T-galactose; 7, 3-glucose; 8, 3-galactose; 9, 4-glucose; 10, 6-galactose; 11, 4,6-glucose; 12, 3,6-galactose.

lyzed by GLC (Hewlett-Packard, model 5790A) on a glass capillary column (15 m × 0.25 mm) of DB-225. The column oven temperature was raised linearly from 140 to 190°C at a rate of 2°C/min.

Xyloglucan Binding Assay. One hundred μg of pea xyloglucan was added to 2000 μg of pea cellulose containing various oligosaccharides (100 μg) in 1 ml of 5 mM acetate buffer (pH 5.5) with 0.01% NaN_3. The suspension was heated at 100°C for 5 min and incubated at 30°C for 12 h with shaking. The mixture was then centrifuged and the precipitate was washed three times with buffer. The insoluble material was incubated with 24% KOH at 40°C for 2 h, and centrifuged. The amount of xyloglucan was determined by the iodine-sodium sulfate method (19) after neutralization of the supernatant. In standard assay conditions, a maximum of 100 μg of pea xyloglucan bound to 2000 μg of pea cellulose.

RESULTS AND DISCUSSION

Regeneration of Cell Wall by Protoplasts during Cultivation. The deposition of β-glucans during protoplast culture could be visualized at cell surfaces by fluorescence microscopy (Fig. 1). Protoplasts were stained with fluorescein-labeled fucose-binding lectin (from *Ulex europeus*) for xyloglucan (12), with Aniline blue for 1,3-β-glucan (8), or with Calcofluor as a nonspecific dye for wall polysaccharide. None of these dyes reacted with the surfaces of freshly prepared protoplasts. Calcofluor staining was detectable after 1 d of culture and increased steadily up to 6 d (Fig. 1A). Xyloglucan and 1,3-β-glucan could be detected with fluorescent labels by d 3 (Fig. 1, B and C). The appearance of exposed protein at random sites of the protoplast surfaces, as

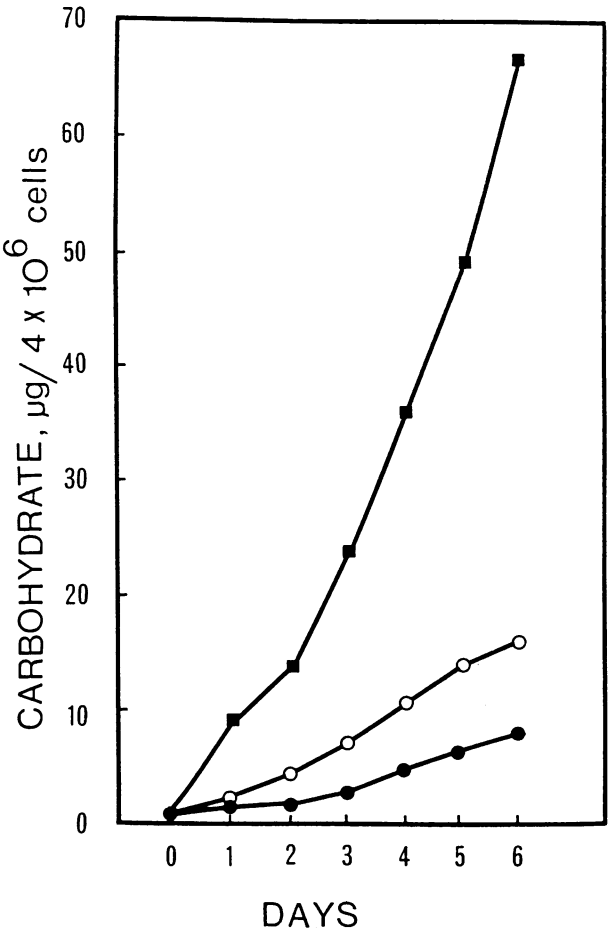

FIG. 3. Formation of xyloglucan, cellulose and 1,3-β-glucan by cultured pea protoplasts. (●), Xyloglucan; (○), cellulose; (■), 1,3-β-glucan. Protoplast culture (4 × 10^7 cells, 2 ml) was poured into ethanol to make 70% ethanol and boiled for 5 min. The precipitate was washed four times with 70% ethanol, and extracted with 24% KOH. Xyloglucan and 1,3-β-glucan contents were determined from the extracts, and cellulose content was from the insoluble fraction.

