The Institute of Paper Chemistry

Appleton, Wisconsin

Doctor's Dissertation

A Study of Cell Wall Regeneration by Douglas-Fir *[Pseudotsuga menziesii* (Mirb.) Franco] Protoplasts from Suspension Cultures

Kim William Robinson

June, 1980

A STUDY OF CELL WALL REGENERATION BY DOUGLAS-FIR [*Pseudotsuga menziesii* (Mirb.) FRANCO] PROTOPLASTS FROM SUSPENSION CULTURES

A thesis submitted by

Kim William Robinson

B.S. 1973, State University of New York,

College of Environmental Science and Forestry

M.S. 1976, Lawrence University

in partial fulfillment of the requirements
of The Institute of Paper Chemistry
for the degree of Doctor of Philosophy
from Lawrence University,
Appleton, Wisconsin

June, 1980

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

An examination was made of the constituents deposited in the regenerating cell wall of nondividing Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] protoplasts.

Suspension cultures of Douglas-fir cells derived from both needle and hypocotyl tissues were treated with cell wall degrading fungal enzyme preparations to generate uniform populations of protoplasts. Both L-(U-^{14}C)-proline and D-(U-^{14}C)-glucose were separately fed to the protoplasts and their uptake into the regenerating cell wall was monitored for up to 6 days. Proline was observed to incorporate into the wall immediately, but the glucose uptake was initiated only after about 30 hours. Uptake of the two precursors suggested that both protein and polysaccharide components were deposited during the period studied. The beginning of glucose incorporation coincided with a measurable linear increase in cell wall weight.

The major sugars detected in the hydrolyzed regenerating wall were glucose, galactose, mannose and arabinose; glucose was dominant. All of the sugars except mannose were deposited only after two days of protoplast culturing. X-ray diffraction analysis of wall samples revealed amorphous patterns, suggesting the absence of crystalline cellulose. Further analysis of acetolysis demonstrated that cellulose was not present. Microscopical techniques, however, revealed the probable deposition of callose and pectin. Wall-bound protein was extracted by two different methods, one of which removed two proteins which isoelectrically focused at pH 4.2 and 4.4 but sedimented as one during ultracentrifugation. Amino acid and sugar analysis revealed that the regenerated wall glycoprotein was abundant in glycine, alanine, glucose, and galactose.

The general conclusion of this study is that the regenerated cell wall of Douglas-fir protoplasts originating from suspension cells was synthesized in response to the stress of cell wall removal. Callose, usually associated with wound tissue,

was detected in the material found on the protoplast surface. The cell wall glycoprotein isolated from the regenerating wall was compared with and found to be similar to a wound-induced cell wall glycoprotein reported in Phaseolus vulgaris, [Brown, R. G. and Kimmins, W. C., Phytochem. 17:29-33(1978)]. Given the absence of cell division and cellulose, it appears that the regenerated wall material of these cultured protoplasts differs considerably from normal cell walls.

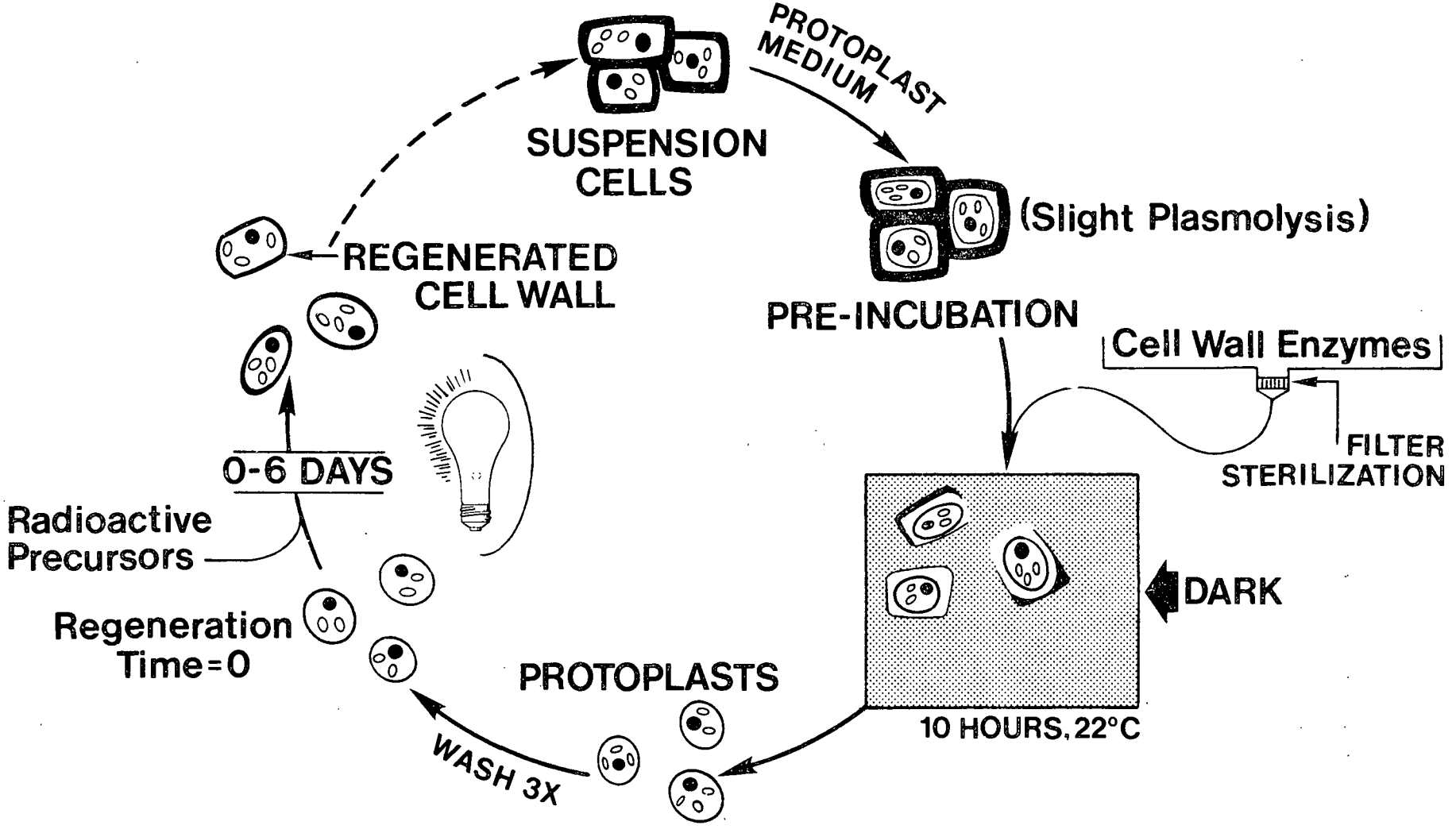
INTRODUCTION

Cellulose is the most abundant organic material in our biosphere and it is estimated that about 9 x 10^{10} metric tons of cellulose are produced annually (1). Cellulose is the foundation of the forest products, agricultural, apparel, and petrochemical industries. Relatively little is understood of the fundamental processes involved in cellulose synthesis and cell wall biogenesis despite the fact that numerous investigations have been performed (2). Theories abound, for instance, attempting to describe the molecular orientation and microfibril synthesis of cellulose (3-10). Due to limitations of the natural systems, new approaches were needed to overcome some of the inherent impasses (11).

Tomato-fruit protoplasts were reported to synthesize a new cell wall subsequent to the removal of wall-degrading fungal enzymes (12). If this observation and others (13,14) using whole plants were accurate, then protoplasts could represent a synchronous culture of cells which simultaneously deposit a cell wall and, therefore, may be a valuable tool for furthering the understanding of cell wall synthesis (15).

