Planta (1981) 152:105–114

Planta

Cellulose and 1,3-glucan synthesis during the early stages of wall regeneration in soybean protoplasts

Anita S. Klein[1]*, David Montezinos[1, 2], and Deborah P. Delmer[1]**

[1] MSU-DOE Plant Research Laboratory and Department of Biochemistry, Michigan State University, East Lansing, MI 48824, and
[2] Department of Biology, Wayne State University, Detroit, MI 48202, USA

Abstract. Protoplasts isolated from cultured soybean cells (*Glycine max* (L.) Merr., cv. Mandarin) were used to study polysaccharide biosynthesis during the initial stages of cell wall-regeneration. Within minutes after the protoplasts were transferred to a wall-regeneration medium containing [^{14}C]glucose, radioactivity was detected in a product which was chemically characterized as cellulose. The onset and accumulation of radioactivity into cellulose coincided with the appearance fibrils on the surface of protoplasts, as seen under the electron microscope. At these early stages, a variety of polysaccharide-containing polymers other than cellulose were also synthesized. Under conditions where the protoplasts were competent to synthesize cellulose from glucose, uridine diphosphate-[^{14}C]glucose and guanosine diphosphate-[^{14}C]glucose did not serve as effective substrates for cellulose synthesis. However, substantial amounts of label from uridine diphosphate glucose were incorporated into 1,3-glucan.

Key words: Cell wall formation – Cellulose – *Glycine* – Protoplasts – Glucan.

Introduction

Plant protoplasts offer promising opportunities to study the synthesis, secretion and assembly of the primary cell wall. In the "normal" cell wall, polysaccharides and proteins are tightly interwoven and may be covalently crosslinked. In contrast, the individual components of the regenerating wall of protoplasts tend to be loosely associated and this wall is therefore more convenient for cytological investigations of cellulose-fibril deposition and other synthetic studies (for a recent review see Willison 1976).

The deposition of fibrils on the surface of protoplasts, from a variety of plant species, has been visualized in many cytological studies (Willison 1976). Criteria such as positive Calcofluor staining, and cellulase digestion have frequently been used as evidence of the cellulosic nature of these fibrils; however, these criteria are not entirely rigorous: Calcofluor is not specific for β-1,4-glucan (Darken 1961); most available cellulase preparations are heavily contaminated with other polysaccharide-degrading enzymes (Anderson and Ray 1978); and polysaccharides other than cellulose may appear fibrillar (see Preston 1974).

An alkali-insoluble, fibrous material, isolated from tobacco mesophyll protoplasts which had been cultured for 6 d, displayed an X-ray diffraction pattern which showed some similarity with that of cellulose, but was only weakly crystalline (Hearth and Meyer 1977). In contrast, Takeuchi and Komamine (1978) reported that the regenerated wall of *Vinca rosea* (*Catharanthus roseus*) protoplasts was completely soluble in 24% KOH and contained 1,3- and 1,4-linked glucose residues; they interpreted these data to mean that the regenerated wall of *Vinca* did not contain cellulose.

The time of onset of microfibril deposition during wall regeneration may vary with the material chosen for observation. Burgess et al. (1978) suggested that protoplasts were not competent to synthesize microfibrils immediately after removal from the wall-degrading enzymes, but microfibrils were produced by tobacco mesophyll protoplasts only after a lag of 8 h. This lag period was even longer for protoplasts isolated from other plants, and the authors suggested that this lag period might be required for the protoplasts to assemble the cellulose-synthesizing machinery. In contrast, Williamson et al. (1977) reported that fibril

* *Current address:* Laboratory of Genetics, University of Wisconsin, Madison, WI 53706, USA
** To whom reprints requests should be addressed
Abbreviations: ECM = extracellular material; GLC = gas liquid chromatography; GDP-glucose = guanosine diphosphate glucose; UDP-glucose = uridine diphosphate glucose; U = enzyme units as defined by Sigma Chemical Corp., St. Louis, Mo., USA

0032-0935/81/0152/0105/$02.00

deposition began on the surface of *Vicia hajastana* protoplasts within minutes after the cell-wall degrading enzymes had been removed.

Our initial interest was to utilize protoplasts to study the metabolic pathway of cellulose synthesis. We felt, however, that it was first necessary to establish chemical evidence for the synthesis of cellulose as a part of wall regeneration, and to determine just how soon cellulose synthesis was initiated during wall regeneration. In this paper we present the results of studies on the time course of cellulose biosynthesis in protoplasts isolated from soybean cells grown in suspension cultures. In addition, we examined the spectrum of polysaccharide-containing polymers other than cellulose, synthesized during the first 3 h of wall regeneration. Finally, we investigated whether intact protoplasts which were competent to synthesize cellulose from glucose could also utilize nucleotide sugars as substrates for cellulose biosynthesis.

Materials and methods

Cell cultures. A liquid suspension of cells of *Glycine max* (L.) Merr cv. Mandarin, designated SB-1, was obtained from Dr. Gerhard Weber (University of Utah, Salt Lake City, Ut., USA). The cultures were maintained in 53 ml of 1B5 medium (Gamborg 1975) in 125-ml Erlenmeyer flasks, at 27° C in the dark on a gyratory shaker at 125 rpm. The cells were subcultured by a 1:3 dilution at 3–4-d intervals. The cell line was also maintained as a callus culture on the same medium solidified with agar (0.9%); calli were subcultured monthly. Periodically calli were used to start fresh suspension cultures.

Chemicals and reagents. D-[(U)-^{14}C]glucose, UDP-[(U)-^{14}C]glucose, and GDP-[(U)-^{14}C]glucose, were obtained from New England Nuclear (Boston, Mass., USA), or from ICN Pharmaceuticals (Irvine, Cal., USA). The *Streptomyces* cellulase preparation was a generous gift of Dr. E.T. Reese (U.S. Army Laboratories, Natick, Mass., USA). Alkaline phosphatase (Type III), nucleotide pyrophosphatase (Type II), hog pancreatic α-amylase (Type I A), Sephadex G25 and Ficoll 400 were purchased from Sigma Chemical Corp. (St. Louis, Mo., USA). The Ficoll was purified before use according to Boller and Kende (1979). Thompson-Haywood Chem. (Kansas City, Ka., USA) provided 2,6-dichlorobenzonitrile.

Cell-wall-degrading enzymes were partially purified, as described by Gamborg (1975), with minor modifications. Cellulase R-10 (Onozuka; Kinki Yakult Mfg. Co., Nishinomiya, Japan), Macerase (Calbiochem, Irvine, Cal., USA) or Cellulysin (Calbiochem), were each dissolved in cold water (10–15 g/100 ml) and clarified by centrifugation. The supernatant was applied to a Sephadex G-25 column (90 cm long, 5.5 cm diameter) and eluted with water. Fractions were monitored for protein with Bradford's reagent (Bradford 1976); those which contained protein were retained and lyophilized. The resulting material (less than 40% by weight of the commercial preparation) was used in protoplasts isolation.