Table I. *Solubilization of Xyloglucan and 1,3-β-Glucan from 6-d-Cultured Pea Protoplasts (8 × 10^7)*

Fraction	Polysaccharide Formed		
	Total	Xyloglucan	1,3-β-Glucan
		μg	
Medium	808	68	121
Cells			
0.1 M EDTA (pH 7.0)	610	28	574
4% KOH/0.1% $NaBH_4$	486	40	289
24% KOH/0.1% $NaBH_4$	188	28	130
Insoluble	310	0	0
Total	2402	164	1114

stained with the protein-specific fluorescent probe ANS (25), was detectable within 1 d of culture and appeared to remain at about the same quantitative level for up to 6 d of culture (Fig. 1D). Such newly integrated surface proteins could represent the so-called protoplast proteins related either to cell-wall synthesis or reassembly of the suitably vectorial complexes of β-glucan synthases (4), or 'osmotic shock proteins' which are evoked by high

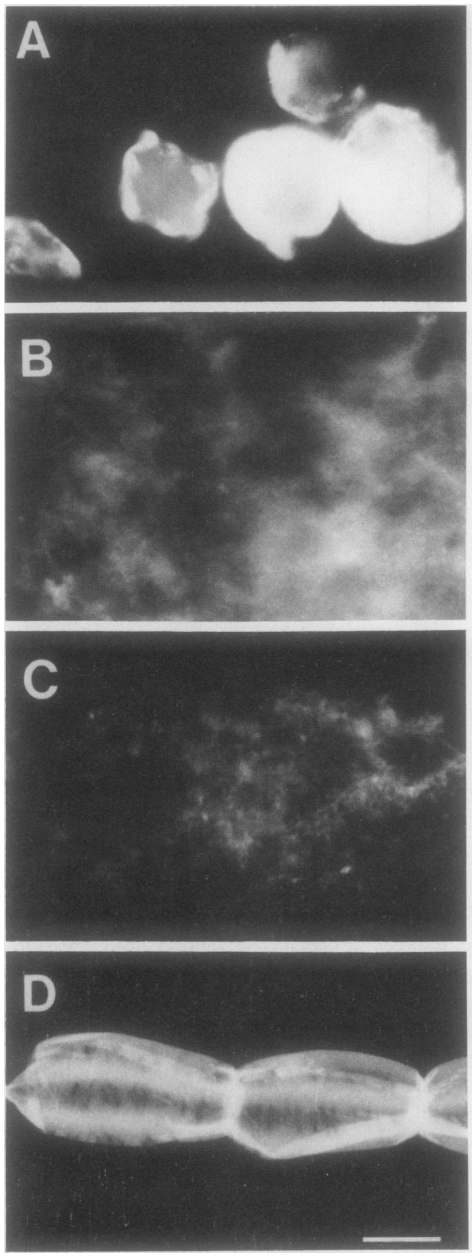

FIG. 4. Effect of sequential extractions on protoplast structure after 6 d culture, as revealed by Calcofluor staining of cell wall surfaces. Protoplasts were extracted with 0.1 M EDTA (pH 7.0) (A), 4% KOH/0.1% $NaBH_4$ (B), and 24% KOH/0.1% $NaBH_4$ (C). Intact tissue was extracted with all solvents (D). Magnification was the same in all photographs (bar is 10 μm).

osmotic strength of the medium of incubation (11).

Methylation analyses of regenerated cell walls and of the secreted polymers in culture medium indicated that numerous sugar linkages occurred (Fig. 2). Large amounts of 3-linked glucose are found in the cell walls, indicating the presence of abundant 1,3-β-glucan (18, 27). 4-Linked glucose, characteristic of cellulose and xyloglucan, is found in the profile of both wall and secreted products. Large amounts of 3,6-linked galactose and the series of other galactose linkages (3-linked, 6-linked, terminal) plus the series of arabinoses (1,5-linked, terminal) were found primarily in the secreted polymers. The galactoses and arabinoses suggest the presence of arabinogalactan (7) and hydroxyproline-rich proteins (20). In addition, terminal fucose and xylose, 2-linked xylose and 4,6-linked glucose, characteristic of xyloglucan, were found in both fractions, suggesting that xyloglucan is indeed formed and partly bound to cell wall and partly secreted into the culture medium.