Earlier studies in this laboratory using freeze-etch technology (16) failed to detect cellulose microfibrils on the plasma membrane surface of Douglas-fir protoplasts from suspension cultures. Therefore, a research program was proposed to examine the regenerating cell wall of Douglas-fir protoplasts from suspension cells to determine if it represented a valid model for normal cellulose synthesis and deposition. The generalized experimental approach used in this thesis was largely biochemical, although many physical, histochemical, and microscopic techniques were also employed. After enzymatic removal of the cell wall, cell division was inhibited and the protoplasts were cultured for up to 6 days in nutrient medium under constant illumination as shown in the scheme on the following page. Both ^{14}C-L-proline and ^{14}C-D-glucose were initially fed to the protoplast cultures, and the

METHOD OF PROTOPLAST GENERATION AND CELL WALL REGENERATION



subsequent uptake of radioisotope in the regenerating material on the cell surface was monitored. Analysis and partial characterization of both the polysaccharide and protein components in the regenerating cell wall were performed.

ARTICLES PROPOSED FOR JOURNAL PUBLICATION

STUDY OF THE REGENERATING CELL WALLS OF DOUGLAS-FIR PROTOPLASTS

ARTICLE I. PROLINE AND GLUCOSE METABOLISM

ARTICLE II. CELL WALL GLYCOPROTEIN CHARACTERIZATION

STUDY OF REGENERATING CELL WALLS OF DOUGLAS-FIR PROTOPLASTS

ARTICLE I. PROLINE AND GLUCOSE METABOLISM

Abstract

Radioactive proline and glucose were fed to Douglas-fir protoplasts and then recovered from the regenerating material at the plasmalemma surface. Differences in the time of initiation of radioisotope incorporation demonstrated that protein was deposited prior to polysaccharide synthesis in the wall. An increase in protein and carbohydrate content was found over the six-day period studied and the protoplasts were not observed to divide. Glucose was the dominant neutral sugar deposited, although significant amounts of arabinose, galactose, and mannose were also found. Analysis of the regenerated cell wall by x-ray diffraction, carbohydrate acetolysis, histochemical staining, and microscopy suggested the absence of cellulose and the probable presence of pectin and callose, a β-(1,3)-glucan. The callose and protein deposition in the wall are both thought to be part of an overall response to cellular stress during and after protoplast formation.

Introduction

Since E. C. Cocking's (1960) isolation of higher plant protoplasts with fungal enzyme preparations, the botanical literature has been avidly concerned with the nature of protoplasts and the regenerated cell wall. Several reviews on protoplasts and cell wall regeneration are available (Willison, 1976; Preston, 1974; Cocking, 1972; Ruesink, 1971).

Until the early seventies, predominantly light and electron micrographs of cell cross sections were used to elucidate gross aspects of cell wall regeneration. In 1972, Willison and Cocking used high resolution freeze-etching technology to describe microfibril synthesis on the protoplast membrane. This approach provided

workers with the opportunity to use protoplasts as tools by which biogenesis of the cell wall might be understood. The tacit hope was that protoplast wall regeneration could be used as a model of *in vivo* cell wall synthesis. Most of the freeze-etch studies have, in fact, revealed that fibrils are deposited on the plasmalemma. In many cases a lag was observed between enzyme removal and the initiation of cell wall regeneration. Lags of 24 hours (Willison and Cocking, 1975) to 72 hours (Robenek and Peveling, 1972) were commonly observed, although wall production was also reported to begin immediately (Klein and Delmer, 1979). The general assumption underlying this approach is that the fibrils observed are cellulose and that the microfibrillar structure reflects native cellulose, i.e., Cellulose I.

Biochemical studies on the metabolism of the regenerating cell wall were nonexistent until recent years. Efforts were usually frustrated by the obvious difficulties of amassing sufficient cultured protoplasts and regenerated cell wall material for analysis.

Chemical evaluations of the regenerated cell wall have generally proved helpful to better appreciation of the protoplasts' response to wall removal. Takeuchi and Komamine (1978a) report that the dominant neutral sugar in the regenerated wall from *Vinca rosea* protoplasts is 1,3-linked glucose. The disaccharide, laminaribiose, is the repeating unit of callose which is generally considered to be a wound-induced polysaccharide. Herth and Meyer (1977) observed fibrillar deposition on the plasma membranes of tobacco protoplasts, and x-ray analysis failed to detect significant levels of crystallinity. Upon partial acid hydrolysis or 20% alkali extraction, they respectively identified a minor product, cellobiose, and a weak Cellulose II response. These facts suggest that cellulose is not as dominant as the freeze-etch studies would imply.

The disparity in these results may be resolved partially by differences arising from diversity in species, tissue source and history, and culture conditions (Hanke and Northcote, 1974). However, a potential source of confusion relates to identifying cell wall material as cellulose on purely its solubility characteristics (Brett, 1978; Takeuchi and Komamine, 1978b; Asamizu et al., 1977; Burgess and Fleming, 1974; Talmadge, et al., 1973; Updegraff, 1966). Losses due to cellulose degradation, irreversible adsorption of hemicelluloses on polysaccharides (Yllner and Enstron, 1956), and partial insolubility of some β-(1,3)-glucans (Flowers et al., 1968; Robinson, 1979) all could lead to erroneous conclusions regarding the cellulose content of cell walls. Only crystallography, partial hydrolysis (Elbein and Forsee, 1973), and/or acetolysis analysis (Green, 1963) can provide positive confirmation of the cellulosic content.

The aim of the present study was to characterize the regeneration of cell walls on Douglas-fir protoplasts from cell suspension cultures. The approach involved the analysis of various chemical and physical parameters. In addition, the ^{14}C-isotopes of L-proline and D-glucose were used, respectively, to evaluate the relative contributions of the protein and polysaccharide components in the regenerating cell wall. The protoplasts were observed to generate aberrant wall structures in response to cellular wounding.

Materials and Methods

Protoplast Culture

Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] suspension cultures were derived from both needle and hypocotyl explants from greenhouse stock. Needles and hypocotyls were excised from 1-year-old and 1-month-old seedlings, respectively, and placed on solid agar medium. Subculturing was performed monthly, and after 5 to 8 months, the callus clumps were gently broken apart and transferred to 150 mL of the

suspension medium consisting essentially of half strength Murashige-Skoog (M-S) growth medium (1962)*. The medium was supplemented with 1.04 μM NAA, while glycine, casein hydrolyzate, IAA and kinetin were deleted. The cell suspensions were cultured under constant illumination at 22°C in slowly rotating nipple flasks with a monthly addition of 100 mL of medium. After 3 months of suspension culture, cells were considered in the stationary phase and used for protoplast generation.

One day prior to enzyme treatment to remove cell walls, the suspension cultures were transferred to a protoplast medium* buffered at pH 5.8. The medium consisted of mannitol (0.537M), M-S medium, citric acid-phosphate buffer (24.5 mM), ferrous ammonium sulfate (1 mM), and L-ascorbic acid (1 mM). Cellulysin and Macerase (Calbiochem, San Diego, Calif.) were dissolved in the protoplast medium and filter-sterilized, each at a final concentration of 2% (w/v). The enzyme mixture was added to the suspension cultures, which were then incubated at 22°C on a rotary shaker (120 rpm) in the dark. After 10 hours protoplasts were ubiquitous. They were washed with the protoplast medium 3 times and collected by centrifugation at less than 75 x g.

Cell Wall Regeneration

The protoplasts were split into 2 equal volumes and either L-(U-^{14}C) proline or D-(U-^{14}C) glucose (ICN Pharmaceuticals, Inc., Irvine, Calif.) was introduced with respective specific radioactivities of 250 and 240 mCi/mmole. Each radioactive species was applied at a concentration of 0.1 μCi/mL of medium. Aliquots (5.0 mL) of protoplast suspension were aseptically transferred to test tubes with metal closures and rotated (1 rpm) under constant illumination (1.6, 1.2 and 0 μW/cm^2 at peak wavelengths of 455, 640 and 735 nm, respectively). Samples were evaluated at cell wall regeneration times of 0, 1/2, 1, 2, 3, 4, 5, and 6 days. Each sample time reported in the isotope experiments represented an average of 4 replications.