Protoplast isolation. All manipulations were performed under sterile conditions. Two-d-old suspension cultures were transferred to conical tubes and allowed to settle under gravity. The cells were washed twice with B5 salts and vitamins (Constabel 1975). One volume of 3% (w/v) Cellulysin (or Cellulase) and 1% (w/v) Macerase in 10% (w/v) sorbitol, at pH 5.5 in water, was added to the cells. The suspension of cells was transferred to a Petri dish (100 mm diameter, 20 mm high), and was incubated for 2.5 h on a reciprocal shaker (ca. 30 rpm), at 27° C, in the dark.

The suspension was filtered through Nitex Cloth (linear pore size, 48 μm; Tabler, Ernst & Traber, Des Plaines, Ill., USA), then protoplasts and cell debris were collected by centrifugation (8 min, 120 *g* in a clinical centrifuge). The protoplasts were purified by flotation upward through a Ficoll 400 gradient, in a manner similar to that described by Boller and Kende (1979). The protoplasts and debris were resuspended in 19% Ficoll (w/w in wall-regeneration medium, see below). This was overlaid with 16% and 13% Ficoll, and finally with wall-regeneration medium. The gradient was centrifuged for 20 min at 170 *g*. Protoplasts were collected from the interface between 13% Ficoll and wall-regeneration medium.

At this stage, the preparation consisted primarily of protoplasts; no intact cells or wall debris could be detected by routine light microscopy. Damaged and lysed protoplasts were trapped in the denser Ficoll layers. Typically, 20 ml packed cell volume yielded between $1 \cdot 10^7$ and $4 \cdot 10^7$ protoplasts. Protoplasts were washed three times with wall-regeneration medium and collected each time by centrifugation (3 min, 120 *g*). Protoplasts were resuspended at final concentrations between $3 \cdot 10^5$ and $2 \cdot 10^6$ ml^{-1} in wall-regeneration medium supplemented with the appropriate labeled sugar; 3–5 ml of protoplast suspension were transferred to individual plastic Petri dishes (60 mm diameter, 15 mm high) for incubation.

Protoplast wall-regeneration medium is that described by Constabel (1975) for soybeans, except glucose was replaced with an equimolar amount of sorbitol.

Isolation of newly synthesized polymers. For the time-course experiments (Figs. 2, 3), incubations were terminated by the addition of four volumes of ethanol to give a final ethanol concentration of 80% (v/v). In other experiments protoplasts were collected by centrifugation (3 min, 120 *g*) and were washed four times with cold wall-regeneration medium, then resuspended in 80% ethanol. This was designated the protoplast fraction. The first supernatant (spent wall-regeneration medium) was brought to 80% ethanol and the precipitate, consisting primarily of polysaccharide and protein, was collected by centrifugation (10 min, 10,000 *g*). This was designated the extracellular fraction (ECM). The various precipitates were washed four times each with 80% ethanol and with chloroform methanol (1:1). To measure the radioactivity in soluble cytoplasmic pools, washed protoplasts were disrupted in 80% ethanol and stored at 4° C overnight. The precipitate was collected by centrifugation and the first supernatant was retained: this constituted the 80% ethanol-soluble cytoplasmic contents.

When protoplasts were labeled with GDP-[^{14}C]glucose, the spent wall-regeneration medium was adjusted to pH between 8 and 9 with NaOH; two units (U) alkaline phosphatase and 0.5 U nucleotide pyrophosphatase were added per ml of spent protoplast culture medium and incubated at 37° C for 20 min. More than 95% of the unused GDP-glucose was degraded to free glucose. The sample was suspended in ethanol to give a final concentration of 80% (v/v), and treated as described above. This procedure was necessary because unacceptable levels of GDP-glucose are precipitated in 80% ethanol. This was not a problem, however, when UDP-glucose was used.

Determination of the radioactivity in crystalline cellulose. Carrier cellulose (1 mg) was added to the ethanol precipitate and the sample was boiled in 2 ml of acetic-nitric acid reagent (Updegraff 1969) for 60–90 min. Water (3 ml) was added to reduce the viscosity and the insoluble material was collected by centrifugation (10 min, 10,000 *g*). The supernatant was removed and the pellet was washed four times with 5–10 ml of water. For direct determination of

radioactivity the pellet was dissolved in 10 ml Biosolve (Beckman Scientific Co., Irvine, Cal., USA) or ACS (Amersham, Arlington Heights, Ill., USA) scintillation mixture.

Analysis of neutral sugars. The neutral-sugar content of cell walls and the distribution of radioactivity in neutral-sugar residues of the ethanol-precipitable material was determined after hydrolysis in 2N trifluoroacetic acid at 120° C for 90 min. Crystalline cellulose is not readily hydrolyzed by this procedure. Neutral sugars were reduced and acetylated by the procedure of Albersheim et al. (1967). Following acetylation, sugar derivatives were redissolved in dichloromethane and analyzed by gas liquid chromatography (GLC). Separations were performed on a model 8350 gas chromatograph (Hewlett Packard, Avondale, Pa., USA) equipped with a variable effluent stream splitter. Nitrogen was used as a carrier gas (22 ml/min). The glass columns (180 cm length, 0.2 cm internal diameter, configuration 8), were packed with Support SP 2340 (Supelco, Bellefonte, Pa., USA). The alditol acetates were separated with temperature programming at 6° C/min, from 140 to 225° C. Acetylated sugar derivatives of the cell wall were identified by relative retention time. Radioactivity in samples derived from protoplasts was identified by co-elution with alditol acetates of a standard sugar mixture: 50% of the effluent stream went to the flame ionization detector, and the remainder was diverted to a heated collection port. The alditol acetates condensed in Uniform Drop Size Pipets (Scientific Products, Romulus, Mich., USA) fitted to the collection port; the condensed material was rinsed into vials with toluene-based scintillation mixture (4.2 g/l 2,5-diphenyloxazole P-bis[2-(5-phenyloxazolyl]-benzene 52·5 mg/l 2,5-diphenyloxazole), and radioactivity was determined by scintillation counting.

Methylation analyses. The linkages of (radioactive) neutral-sugar residues were determined by methylation analysis according to the method of Hakamori (1964), essentially as described in Maltby et al. (1979). After methylation samples were transferred to screw-cap culture tubes (15 mm diameter, 150 mm long, with Teflon-lined caps). Chloroform:methanol 2:1 (v/v; 7 ml) were added to each, then 10 ml of water were added to extract unreacted methyliodide. The extraction was repeated seven times; each time the upper phase of methanol and water was removed, but any insoluble material at the interface and the lower layer were retained. After the extractions, the lower layer and interface were transferred to a conical Microflex tube (Kontes, Vineland, N.J., USA) and dried under nitrogen. After acetylation, the remaining acetic anhydride was evaporated under nitrogen as an azeotrope with toluene, and the gelatinous residue was washed once with toluene. Dichloromethane, 0.5 ml, was added to each sample; the suspension was resuspended and centrifuged in a bench top centrifuge (1 min, 625·*g*), and the supernatant transferred to a clean vial. Chromatography was done on an aerograph, model 2100 (Varian, Louisville, Ky., USA), equipped with an effluent stream splitter (fixed ratio 10:1, collector to flame ionization detector). Helium was used as the carrier gas (30 ml min^{-1}). Separations were performed on glass columns (180 cm long, 0.2 cm internal diameter) packed with Gas-Chrom Q (100–200 mesh; Applied Science Laboratory, State College, Pa., USA) coated with 0.2% poly(ethyleneglycol adipate), 0.2% poly(ethyleneglycol succinate) and 0.4% silicone XE-1150 (Applied Science). The alditol acetates of permethylated sugars were separated with temperature programming (115° C, with a 5-min hold, then 4° min^{-1} to 190° C). The amount of labeled material in samples derived from protoplasts is too low to allow direct identification of sugar derivatives by GLC. Instead fractions were identified by co-elution with known sugar derivatives and the effluent was collected in tips of Pasteur pipets [23 cm (9 in); Scientific Products]. The identity of permethylated sugar derivatives was verified by combined gas chromatography-mass spectrometry of samples which were reduced with sodium borodeuteride; analyses were performed on a Hewlett-Packard gas chromatograph mass spectrometer model 5985A at the MSU mass spectrometry facility. Radioactivity was determined as described above.