Based on chemical analysis, the synthesis of cellulose and xyloglucan as well as of 1,3-β-glucan took place during protoplast culture (Fig. 3) and accounted for about two-thirds of the total polysaccharide formed. The weight ratio of total xyloglucan to cellulose (about 1:2) was approximately the same as that observed for the primary cell wall of pea stems (12). 1,3-β-Glucan, callosic material, was formed in much greater amounts than either cellulose or xyloglucan, although 1,3-β-glucan is only barely detectable in the intact tissues of pea stems. Aniline blue staining is faint for 1,3-β-glucan formed by protoplasts during the early stages of wall regeneration (Fig. 1), although the fluorochrome is quite powerful for staining callose formed in wound tissues (21). It may be that the organization or three-dimensional structure of the newly-formed 1,3-β-glucan in protoplasts is different from that of tissue wound callose.

Recovery and Solubility of Xyloglucan in Protoplasts. Table I shows the distribution of total polysaccharide, xyloglucan, and 1,3-β-glucan in fractions of pea protoplasts cultured for 6 d. In this experiment, about one-third of the total polysaccharide was secreted into the medium. Of the amount remaining associated with protoplasts, 20% was alkali-insoluble (cellulose) as was the case in the original tissue (12). Xyloglucan represented only a relatively minor component of polysaccharides in the protoplast preparation (6% of total), compared to the donor tissue (16% of total) (12). Much of the protoplast xyloglucan was secreted into the medium or was freely soluble in EDTA or dilute alkali whereas, in intact tissue, almost all of the xyloglucan forms a tightly bound complex with cellulose from which it can only be released by endo-1,4-β-glucanase digestion or concentrated alkali (12). 1,3-β-Glucan was the major component of the polysaccharide formed by protoplasts, and most (90%) remained associated with the cells.

Protoplasts could be extracted with EDTA solutions, which removed half of the 1,3-β-glucan and one-quarter of the xyloglucan (Table I) and still retain recognizable cell shapes as cell-wall ghosts when stained with Calcofluor (Fig. 4A). The stained wall material appeared to be deposited in irregular patches. Extraction with dilute alkali removed much of the remaining xyloglucan and 1,3-β-glucan (Table I) and resulted in destruction of wall architecture and dispersal of residual polysaccharide (Fig. 4, B and C). In contrast, the original tissue could be extracted with concentrated alkali and the cellulose wall framework still retained its visual integrity and oriented microfibrils (Fig. 4D).

It appears that the hemicelluloses, mainly 1,3-β-glucan and to a lesser extent xyloglucan, which are deposited in new walls by pea protoplasts in culture eventually contribute to the cell's ability to withstand turgor without bursting and help to maintain the integrity of cell shape (Fig. 4). The cellulose deposited concurrently (Fig. 3; Table I) is evidently not oriented or sufficiently fibrous to alone establish cell shape in protoplasts when polysaccharides are removed by alkali (Fig. 4). Protoplast cellulose is probably less crystalline than tissue cellulose (17). The protoplasts differ from cells *in situ* not only in forming a weaker wall framework, but also in forming xyloglucan that is less firmly bound to cellulose, plus large amounts of relatively soluble 1,3-β-glucan (Table I).

Effect of Xyloglucan on the Regeneration of Cell Wall. To determine when a cell wall was established around the protoplasts with sufficient strength to withstand cell turgor, the survival of intact protoplasts was investigated following a 10-fold dilution of the culture medium with water. The determination of cell number became technically difficult as the culture period proceeded because, after about 2 d, the cells aggregated and were

Table II. *Recovery of Xyloglucan from Pea Protoplasts (8×10^7) Cultured in Medium Containing 0.1% [^{14}C] Xyloglucan*

Protoplasts were cultured for 6 d in the presence of radioactive xyloglucan (^{14}C-labeled, 14 dpm/μg). Newly formed xyloglucan is estimated from the difference between total recovered xyloglucan and supplied [^{14}C]-xyloglucan (amount calculated from dpm, using specific activity).