*See Appendix I.

Parallel controls were conducted during the wall regeneration studies to check that radioactivity uptake by the walls was both an enzyme-mediated and cytologically organized event. The first control was to establish whether physical sorption played a significant role in final cell wall radioactivity. After radioisotope was added to the protoplasts as above, cells were immediately placed in a boiling water bath for 10 minutes to inhibit enzyme activity. The second control was to establish the role of protoplast integrity in the uptake of radioactivity. After radioisotope addition, the protoplasts were ball-milled and reduced to debris. In both controls the cell suspensions were returned to the rotary device and treated as above with the exception that only duplicate samples were evaluated.

Cell Wall Isolation

Regenerated cell walls were separated from cytoplasmic materials by using a modification of Lamport's method (1965). Cells were washed with 1M NaCl and homogenized for 5 minutes in a veined Virtis mixer jar using 3 mm glass beads. The washing and centrifugation schedule is outlined in Table I. Clean cell walls were evaluated for activity in a scintillation counter (Smeltzer and Johnson, 1977) and counted to a ± 2% statistical error. Walls were retrieved from the dioxane-based cocktail by two washes with acetone followed by one with water.

Analytical Techniques

Several methods were employed to characterize the chemical constitution of the regenerated cell wall. For evaluation of the carbohydrate fraction, alditol-acetates of hydrolyzed cell wall polysaccharides were made according to the method of Borchardt and Piper (1970). A Packard Model 417 gas-liquid chromatograph (GLC) equipped with a flame ionization detector and a data system was used.

TABLE I

The washing and centrifugation schedule used to separate cytoplasmic and radioactive materials from the regenerating cell walls. The cells were first ball-milled at slow speed for 5 minutes in 1N NaCl to separate the cell walls, then transferred to 40 mL centrifuge tubes and washed according to this schedule.

Washing Solvent	Centrifugal Force, x g	Centrifugation Time, minutes
1M NaCl	800	0.5
Water[a]	800	0.5
Water	800	0.5
Water	29,000	20.0
Acetone	29,000	10.0

[a]Double distilled water used throughout all experiments.

The crystallinity of the regenerated cell wall was determined by x-ray diffraction (Atalla and Whitmore, 1978). Samples were pelletized, scanned, and compared with the spectra of β-(1,4)-glucan(cellulose) and β-(1,3)-glucan (bacterial callose). The β-(1,4)-linked polymer is the most abundant constituent in normal cell walls, while the β-(1,3)-glucan is commonly synthesized in response to wounding (Clark and Stone, 1963).

Regenerated wall material and filter paper (control) were separately assayed for cellulose by the acetolysis procedure of Braun (1943). Detection of octaacetyl-α-cellobiose using this method is a positive indication of the presence of cellulose. The acetolysis products were evaluated by both thin-layer chromatography (TLC) on silica gel plates and by GLC on an OV-17 column using octaacetyl-α-cellobiose and glucose pentaacetate as standards. (See Appendix VIII for further details.)

Cell walls regenerated on protoplasts were evaluated for amino acid composition. Samples were hydrolyzed in 1 mL of constant-boiling HCl and 0.5 μL of mercaptoethanol for 22 hours at 110 ± 0.5°C. Humin was removed by mild centrifugation, and

the supernatant was dried over NaOH pellets *in vacuo* at 60°C (Brenner *et al.*, 1965). Amino acid composition was determined on a Beckman Model 119CL amino acid analyzer equipped with a Beckman Model-126 computer.

Microscopy

Hypocotyl-derived cell populations from the original suspension cultures, isolated protoplasts, and cells with regenerated walls were separately fixed in 2% glutaraldehyde plus 2% acrolein in protoplast medium for 3 hours at room temperature, and some samples were post-fixed in 1% OsO_4. Of those treated with osmium, some were dehydrated in an alcohol series, plated on a glass-supported polylysine film (Tsutsui *et al.*, 1976), dried over argon gas *in vacuo*, and viewed in a JEOL JSM-U3 scanning electron microscope (SEM) at 20 kV. Others were dehydrated in an acetone series and embedded in Spurr resin (E. F. Fullam, Inc., Schenectady, N.Y.). Both 10 and 0.1 μm sections were made. The thicker sections were used in light microscopy for the detection of lignin and callose in the cell wall region using a Zeiss photomicroscope equipped with a UV source. Lignin was detected by autofluorescence and callose by a specific reaction with a 0.01% solution of aniline blue (Eschrich and Currier, 1964; Currier and Strugger, 1956).

The ultrathin sections were post-stained with uranyl acetate (5% in 50% ethanol) and lead citrate (0.5% in water). Samples were examined on an R.C.A. Model EMU-3F transmission electron microscope (TEM).

Samples not osmium-treated were reacted separately with several electron-dense stains. The hydroxylamine-iron staining scheme (McCready and Reeve, 1955; Gee *et al.*, 1959) was employed to detect the presence of pectin in the wall. As a control of staining reactivity, other samples were similarly treated but boiled in 0.5% aqueous ammonium oxalate for 24 hours prior to staining. The second staining

sequence evaluated the availability of (1,4)-sugar linkages in the wall. The reaction involved periodic acid oxidation of adjacent sugar hydroxyls followed by reaction with thiosemicarbazide and osmium reduction (Seligman et al., 1965). Both staining sequences were followed by osmium fixation, embedding, and ultrathin-sectioning (0.1 μm) as before.

Results

Radioactivity from ^{14}C-Proline and ^{14}C-Glucose in the Regenerating Cell Wall

The biosynthesis of protein and polysaccharide material in the regenerating cell walls of Douglas-fir needle-derived protoplasts was demonstrated by the respective uptake of radioactive L-proline and D-glucose into polymeric forms as shown in Fig. 1. Proline was rapidly incorporated* into the wall fraction, but a lag was observed in the uptake of glucose.

The hypocotyl-derived protoplasts also incorporated both proline and glucose into the regenerating wall as shown in Fig. 2. On a similar dry weight basis with the protoplasts in Fig. 1, the incorporation of radioactivity was observed to be less for the hypocotyl cells. However, a similar lag was observed in both cultures between the onset of proline incorporation and the beginning of glucose uptake.

The remainder of the results, with the exception of the microscopic study, pertain to experiments involving the needle-derived tissue only.

Minor binding of radioactivity from both precursors was detected in the boiled controls, and this amount was subtracted from the activity reported in Fig. 1 and 2. The second control consisting of cytoplasmic and wall debris indicated that only organized cells were involved in the uptake of radioactivity.

*Incorporation is defined here as an enzyme-mediated association determined through the recovery and detection of radioactivity and is not meant to suggest mechanistic, spatial, or linkage considerations.