Isolation of polysaccharides and protein from cell walls and spent medium of liquid suspension cultures. Cells from 2-d-old suspension cultures were collected on a sintered-glass filter. Ethanol, 3.5 volumes, was added to the spent medium (filtrate) and the mixture was chilled at 4° C overnight. The precipitate, containing polysaccharide and protein, was washed four times with 70% (v/v) ethanol. This material is comparable to the secreted extracellular polysaccharide ("SEPS") and secreted arabino-galactan protein of cultured *Acer pseudoplatanus* cells (Lamport 1977, 1978).

The cells were washed extensively with a solution of B5 salts (at equivalent concentration to that in the culture medium), and were resuspended in cold 10 mM KH_2PO_4-HPO_4 buffer, pH 7, with 4 mM sodium metabisulfite, then disrupted at 4° C by three 2-min cycles of sonication with a Biosonic VI sonifer (intensity 80; Bronwill Scientific, Rochester, N.Y., USA). Cell walls were prepared essentially as described by Talmadge et al. (1973). Following the final acetone wash, the pellets were washed three times in 50% methanol and once in water. The walls were resuspended in 50 mM potassium-phosphate buffer, pH 6.8 (ca. 10 mg/ml) with 100 U α-amylase per ml of suspension, and incubated with 50 µl of toluene for 25 h at 30° C in a gyratory shaker (approx. 125 rpm). The walls were washed three times with water and once with acetone, lyophilized, and stored in a desiccator under vacuum.

Preparation of samples for electron microscopy. Protoplasts were cultured for the times indicated in the figure legends, then resuspended in 1% (w/v) glutaraldehyde in wall-regeneration medium at room temperature (essentially as described in Fowke et al. 1975; Williamson et al. 1976). After 1–2 h the protoplasts were resuspended in 3% glutaraldehyde in the same medium, and held overnight at 4° C. The samples were then washed in 0.05 M sodium-phosphate buffer, pH 6.6, postfixed 2 h in 1% osmium tetroxide in 0.05 M sodium-phosphate buffer, pH 6.6, at 4° C, and after two washes in cold distilled water dehydrated by passage through a graded ethanol series followed by amyl acetate. They were transferred to poly-L-lysine-coated cover slips and allowed to dry as described by Mazia et al. (1975).

The samples were shadowed with platinum-carbon at a 45° angle and then carbon-coated at a 90° angle in a BA 360 vacuum evaporator (Balzers, Liechtenstein). The replicas thus formed were removed from the cover slips by slowly dipping them into 50% hydrofluoric acid, rinsed by transferring them through two changes of distilled water, and cleaned by floating them on ca. 5% sodium hypochlorite (commercial bleach) for 1–3 d. The cleaned replicas were mounted on uncoated 300-mesh copper grids and examined in a 1A electron microscope (Siemans, Chicago, Ill., USA).

Results

Chemical characterization of the newly synthesized cellulose. It has been previously reported that, when plant tissue is treated for 30–60 min at 100° C with a reagent containing acetic and nitric acids, the only polysaccharide which remains insoluble is cellulose (Updegraff 1969). Thus, it seemed possible that in our labeling studies with protoplasts, cellulose synthesis could be conveniently monitored by quantifying the labeled material remaining insoluble after acetic-nitric-acid-treatment. However, it was necessary to confirm the validity of this procedure by establishing

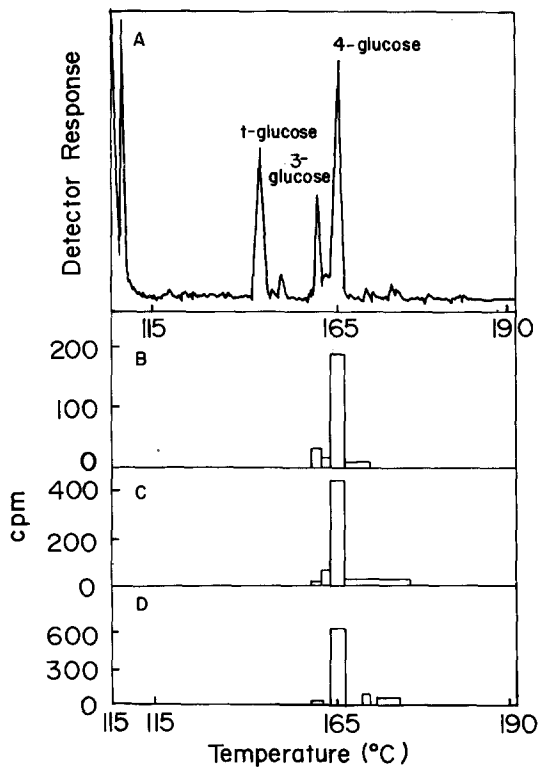

Fig. 1. **A** Gas liquid chromatogram of permethylated alditol acetates from a mixture of laminaribiose, cellobiose and cellulose. Peaks were identified by relative retention times. **B** Elution profile of radioactivity of permethylated derivatives from acetic-nitric-acid-insoluble material obtained from soybean protoplasts which had been incubated for 1 h. **C** Same as B, except the protoplasts were incubated for 3 h. **D** Same as B, except the protoplasts were incubated for 21 h

that all of the radioactivity in such a residue was in fact cellulose.