Fraction	Total Sugar	Total Xyloglucan	Supplied [^{14}C]Xyloglucan	Newly Formed Xyloglucan
			μg	
Medium			2000	0
Cells				
0.1 M EDTA (pH 7.0)	1214	270	258	12
4% KOH/0.1% $NaBH_4$	580	94	63	31
24% KOH/0.1% $NaBH_4$	338	96	1	95
Insoluble	326	0	0	0
Total	2458	460	322	138

impossible to separate on a grid for counting. However, aggregation could be prevented by using a culture medium containing added pea xyloglucan (above 0.05%), or any of several polysaccharides, *e.g.* dextran, carboxymethylcellulose, at concentrations above 0.5% w/v. For this study, 0.1% pea xyloglucan was added to prevent aggregation. Cell number in the culture containing xyloglucan began to increase about 6 d after inoculation and 10% of the protoplasts formed dividing cells after 7 d in culture. Under these conditions, the cultured cells acquired a wall within 3 d which prevented bursting of most cells when osmotic pressure was reduced by diluting the medium.

The association of xyloglucan with cellulose microfibrils could occur on the surface of the plasma membrane during microfibril assembly concurrent with xyloglucan secretion. It must be noted that biosynthesis of 1,3-β-glucan also occurs in plasma membranes (1, 21) and that this β-glucan is the first major polysaccharide synthesized in regenerating walls of protoplasts (Fig. 3). To check whether 1,3-β-glucan interferes with the association of xyloglucan to cellulose during wall regeneration, the capacity of pea xyloglucan to bind to pea cellulose *in vitro* was tested (12) directly in the presence of 1,3-β-glucan derivatives. The results showed that laminari-oligosaccharides (laminaribiose to laminarihexaose) did not interfere with binding capacity.

Table II shows the distribution of total polysaccharide and xyloglucan in sequential fractions of protoplasts cultured in medium containing 0.1% radioactive pea xyloglucan. Polysaccharide deposition in the cell wall fractions was increased by the addition of exogenous xyloglucan to the culture medium (Tables I and II) and furthermore, the radioactive xyloglucan appeared to inhibit secretion of polysaccharides into the culture medium. The increased deposition in the presence of exogenous xyloglucan was due not only to the exogenous xyloglucan that became bound to the cell wall fraction, but also to the retention of other polysaccharides which would have been secreted into the culture medium in the absence of external xyloglucan. This phenomenon has been well documented with *Acer pseudoplatanus* cultured cells, where secretion of polysaccharides into the medium can be prevented by the addition of extracellular polysaccharides (23). The coating of protoplasts with exogenous xyloglucan also appears to mask an adhesive substance which would otherwise induce cell aggregation.

It was evident that newly formed xyloglucan in the cells grown in medium containing 0.1% xyloglucan was more strongly (3-fold) associated with cellulose microfibrils than that in standard medium (Tables I and II). Since the specific activity of exogenous [^{14}C]xyloglucan was very low in the 24% KOH extracts, the exogenous xyloglucan clearly failed to complex with the newly formed cellulose microfibrils. Thus, exogenous xyloglucan prevented both polysaccharide secretion into the medium and cell aggregation, and also promoted a tight association between newly formed xyloglucan and cellulose. It is noteworthy that when xyloglucan and cell wall cellulose were mixed *in vitro*, the amount of xyloglucan bound at full saturation never exceeded 6%, whereas the amount bound *in vivo* was commonly about 70% (12). It appears that the presence of xyloglucan outside the protoplasts led to a coordination of the secretion of xyloglucan and the synthesis of cellulose so that the two remained in close association, as they do *in vivo*.

Acknowledgment—We thank Dr. F. Hoffmann (University of California, Irvine) for valuable advice during the final preparation of this paper.

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