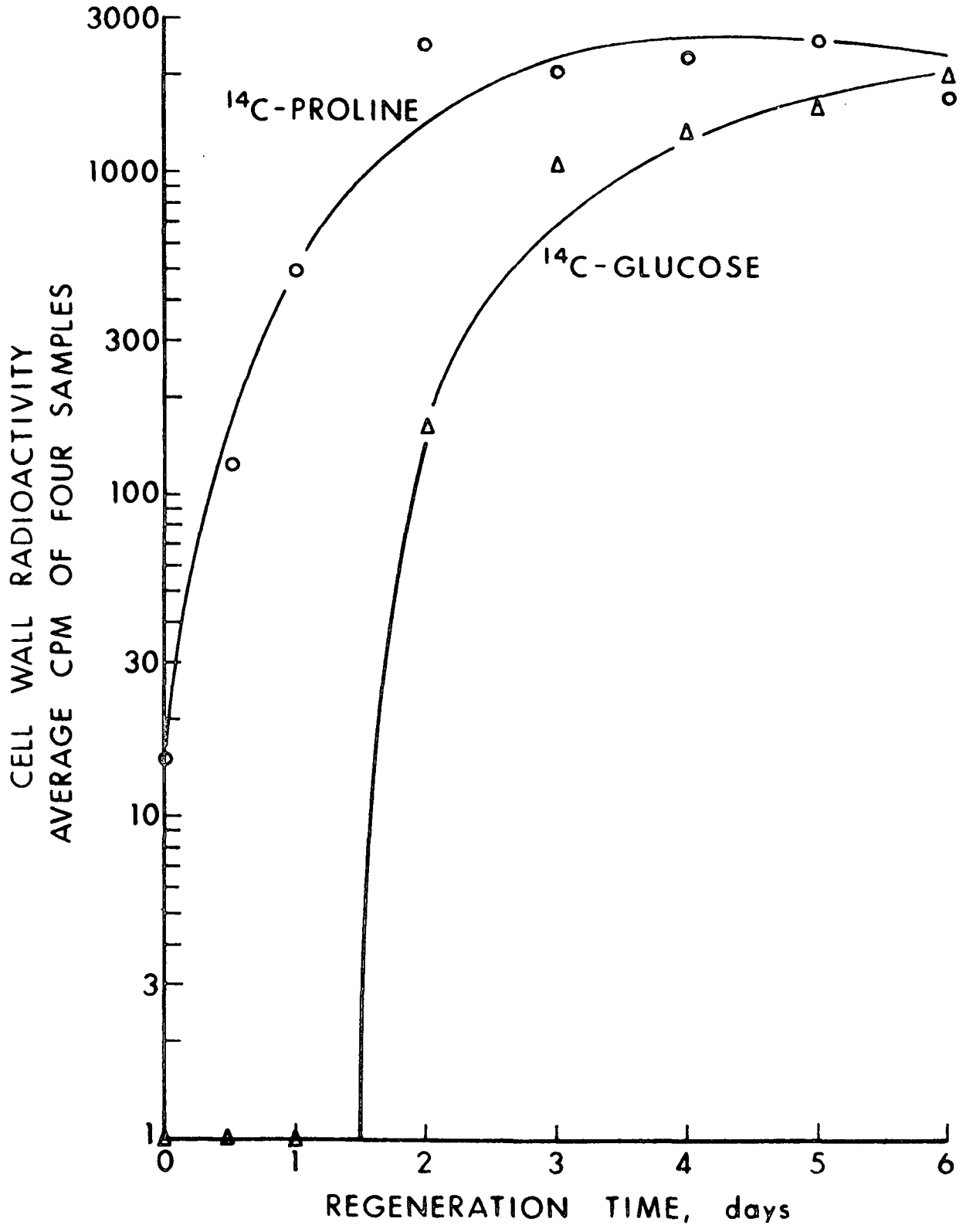


Figure 1. The recovery of radioactivity originating from labelled proline and glucose in the regenerating cell wall of Douglas-fir needle-derived protoplasts. Note the time lag which exists between the uptake of the polysaccharide and protein components. The protoplasts were maintained at constant illumination and remained green over the period studied.

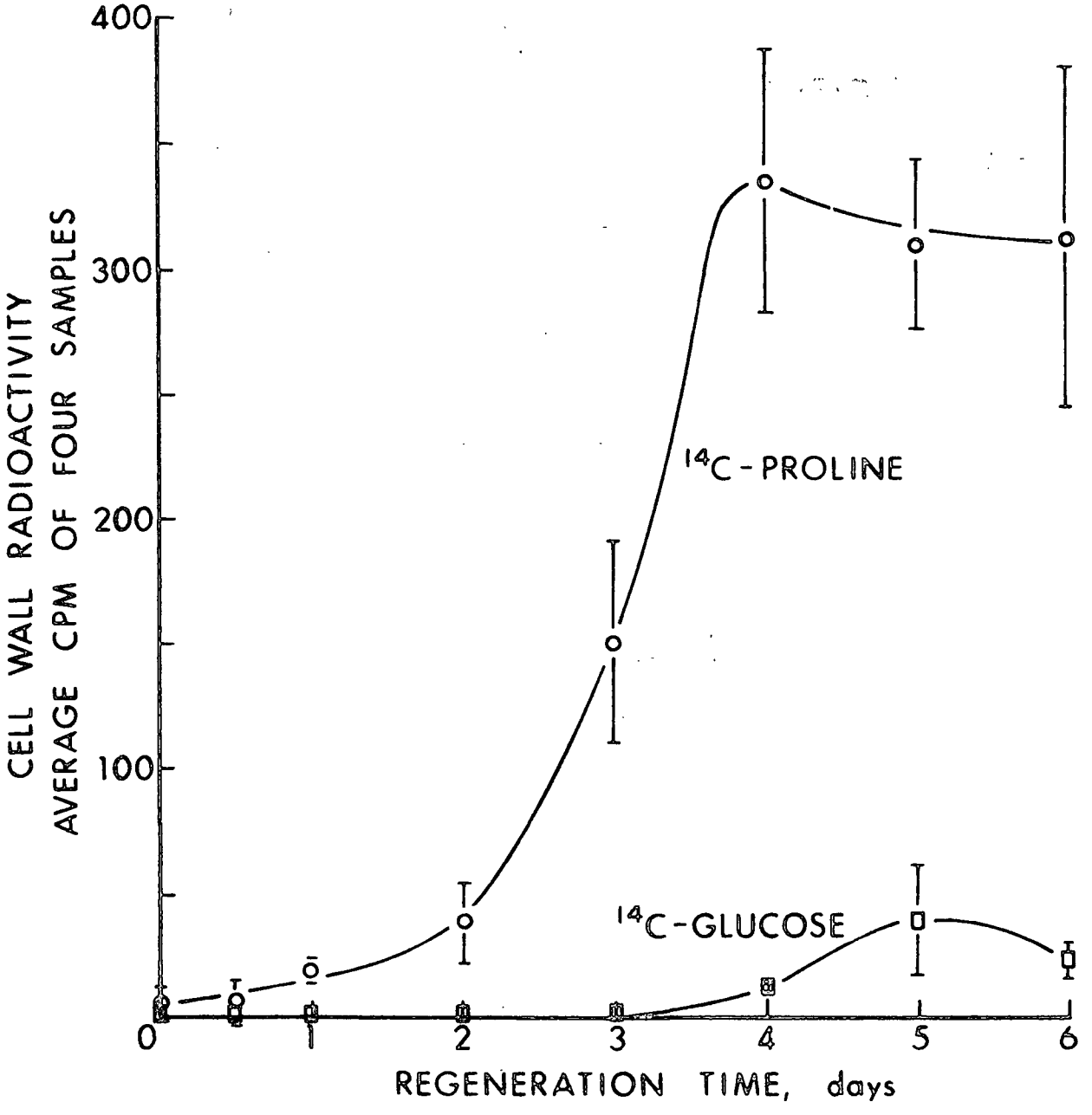


Figure 2. The recovery of radioactivity originating from labelled proline and glucose in the regenerating cell wall of Douglas-fir hypocotyl-derived protoplasts. The plot is as described in Fig. 1 except that the protoplasts are from hypocotyl-derived tissue. A similar lag is observed in the differential uptake of proline and glucose. The protoplasts remained green but did not exhibit the levels of radioactivity found in the needle-derived protoplasts.

Chemical and Physical Analysis of the Regenerated Cell Wall

Wall regeneration for Douglas-fir needle-derived protoplasts appeared to begin at about 30 hours after enzyme removal as indicated by the intercept in Fig. 3. From that time onward the wall exhibited a constant weight increase throughout the period studied.