Acetic-nitric-acid-insoluble material derived from protoplasts incubated with [^{14}C]glucose was hydrolyzed and the resulting monosaccharides were separated by descending paper chromatography (Heiniger and Delmer 1977). Essentially all of the radioactivity cochromatographed with a glucose standard (data not shown). In addition, overnight digestion of acetic-nitric-acid-insoluble material with a *Streptomyces* cellulase preparation released cellobiose and glucose as the only small, labeled saccharides (data not shown). Finally the acetic-nitric-acid-insoluble material was subjected to methylation analysis; at least 85% of the radioactivity cochromatographed with 4-linked glucose (Fig. 1). Similar methylation analyses were obtained irrespective of whether the material was derived from protoplasts incubated with [^{14}C]glucose for 1, 3 or 21 h. In most experiments (Fig. 1C, D), virtually no radioactivity was found in the 3-linked glucose derivative. Small amounts were detected in derivatives with higher retention times. These can be

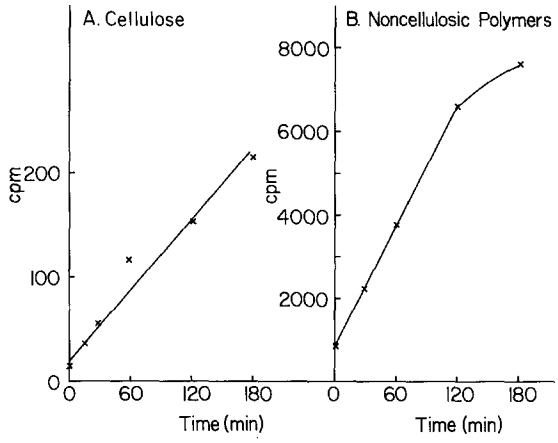

Fig. 2A, B. Short-term incorporation of [^{14}C]glucose into cellulose (**A**) and ethanol-precipitable non-cellulosic polymers (**B**) by soybean protoplasts. Protoplasts ($9 \cdot 10^5$ in 3 ml) were cultured for the times indicated, in wall-regeneration medium supplemented with [^{14}C(U)]glucose (111 kBq/μmol, final glucose concentration 0.75 mM). Each point is the average of duplicates

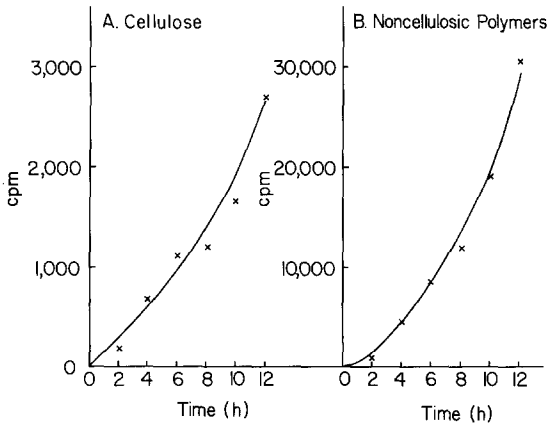

Fig. 3A, B. Long-term incorporation of [^{14}C(U)]glucose into cellulose (**A**) and other polymers (**B**) by protoplasts ($1.65 \cdot 10^6$ in 3 ml) cultured for the times indicated in wall regeneration medium supplemented with [^{14}C(U)]glucose (11.1 kBq/μmol, final glucose concentration 7.5 mM). Each point represents the average of duplicates

attributed to the undermethylation of cellulose. Thus the identity of the radioactive acetic-nitric-acid-insoluble material was confirmed by three separate criteria to be predominantly newly-synthesized cellulose.

Time course of cellulose synthesis. Freshly isolated protoplasts were supplied with [^{14}C]glucose at high specific radioactivity and low concentration in order to label rapidly internal metabolic pools. Under these conditions radioactivity could be detected in newly synthesized cellulose within 15 min (Fig. 2A). Incorporation of label continued over the next 3 h and paralleled the total incorporation of [^{14}C]glucose into noncellulosic polymers (Fig. 2B). It is important to note that in such short-term experiments, the absolute

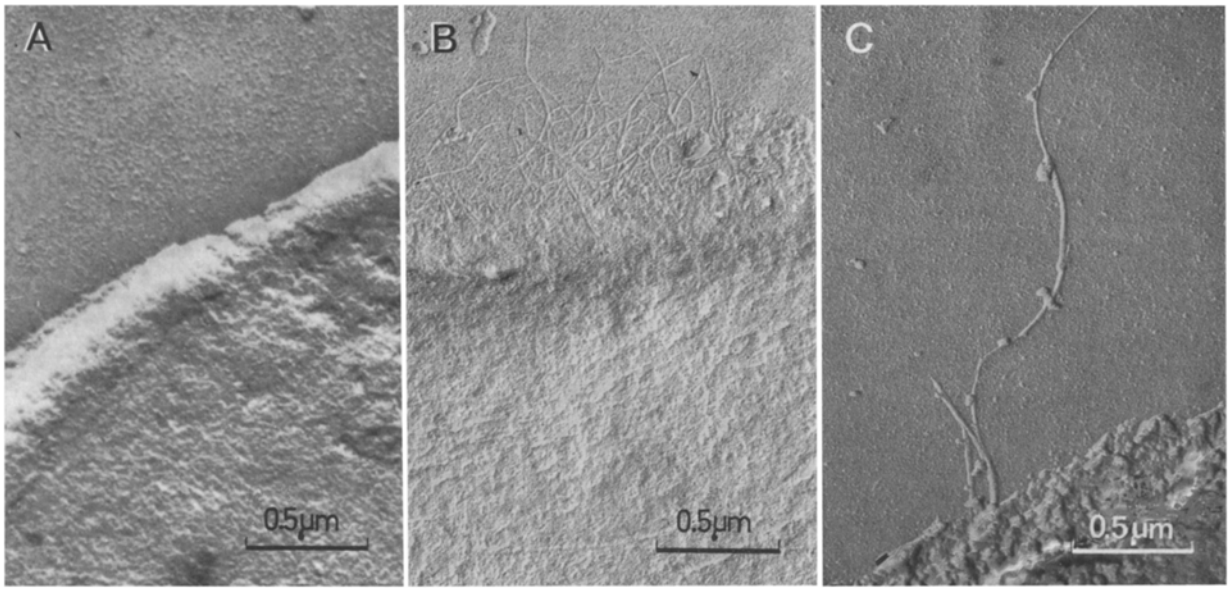

Fig. 4A–C. Deposition of fibrils on the surface of soybean protoplasts with time. **A** Surface of protoplast fixed immediately after isolation (×28,000). **B** Protoplast cultured for 1 h (×31,000). **C** Fibrils on surface of protoplasts cultured for 3 h (×32,000)

magnitude of incorporation into cellulose may not reflect its true rate of synthesis; it is likely that large unlabeled internal carbon pools exist in the soybean protoplasts and make it difficult to label metabolic pools of glucose rapidly to uniform specific activity (Carpita and Delmer, in press). The incorporation of glucose into cellulose was inhibited by 3.3 μM dichlorobenzonitrile, which has been shown to be a specific inhibitor of cellulose biosynthesis (Montezinos and Delmer 1980).

The synthesis of cellulose was also examined over longer time periods (Fig. 3A). Protoplasts were supplied with [^{14}C]glucose at somewhat lower specific activity but at higher final concentrations. Again, the results indicate that cellulose is synthesized without an appreciable lag, and the ratio of incorporation of radioactivity into cellulose and non-celluosic polymers at any given time remains roughly constant (Fig. 3B).

Deposition of microfibrils on the surface of the plasma membrane. Protoplasts were fixed in glutaraldehyde immediately after the final wash of the isolation procedure. Electron micrographs of such samples indicated that the surface of the plasma membrane was devoid of wall material (Fig. 4A). When protoplasts were cultured in wall-regeneration medium for 1 or 3 h fibrils were apparent on the surface of the plasma membrane (Fig. 4B, C). The distribution of fibrils on the surface of protoplasts is not uniform; this may reflect loss of fibrils during fixation.