Total carbohydrate analysis of the regenerated cell wall is displayed in Table II. Glucose is the dominant neutral sugar, followed by mannose, galactose, and arabinose. Only minor amounts of xylose, ribose, fucose, and rhamnose were detected. The percent increase of the individual sugars in the wall is plotted in Fig. 4. The only polysaccharide material actively synthesized at the initiation of wall regeneration (ca. 30 hours) is a polymer containing mannose. The other major carbohydrates are found only after 2 days.

The results from the amino acid analysis of the wall regeneration period studied are displayed in Table III. The general trend of amino acid composition is illustrated in Fig. 5. A more detailed characterization of the protein component found in the regenerated cell wall is reported in part 2 of this investigation (Robinson, Thesis, Part II).

The results from the x-ray diffraction analysis are plotted in Fig. 6. Cell wall material recovered from various stages of wall regeneration is shown along with walls of suspension cells. Native cellulose and bacterial callose (Harada, 1977) are standards. The regenerated and suspension cell walls were not sufficiently crystalline to demonstrate the presence of ordered cellulose. Even after high temperature annealing in glycerol, no increase in order was detected.

Acetolyzates were made of the x-ray analyzed, regenerated wall samples reported above to test chemically for the presence of cellulose. Only the control yielded

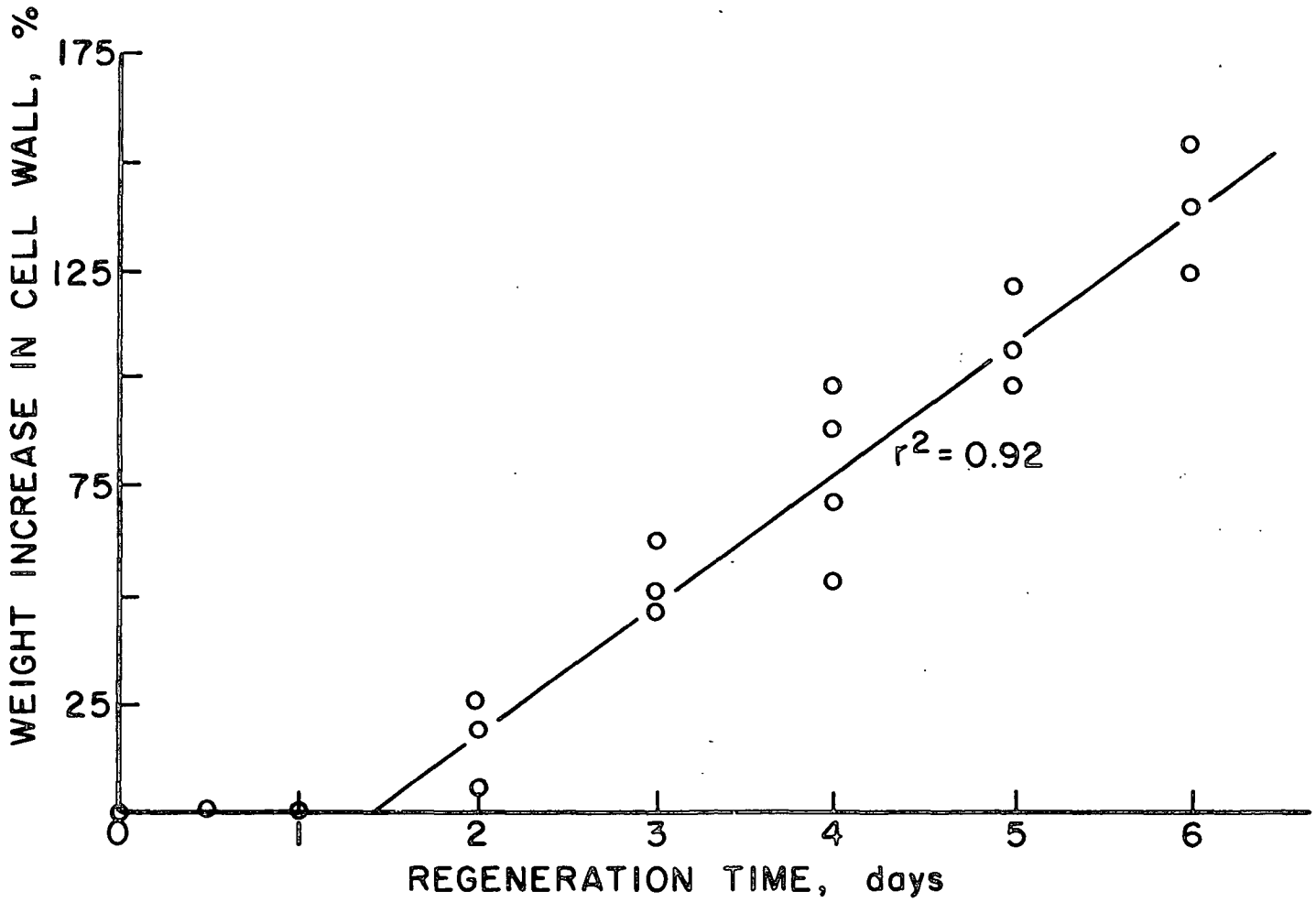


Figure 3. Cell wall weight increase for needle-derived protoplast culture. A linear relationship exists in the deposition of cell wall material after about 30 hours. The constant wall weight increase suggests that no cells divided during the period studied.

the primary cellulose hydrolysis product, octaacetyl-α-cellobiose, thereby confirming the absence of β-(1,4)-glucan in the regenerated cell wall.

TABLE II

The neutral sugars present in the regenerating cell wall. At zero regeneration time the carbohydrate analysis reveals that the residual suspension cell wall is about 13% polysaccharide. The dominant sugar in both the residual and the regenerating wall is glucose.

	Weight, % Regeneration Time, days							
Sugar	0	0.5	1	2	3	4	5	6
Glucose	6.34	4.66	4.98	6.31	16.64	21.98	21.22	27.37
Arabinose	2.88	2.31	2.04	2.46	2.98	3.34	3.11	3.44
Galactose	1.89	1.36	1.30	1.79	2.86	3.50	3.67	4.07
Mannose	0.62	0.77	0.93	1.43	3.62	4.67	5.21	5.29
Xylose	0.63	0.48	0.46	0.38	0.52	0.62	0.52	0.50
Rhamnose	0.43	0.25	0.90	0.30	0.32	0.32	0.25	0.30
Ribose	0.26	0.19	0.17	0.41	0.36	0.30	0.11	0.16
Fucose	0.09	0.03	0.39	0.07	0.09	0.08	0.07	0.08
Total	13.14	10.05	11.17	13.15	25.39	34.81	34.16	41.21

Structural and Histochemical Study

Light and electron microscopy provided insight into the cell wall regeneration process. Shown in Fig. 7 is the cross-section (7a) and cell surface (7b) of Douglas-fir protoplasts (i.e., wall regeneration time = 0 days) which exhibited little evidence of a cell wall. Total removal of the cell walls required a 10 hour incubation period with the fungal enzymes. This situation principally resulted from the large quantities and high concentrations of suspension cells required to conduct an investigation of this scope. Even under these extended conditions, minor amounts of wall residue existed on the otherwise clean cell membranes of some protoplasts (see Fig. 7b).

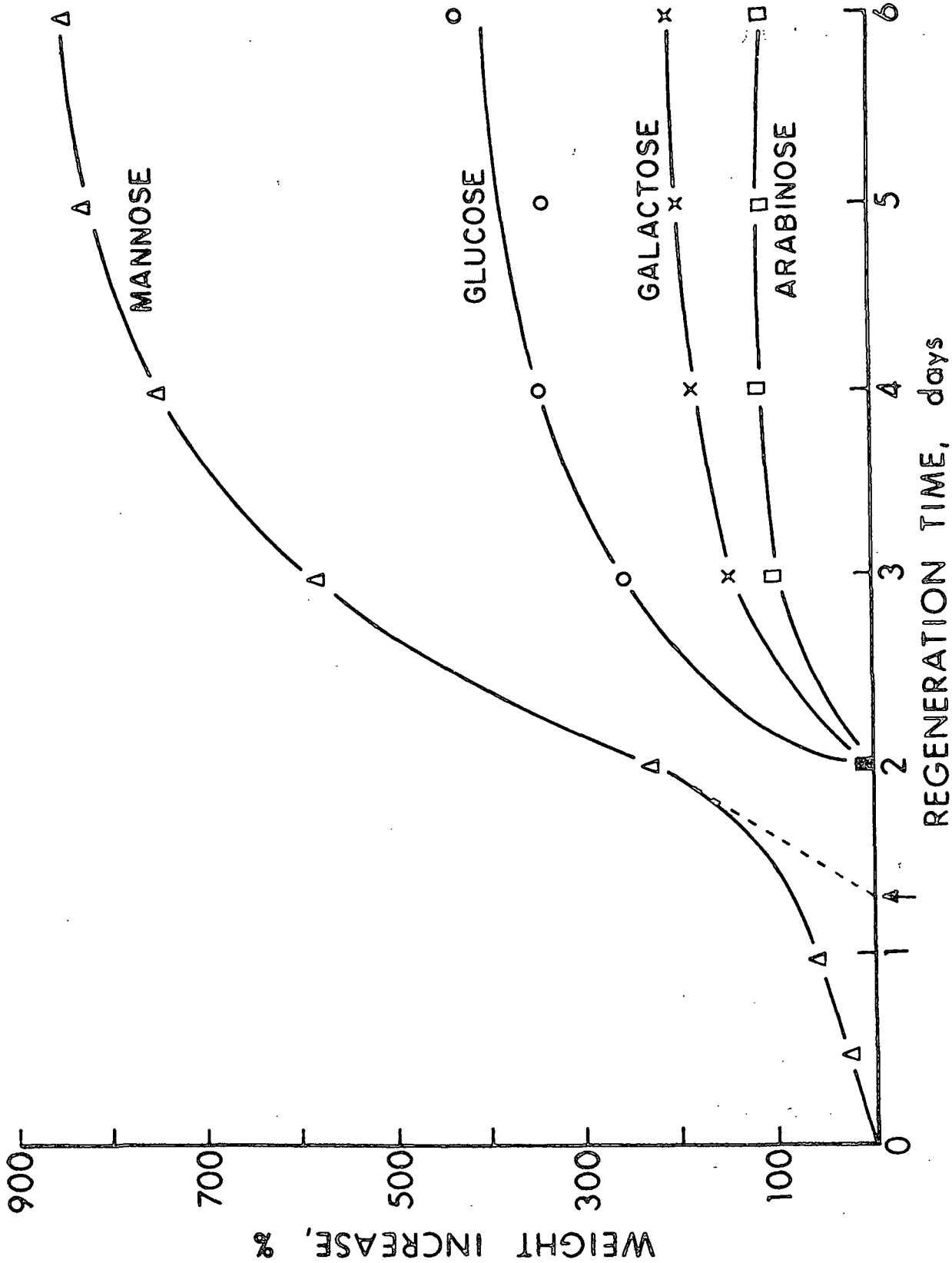


Figure 4. The synthesis of neutral polysaccharides in the regenerating cell wall. The increase in neutral sugars beyond the time of protoplast generation shows the initial formation of a polymer containing mannose. Mannose incorporation is extrapolated to 30 hours to indicate the intersect of cell wall weight increase (Fig. 3) and ^{14}C-glucose uptake (Fig. 1).

TABLE III

The molar percentages of amino acids from acid hydrolyzed cell wall samples. Arranged according to average descending concentration, glycine and alanine alone account for over 25% of the protein fraction. Also, note the fairly good agreement in molar ratios as regeneration proceeds even though the net deposition of protein increases as shown in the bottom row.

Molar Percentage

Amino Acid[a]	Regeneration Time, days								
	0	0.5	1	2	3	4	5	6	Average
Glycine	13.7	13.8	15.8	13.1	13.5	14.3	13.3	13.9	13.9
Alanine	11.4	10.3	11.9	12.1	13.8	14.5	12.9	14.4	12.7
Aspartic acid	10.4	11.2	9.5	9.4	10.5	10.2	9.7	9.9	10.1
Serine	9.7	9.2	9.3	10.6	9.4	9.7	8.5	7.9	9.3
Glutamic acid	7.5	8.8	9.1	7.0	6.7	7.5	8.2	8.1	7.9
Leucine	8.1	7.7	8.7	7.5	7.5	7.8	7.3	7.4	7.8
Proline	5.5	6.5	7.6	6.9	7.2	7.6	6.1	7.3	6.8
Lysine	6.0	4.6	4.1	4.6	4.6	4.4	2.4	3.9	4.3
Threonine	4.2	5.0	4.3	4.4	3.8	3.9	4.5	3.8	4.2
Valine	3.1	4.5	3.8	4.0	3.4	3.4	4.0	3.7	3.7
Phenylalanine	4.7	3.4	3.4	4.4	4.3	3.3	3.1	3.3	3.7
Arginine	4.2	3.4	3.1	3.5	3.5	3.4	3.4	3.0	3.4
Hydroxyproline	3.4	3.4	1.9	2.5	1.9	1.8	4.0	3.4	2.8
Isoleucine	2.1	2.7	2.2	3.0	2.3	2.2	2.5	2.3	2.4
Tyrosine	2.4	2.1	2.2	2.3	2.4	2.1	2.3	2.2	2.3
Histidine	1.9	1.8	1.7	1.8	1.7	1.7	1.6	1.5	1.7
Methionine	1.1	0.4	0.7	1.4	0.7	1.1	0.3	0.7	0.8
Total weight % of cell wall	19.3	15.8	17.4	16.8	26.2	24.8	24.8	31.4	29.3

[a]Neither cysteine nor cystine were detected.