Location of noncellulosic polymers. Differences in the magnitude of incorporation of label into cellulose versus noncellulosic polymers (Figs. 2, 3) illustrate that cellulose biosynthesis is but one of many synthetic activities occurring simultaneously in protoplasts during wall regeneration. These could include the synthesis of other cell wall polysaccharides and (glyco)proteins, and perhaps other intracellular and extracellular polymers, including those normally secreted by plant cells in suspension cultures. The absence of a preexisting wall apparently permits much of the newly synthesized wall material to float away from the surface of the protoplast. Furthermore, the regenerating wall cannot be separated from cytoplasmic organelles during these first few hours by conventional physical techniques. Such separations rely on the size difference between cytoplasmic organelles and the pieces of cell wall: cell wall polymers are as a rule tightly interwoven and wall fragments may approach the dimensions of intact cells, sedimenting at low centrifugal force. In contrast, fragments of the regenerating wall are probably small and loosely organized. Thus, during the early stages of wall regeneration, it is difficult to separate cytoplasm from cell wall, and distinctions between cell wall constituents and extracellular (secreted) material are also blurred. With these reservations in mind, we collected prelabelled intact protoplasts together with the adhering new cell wall material, and then isolated total ethanol-precipitable material from the remaining, extracellular culture medium (ECM).

Neutral sugar composition of cell wall components and newly synthesized noncellulosic polymers. The neutral sugar composition of soybean cells and ex-

Table 1. Distribution of neutral sugars (**A**) in cell walls and secreted polymers from soybean cell cultures (mol%).
Neutral sugar compositions of the cell walls and secreted polysaccharides were determined as described in the Materials and Methods. The alditol acetates were identified by comparison to the retention times of derivatives of standard sugar mixtures. Values are expressed as mol-% composition, after correction for differences in recovery for each sugar (based on derivatives of standard sugar mixtures, as described in Meinert and Delmer 1977).
(**B**) Neutral sugars in newly synthesized non-cellulosic polymers from protoplasts. Protoplasts ($1 \cdot 10^5$ ml^{-1}, 5 ml per sample) were cultured for 3 h in wall-regeneration medium supplemented with [^{14}C(U)]glucose (370 kBq/μmol, final concentration 1 mM). At the end of the incubation, the protoplasts were separated from the incubation medium. Polymers were collected by ethanol precipitation; the distribution of radioactivity in the alditol acetates derived from the neutral sugar residues in these polymers was determined. The amount of radioactivity in each neutral sugar is the value from representative GLC separations, corrected for differences in recovery between derivatives as determined for standard sugar mixtures. Mol% values are the mean of 3 individual analyses for replicate samples

A. Neutral sugars	Cell walls (mol-%)	Secreted polymers (mol-%)
Rhamnose	9	–
Fucose	3	2
Arabinose	18	14
Xylose	21	32
Mannose	3	7
Galactose	20	26
Glucose	23	19

B.	Protoplasts		Extracellular material	
	cpm	mol-%	cpm	mol-%
Rhamnose+fucose	32	7	236	8
Arabinose	106	22	502	21
Xylose	46	10	304	13
Mannose	115	24	321	14
Galactose	50	10	444	21
Glucose	126	27	515	23

tracellular polymers is presented in Table 1A. This analysis gives the composite neutral-sugar profile for all of the polysaccharides and glycoproteins in the fraction, except cellulose. The distribution of sugars is different between the two fractions: rhamnose is present only in the cell wall; glucose accounts for the largest proportion of the neutral sugars in the wall. Xylose, followed by galactose, are the predominant sugars of extracellular polymers. Ethanol-precipitable materials were isolated from protoplasts and from the culture medium, and the radioactivity present in different neutral sugar residues was determined after acid hydrolysis with 2M trifluoroacetic acid (Table 1 B). These data represent the neutral-sugar composition of newly-synthesized polysaccharide-containing polymers other than cellulose. During the 3 h of protoplast culture, tracer quantities of [^{14}C]glucose were converted to polymeric materials containing a variety of pentoses and hexoses such as were found in the cell wall and extracellular polysaccharides from intact cells. There were some differences in the distribution of label between the protoplast fraction and the ECM; for example, the mol-% of mannose-labeled material was higher in the protoplast fraction than in the extracellular fraction. The opposite distribution of label was found for galactose; polymers in the ECM contained relatively more labeled galactose. The differences in the distribution of the label indicate some quantitative differences with perhaps underlying qualitative differences in the polymers associated with the ECM versus the protoplast fractions.

Methylation analyses of newly synthesized polymers. Gas liquid chromatography of the permethylated alditol acetates from cell walls of dividing, suspension-cultured cells (Fig. 5A), and of the secreted polymers found in the culture medium (Fig. 6A) illustrates the broad spectrum of sugar linkages present (in all of the polymers combined in these fractions). There is more terminally-linked arabinose and 3,6-linked galactose in the secreted polymers, characteristic of soluble arabino-galactan hydroxyproline-rich proteins (Lamport 1977; Clarke et al. 1979). 4-Linked hexose, characteristic of cellulose and starch, is prominent in the profile of the derivatives of cell walls.

Since large amounts of polysaccharide-containing polymers other than cellulose were synthesized during wall regeneration, it was of interest to examine the distribution of linkages in these with respect to the normal components of the cell wall and of the secreted polysaccharides. As noted above, after only a few hours of culture under wall-regenerating conditions, it is not possible to resolve a true cell-wall fraction; instead, the distribution of label from [^{14}C]glucose in permethylated derivatives of the newly synthesized polymers was examined in the total protoplast fraction (Fig. 5B) and in the ECM (Fig. 6B). Although these data are not corrected for differences in recovery, it is evident that a variety of differently linked hexoses and pentoses are labeled in both fractions; the distribution of radioactivity in the derivatives for the various sugars is generally consistent with the neutral-sugar composition of the newly synthesized polymers (Table 1 B). The proportion of label in individual radioactive derivatives obtained from the protoplast fraction is different from that in the ECM. Also, the distribution of label in each differs considerably from the profiles of sugars in cell walls and in the secreted material. For example, labeled 3-linked glucose, the repeating unit of β-1,3-glucan (callose),

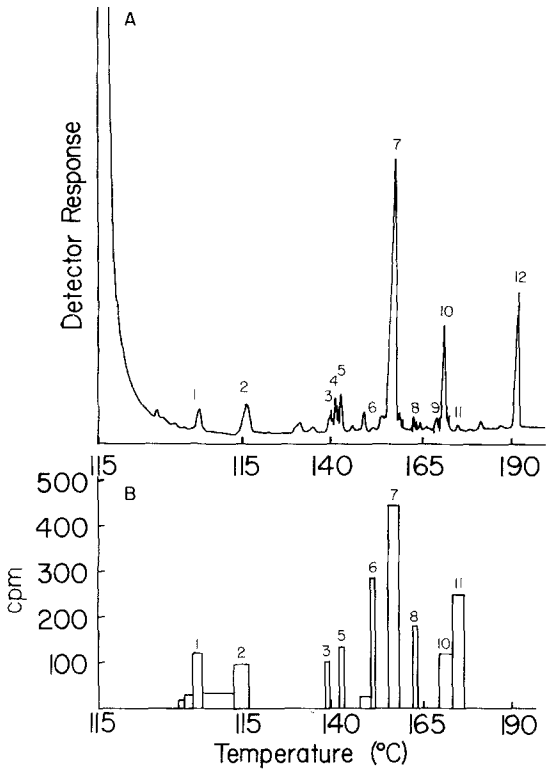

Fig. 5. A GLC elution profile of permethylated derivatives of soybean cell walls. Cell walls were isolated from 2-d-old suspension culture cells and derivatized as described in Materials and Methods. Peaks were identified by relative retention times, comparison with standards, and/or mass-spectral analysis (data not shown). Peak numbers refer to the permethylated derivatives of 1: *t*-arabinose; 2: *t*-xylose; 3: 1,5-arabinose; 4: *t*-galactose 5: 2- and 4-xylose; 6: 3-glucose; 7: 4-hexose (4-glucose, 4-mannose and 4-galactose are not resolved on this column); 8: 6-galactose; 9: 4,6-galactose; 10: 4,6-glucose + plasticizer; 11: 3,6-galactose; 12: inositol (internal standard). **B** Elution profile of radioactivity of permethylated derivatives obtained from the whole-protoplast fraction. Protoplasts ($9.0 \cdot 10^6$ in 5 ml) were cultured for 3 h in wall-regeneration medium supplemented with [^{14}C(U)]glucose (370 kBq/μmol; final glucose concentration 1 mM). Protoplasts were collected by centrifugation and methylated. Chromatography was performed on duplicate samples and fractions were identified by co-elution with cold standards. Similar results were obtained in a duplicate sample and in two other experiments

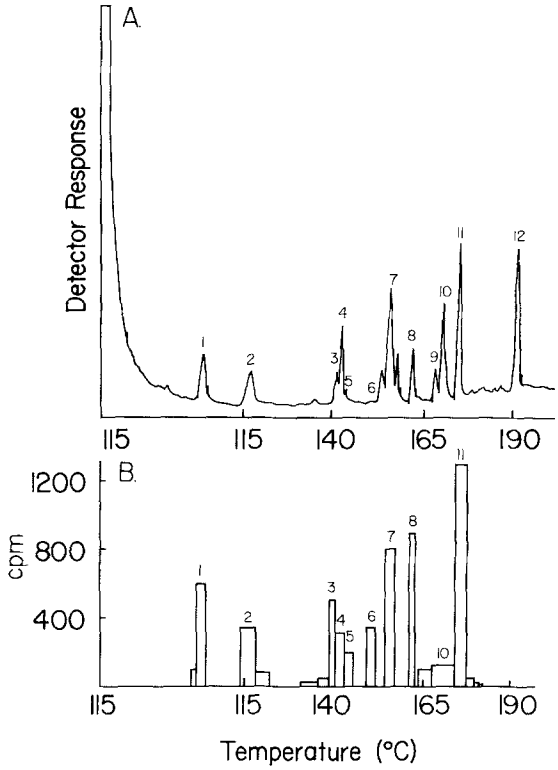

Fig. 6. A GLC elution profile of permethylated derivatives of polymers secreted into the culture medium of soybean cell suspensions. The material was isolated, derivatized and chromatographed as described in Materials and Methods. Peak identifications as in Fig. 5. **B** Elution profile of radioactivity in derivatives prepared from the ECM isolated from wall regeneration medium after 3 h of protoplast culture (see Fig. 5B)

was identified in both fractions; this result indicates that the very early stages of wall regeneration may include the synthesis of abnormal polymers since little or no 3-linked glucose is found in normal soybean cell walls (Fig. 5A), or in secreted polymers (Fig. 6A). In addition, 3,6-linked galactose is prominent in the newly synthesized polymer from the protoplasts but almost absent in the normal cell wall; this galactose could be part of an arabinogalactan protein synthesized in the cytoplasm but which is destined to be secreted into the culture medium. These results indicate that a wide range of complex polymers are synthesized during the first 3 h of wall regeneration.

Utilization of nucleotide sugars. A large volume of mostly circumstantial evidence indicates that the nucleotide sugar, uridine diphosphate glucose (UDP-glucose) is the high-energy sugar donor for cellulose biosynthesis, although guanosine diphosphate glucose (GDP-glucose) has also been proposed as a substrate (for a review, see Delmer 1977; Hopp et al. 1978). Thus, having shown that the soybean protoplasts are competent to synthesize cellulose from glucose, we were interested in determining whether they could utilize either of these nucleotide sugars for cellulose synthesis. Protoplasts were cultured (3 h) in wall-regeneration medium containing 1 mM unlabeled glucose, supplemented with either GDP-[^{14}C]glucose or UDP-[^{14}C]glucose (Table 2). Only a small fraction of the supplied label (0.05%) became associated with 80%-ethanol-soluble cytoplasmic pools; at the end of the incubation very little of the supplied label (less than 1%) was hydrolyzed to free glucose (data not shown). However, substantial amounts of label were incorporated into ethanol-precipitable material; the bulk of this material was not associated with the protoplasts but was isolated from the culture medium.

Table 2. Utilization of nucleotide sugars by soybean protoplasts. Protoplasts were cultured for 3 h in wall-regeneration medium supplemented with either uridine diphosphate [^{14}C(U)]glucose or guanosine diphosphate [^{14}C(U)]glucose; 1 mM unlabeled glucose was included in the medium. Data from a similar experiment with [^{14}C]glucose are included for comparison. Each value is the average of duplicates in one experiment; similar results were obtained in other experiments. The number of protoplasts per sample is indicated

Label supplied[a]	Fraction labeled	Noncellulosic polymers (cpm)	Cellulose (cpm)
UDP-[^{14}C]glucose (37 kBq/μmol)	Protoplasts[b]	5,800	148
	ECM[c]	30,000	64
GDP-[^{14}C]glucose (18.5 kBq/μmol)	Protoplasts[d]	300	0
	ECM	106,000	0
[^{14}C]glucose (370 kBq/μmol)	Protoplasts[b]	562,000	14,400
	ECM	308,000	1,800

[a] Final concentration in all cases 1 mM
[b] $9.5 \cdot 10^6$ in 5 ml
[c] Extracellular material
[d] $4.5 \cdot 10^6$ in 5 ml

In contrast, when protoplasts were incubated under similar conditions with [^{14}C]glucose, nearly two thirds of the newly-synthesized polymers remained associated with the protoplast fraction.

The majority of the label incorporated from the nucleotide sugars was solubilized by treatment with the acetic-nitric-acid reagent, providing some evidence that the products were not crystalline cellulose (Table 2). To identify the form of the label, originating from GDP-glucose or UDP-glucose, in these noncellulosic polymers we carried out methylation analyses of the radioactive products and identified the labeled derivatives as previously described. When UDP-[^{14}C]glucose was supplied, over 90% of the radioactivity was found in 3-linked glucose; none was detected in terminal-glucose; and from 0 to 10% of the radioactivity was detected in 4-linked glucose. This labeling pattern was found in both the ECM and the protoplast fraction. When GDP-[^{14}C]glucose was the source of radioactivity, both terminal and 4-linked glucose residues were labeled in very similar amounts; but as indicated above this material cannot be regarded as crystalline cellulose since it was not resistant to acetic-nitric-acid digestion. This product has not, as yet, been further characterized. Thus, under conditions in which protoplasts actively synthesized cellulose, UDP-glucose and GDP-glucose did not serve as effective substrates for synthesis of crystalline cellulose when supplied to the outer surface of the plasma membrane.