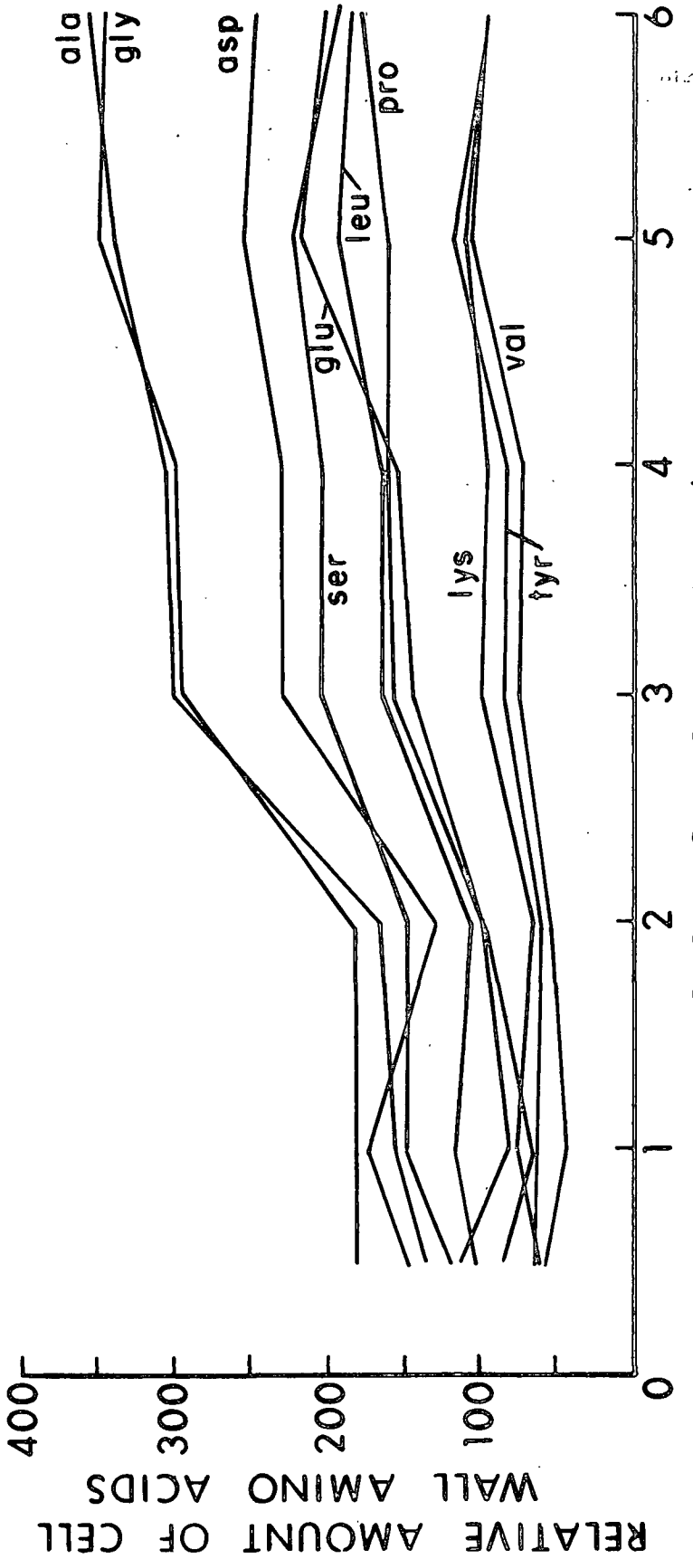


Figure 5. Relative concentration of selected amino acids present in the acid hydrolyzates of regenerated cell wall samples. An orderly trend is observed in the relative increase in amino acids, suggesting that protein is accumulated in the wall during the period studied. The amino acids not shown here are listed in Table III and were observed to exhibit similar trending but were omitted on this plot for clarity.

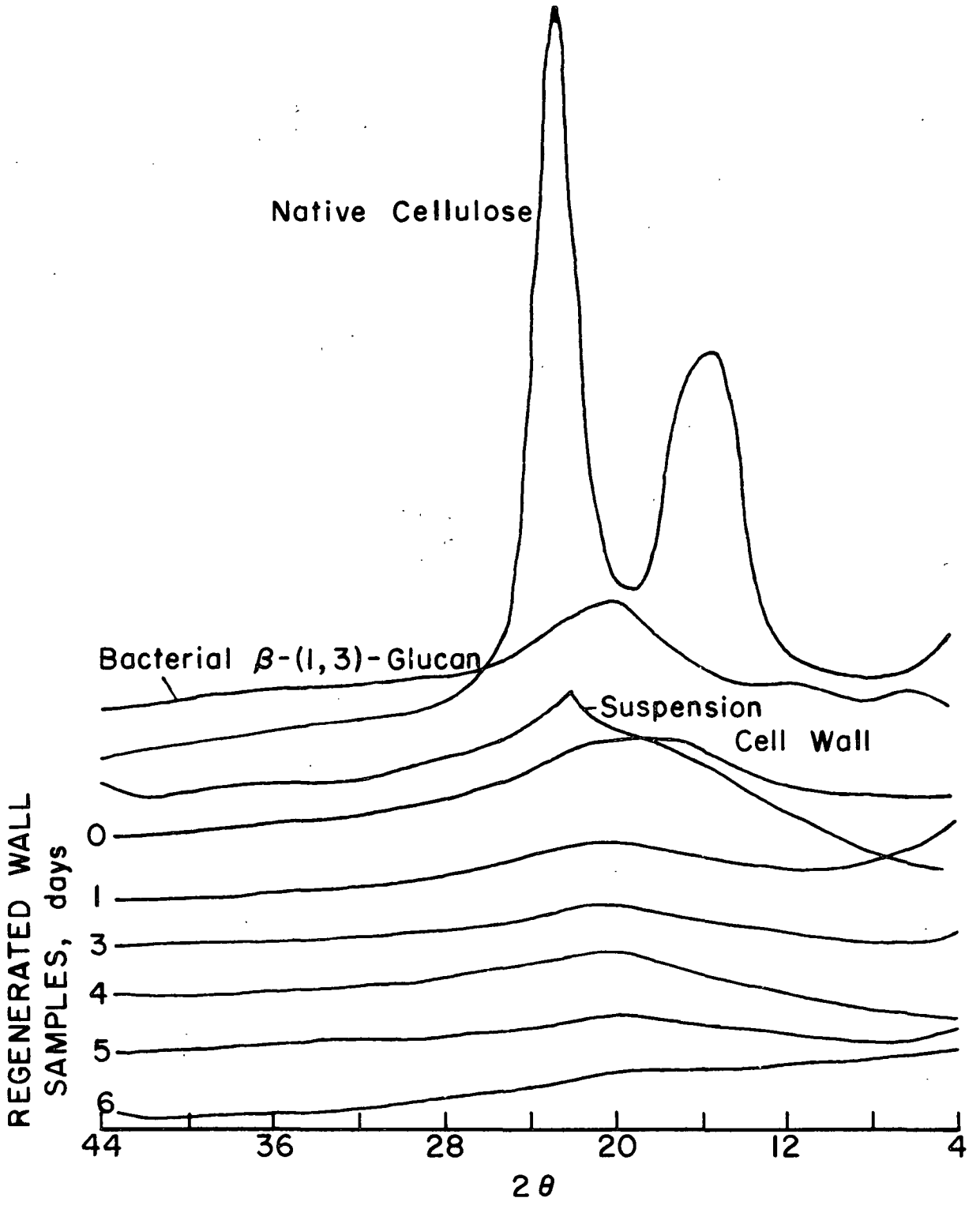


Figure 6. X-ray diffractograms of regenerating and suspension cell walls compared with those of native cellulose and bacterial β-(1,3)-linked glucan (i.e., curdlan, Harada, 1977). The general shift of ordering by the regenerating walls is to 20°, the most ordered point of the native β-(1,3)-glucan and not to the 16° and 23° region which is characteristic of cellulose. High temperature glycerol annealing of the regenerating cell wall did not increase crystallinity or shift the diffraction pattern (not shown), confirming the absence of crystalline cellulose.