Discussion

Using [^{14}C]glucose as a tracer, we have examined the onset and time course of cellulose synthesis during the first few hours of wall regeneration by soybean protoplasts. Within minutes after the protoplasts were isolated from wall-degrading enzymes and transferred to wall-regeneration medium, label was detected in an acetic-nitric-acid-insoluble polymer. By three separate criteria, we established that the labeled product was in fact cellulose. These results, provide clear chemical evidence that protoplasts are capable of synthesizing cellulose during the first hours of wall regeneration. Cytological studies indicated that, immediately after isolation, the surface of the plasma membrane was devoid of wall material, implying that the radioactive glucose was incorporated into new cellulose chains, not preexisting cellulose fibrils. We interpret these data to mean that the cellulose-synthesizing machinery remains intact during protoplast isolation from rapidly growing cultured cells. The time course of incorporation of label into cellulose was correlated with additional electron-microscopic observations. After 1 h of culture in wall-regeneration medium, fibrils were apparent on the surface of the plasma membrane.

There have been few reports on the chemical composition of the non-cellulosic components of the regenerating cell wall. Hanke and Northcote (1974) reported that during the first 20 h of wall regeneration by soybean protoplasts, exogenous [^{14}C]glucose was incorporated primarily into protein, starch and cellulose, with a small amount incorporated into an acidic pectin. They suggested that little other cell-wall material was synthesized during the first day of wall regeneration. Asamizu and Nishi (1980), however, indicated that the regenerating wall of carrot protoplasts contained newly synthesized pectins, hemicelluloses and cellulose within this same time span. Our data are similar to those of Asamizu and Nishi since we have observed synthesis of a broad spectrum of polysaccharide-containing polymers. As it is not feasible to isolate a pure cell-wall fraction during the first hours of wall regeneration we examined 80% ethanol-precipitable material in the whole-protoplast fraction and in material isolated from the culture medium (ECM) after 3 h incubation. We assume that the ECM is representative of polymers synthesized in the cytoplasm from [^{14}C]glucose and then secreted. Some of these polymers may normally be secreted to the medium (e.g. arabino-galactan protein). However, others may represent polymers destined for the regenerating wall but because of the lack of a substantial wall matrix are instead released into the culture medium

(Willison 1976). The spectrum of labeled polysaccharide-containing polymers synthesized during the first 3 h of wall regeneration indicates that many types of polymers are synthesized during the very earliest stages of wall regeneration; some of these appear to resemble in quality the noncellulosic polymers of the normal cell wall and of the extracellular material isolated from liquid suspension cultures of whole cells. Arabinose and galactose are normally components of pectins, hemicelluloses, extensin and related arabinogalactan hydroxyproline-rich proteins, and hydroxyproline-poor glycoproteins (Brown and Kimmins 1979). Noncellulosic glucose could be derived from starch, xyloglucan or 1,3-glucan. Glucose accounts for less than 30% of the labeled sugar residues in the protoplast fraction, indicating that starch synthesis is not the dominant synthetic activity during the early stages of wall regeneration. Some glucan containing 3-linked residues was also synthesized. This polysaccharide may be an abnormal product associated with the early stages of wall regeneration since little or no 3-linked glucose was found in the methylation analysis of the cell wall or extracellular polysaccharides isolated from cultured soybean cells. Takeuchi and Komamine (1978) also reported that the regenerating wall of *Vinca* protoplasts showed some differences from the cell wall of the original culture; the regenerating cell wall did not contain cellulose but instead was composed of noncellulosic glucans containing 1,3- and 1,4-linkages.

Putative cellulose-synthetase complexes have been observed in the plasma membrane of algae and higher plants (Brown and Montezinos 1976; Mueller and Brown 1980; Giddings et al. 1980). It is not clear whether the active sites of such complexes would be freely exposed to extracellular, activated forms of glucose which might serve as substrates for cellulose synthesis. Previous studies with cultured soybean cells (Brett 1978) and pea stem segments (Anderson and Ray 1978) indicated that β-1,3-glucan was synthesized when the cultured cells or pea slices were supplied UDP-glucose. In the pea stem segments, incorporation occured at cut surfaces probably at the plasma membrane; incorporation by cultured cells was stimulated by wounding. Raymond et al. (1978) reported similar results with pea stem slices; however, they indicated that glucans with β-1,4-linkages were also synthesized from UDP-glucose and that total incorporation was proportional to the cut surface. To provide additional information on the utilization of nucleotide sugars by putative plasma-membrane glucan synthetases, we supplied UDP-[^{14}C]glucose and GDP-[^{14}C]glucose to intact protoplasts. In this system the radioactive substrate can be uniformly supplied to the exterior surface of the plasma membrane because (i) protoplasts exist as single intact cells and (ii) the cell wall, which may interfere with diffusion of charged substrates, is absent. When soybean protoplasts were incubated with UDP-[^{14}C]glucose or GDP-[^{14}C]glucose, under conditions in which it was demonstrated that they were competent to synthesize cellulose from glucose, neither nucleotide sugar was taken up by the protoplasts into soluble cytoplasmic pools, nor did either nucleotide sugar serve as an effective substrate for the synthesis of crystalline cellulose. Delmer et al. (1977) obtained similar results with intact fibers from cultured cotton ovules. However, label was incorporated by soybean protoplasts from GDP-glucose into ethanol-precipitable polymers containing terminal and 4-linked glucose residues. This material was not resistant to acetic-nitric-acid digestion and thus cannot be considered cellulose. However, we cannot as yet rule out the possibility that this product may represent some nascent form of cellulose. Likewise label from UDP-glucose was incorporated into polymer(s); but in this case over 90% of the label was in 3-linked glucose residues. Thus, we conclude that if either UDP-glucose or GDP-glucose is a precursor to cellulose in these protoplasts the active site cannot effectively accept the substrate and catalyze the synthesis of crystalline cellulose when such substrates are supplied to the outer face of the plasma membrane.

In summary, the results provide chemical evidence that soybean protoplasts are competent to synthesize cellulose after they are transferred to a medium favoring wall regeneration and without any appreciable lag period. At the same time protoplasts begin to synthesize other polymers, some of which may be incorporated into the regenerating cell wall. Although soybean protoplasts were competent to synthesize cellulose, they did not utilize supplied nucleotide sugars for cellulose synthesis.

We would like to thank Dr. Larry Fowke, University of Saskatchewan, Saskatoon, Canada for providing detailed instructions for protoplast fixation; Dr. Gerhard Weber, University of Utah, Salt Lake City, USA, for the SB-1 cell line and instruction on culture conditions; and the MSU Mass Spectrometry Faculty for assistance in the use of their facilities. We also thank Dr. Anthony Bacic for helpful discussions and Carl Kulow, Kathy Johnson and Beth Schmidt for general assistance. This work was supported by U.S. Department of Energy Contract DE-AC02-76ERO1338.

References

Albersheim, P., Nevins, D.S., English, P.D., Karr, A. (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohydr. Res. **5**, 340–345

Anderson, R.L., Ray, P.M. (1978) Labeling of the plasma membrane of pea cells by a surface localized glucan synthetase. Plant Physiol. **61**, 723–730

Asamizu, T., Nishi, A. (1980) Regenerated cell wall of carrot protoplasts isolated from suspension-cultured cells. Physiol. Plant. **48**, 207–212

Boller, T., Kende, H. (1979) Hydrolytic enzymes in the central vacuole of plant cells. Plant Physiol. **63**, 1123–1132

Bradford, M. (1976) A rapid and sensitive assay for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. **72**, 249–259

Brett, C.T. (1978) Synthesis of β(1-3)-glucan from extracellular uridine diphosphate glucose as a wound response in suspension-cultured soybean cells. Plant Physiol. **62**, 377–382

Brown, R.G., Kimmins, W.C. (1979) Linkage analysis of hydroxyproline-poor glycoprotein from *Phaseolus vulgaris*. Plant Physiol. **63**, 557–561

Brown, R.M., Jr., Montezinos, D. (1976) Cellulose microfibrils: visualization of biosynthetic and orienting complexes in association with the plasma membrane. Proc. Natl. Acad. Sci. USA **73**, 143–147

Burgess, J., Linstead, P.S., Bonsall, V.E. (1978) Observations of the time course of wall development at the surface of isolated protoplasts. Planta **139**, 85–91

Carpita, N., Delmer, D.P. (in press) Concentration and metabolic turnover of UDP-glucose in developing cotton fibers. J. Biol. Chem.

Clarke, A.E., Anderson, R.L., Stone, B.A. (1979) Form and function of arabinogalactans and arabinogalactan-proteins. Phytochemistry **18**, 521–540

Constabel, F. (1975) Isolation and culture of plant protoplasts. In: Plant tissue culture methods. pp. 11–21, Gamborg, O.L., Wetter, L.R., eds. Nat. Res. Counc. of Canada, Saskatoon, Sask.

Darken, M.A. (1961) Applications of fluorescent brighteners in biological techniques. Science **133**, 1704–1705

Delmer, D.P. (1977) The biosynthesis of cellulose and other plant cell wall polysaccharides. Rec. Adv. Phytochem. **11**, 45–77

Delmer, D.P., Heiniger, U., Kulow, C. (1977) UDP-glucose: glucan synthetase in developing cotton fibers. I. Kinetic and physiological properties. Plant Physiol. **59**, 713–718

Fowke, L.C., Bech-Hansen, C.W., Gamborg, O.L., Constabel, F. (1975) Electron-microscope observations of mitosis and cytokinesis in multinucleate protoplasts of soybean. J. Cell Sci. **18**, 491–507

Gamborg, O.L. (1975) Callus and cell culture. In: Plant tissue culture methods, pp. 1–10, Gamborg, O.L., Wetter, L.R., eds. Nat. Res. Counc. of Canada, Saskatoon, Sask.

Giddings, T.H., Jr., Bower, D.L., Staehelin, L.A. (1980) Visualization of particle complexes in the plasma membrane of *Micrasterias denticulata* associated with the formation of cellulose fibrils in primary and secondary walls. J. Cell Biol. **84**, 327–339

Hakamori, S. (1964) A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. **55**, 205–208

Hanke, D.E., Northcote, D.H. (1974) Cell wall formation by soybean callus protoplasts. J. Cell Sci. **14**, 29–50

Heiniger, U., Delmer, D.P. (1977) UDP-glucose: Glucan synthetase in developing cotton fibers II Structure of the reaction product. Plant Physiol. **59**, 719–723

Herth, W., Meyer, Y. (1977) Ultrastructural and chemical analysis of the wall fibrils synthesized by tobacco mesophyll protoplasts. Biol. Cel. **30**, 33–40

Hopp, H.E., Romero, P.A., Daleo, G.R., Pont Lezica, R. (1978) Synthesis of cellulose precursors: the involvement of lipid-linked sugars. Eur. J. Biochem. **84**, 561–571

Lamport, D.T.A. (1977) Structure, biosynthesis and significance of cell wall glycoproteins. Rec. Adv. in Phytochem. **11**, 79–115

Lamport, D.T.A. (1978) Cell wall carbohydrates in relation to structure and function In: Frontiers in plant tissue culture, pp. 235–244, Thorpe, T.E. ed, Int. Assoc. for Plant Tissue Culture, Calgary, Alta., Canada

Maltby, D., Carpita, N.C., Montezinos, D., Kulow, C., Delmer, D.P. (1979) β-1,3-Glucan in developing cotton fibers: structure, localization, and relationship of synthesis to that of secondary wall cellulose. Plant Physiol. **63**, 1158–1164

Mazia, D., Schatten, G., Sale, W. (1975) Adhesion of cells to surfaces coated with polylysine. J. Cell Biol. **66**, 198–200

Meinert, M., Delmer, D.P. (1977) Changes in biochemical composition of the cell wall of the cotton fiber during development. Plant Physiol. **59**, 1088–1097

Montezinos, D., Delmer, D.P. (1980) Characterization of inhibitors of cellulose synthesis in cotton fibers. Planta **128**, 303–311

Mueller, S.C., Brown, R.M., Jr. (1980) Evidence for an intramembrane component associated with a cellulose microfibril synthesizing complex in higher plants. J. Cell Biol. **84**, 315–326

Preston, R.D. (1974) The physical biology of plant cell walls. Chapman and Hall, London

Raymond, Y., Fincher, G.B., Maclachlan, G.A. (1978) Tissue slice and particulate β-glucan synthetase activities from *Pisum* epicotyls. Plant Physiol. **61**, 938–942

Takeuchi, Y., Komamine, A. (1978) Composition of cell wall formed by protoplasts isolated from *Vinca rosea* suspension culture. Planta **140**, 227–232

Talmadge, K.W., Keegstra, K., Bauer, W.D., Albersheim, P. (1973) The structure of plant cell walls. I. Macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of pectic polysaccharides. Plant Physiol. **51**, 158–173

Updegraff, D.M. (1969) Semi-micro determination of cellulose in biological materials. Anal. Biochem. **32**, 420–424

Williamson, F.A., Fowke, L.C., Constabel, F.C., Gamborg, O.L. (1976) Labelling of concanavalin a sites on the plasma membrane of soybean protoplasts. Protoplasma **89**, 305–316

Williamson, F.A., Fowke, L.C., Weber, G., Constabel, F.C., Gamborg, O.L. (1977) Microfibril depostion on cultured protoplasts of *Vicia hajastana*. Protoplasma **91**, 213–218

Willison, J.H.M. (1976) Synthesis of cell walls by higher plant protoplasts. In: Microbial and plant protoplasts, pp. 283–298, Peberdy, J.F., ed. Academic Press, New York

Received 16 July 1980; accepted 10 February 1981