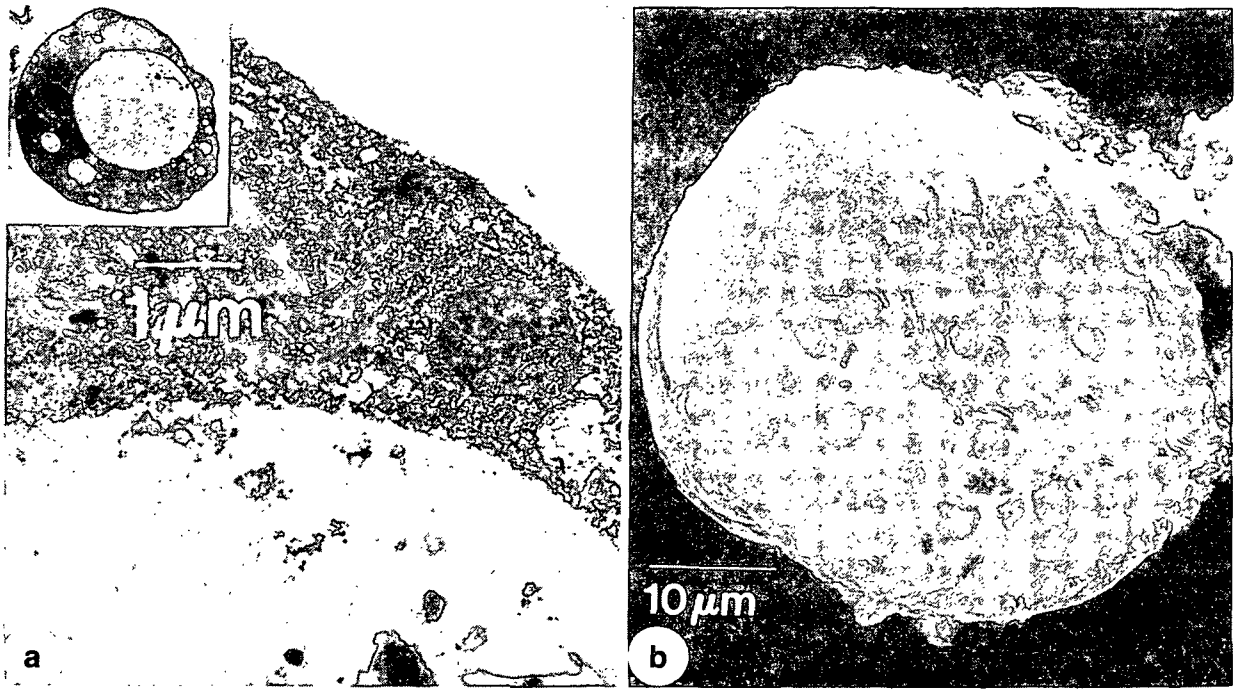


Figure 7. A cross-section (a) and surface view (b) of Douglas-fir protoplasts. In (a) the cell wall has been removed (14,000X) and the cell shows the characteristic protoplast spherical shape (inset). However, (b) shows that some residual wall remains on the protoplast even though what may appear to be cell organelles are observed beneath the clean and skinlike membrane surface (1700X).

Since the protoplasts did not exhibit a measurable cell wall (Fig. 7a), the remainder of the microscopy study involved detecting qualitative similarities and differences which existed between the suspension cell walls and the regenerated cell walls. After 6 days of protoplast culture for wall regeneration, bubblelike structures were observed on the cell surface of some protoplasts as shown in Fig. 8. The balloonlike materials on the cell surface suggested callose formation (Fahn, 1974) and a positive reaction to aniline blue in the wall region of cells from the same population was detected. A summary of these and other results are displayed in Table IV.

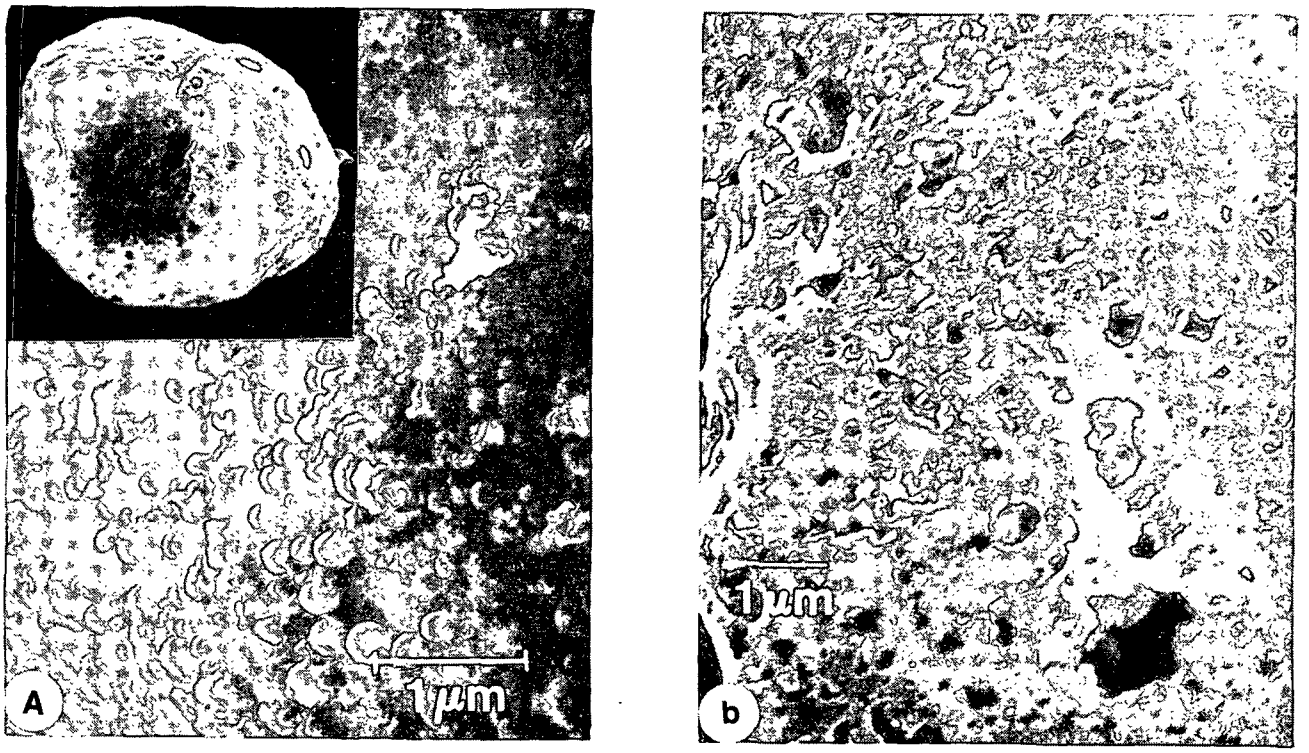


Figure 8. (a) Surface view of a cell after 6 days wall regeneration. Shown is a cell which has regenerated a cell wall. (b) The wall consists of bubble-like material suggestive of callose (20,000X).

TABLE IV

A summary of the microscopy study. The suspension and regenerated cell walls were compared and observed to differ in their response to callose and lignin detection. Both types of cell walls contain pectin and (1,4)-linked polysaccharides.

	Suspension Cell Walls	Regenerated Cell Walls
Cellulose microfibrils	N.D.	-[b]
Callose (β-1,3-glucan)	N.D.	+[c]
Lignin	+[d]	-
1-4 Linked carbohydrates	+[e]	+
Pectin	+[f]	+

[a]Not determined.
[b]As detected by standard shadowing and freeze-fracture techniques.
[c]As detected by reaction with aniline blue.
[d]As detected by autofluorescence.
[e]As detected by oxidation with periodic acid, followed by reaction with thiosemicarbazide, and reduction of of osmium tetroxide.
[f]As detected by forming hydroxamic acids and then reacting with ferric chloride.

Discussion

Douglas-fir protoplast cultures were incubated with radioactive L-proline and D-glucose in order to observe their relative metabolic involvement in the regenerating cell wall. Reproducibly characteristic was the initiation of proline incorporation prior to glucose uptake (see Fig. 1 and 2). Although lags in microfibril synthesis have been reported (Burgess and Linstead, 1979; Grout, 1975; Marchant and Hines, 1972), this is the first study which demonstrates a potential relationship between protein and carbohydrate components in the regenerating cell walls of higher plants.

The rate of wall protein and polysaccharide anabolism appears to vary in Douglas-fir cultures from the needle-derived to hypocotyl-derived tissues. It is unclear, however, whether this response is in any way related to some fundamental and characteristic differences between the two cell types or is just indicative of variation in the lag associated with wall regeneration. Observed differences might, in fact, disappear if a statistical study were undertaken.

Cell wall weight increased linearly over the period of synthesis studied (see Fig. 3). As expected, wall weight increase is simultaneous with ^{14}C-glucose uptake, clearly suggesting that glucose incorporation is indicative of cell wall regeneration. In addition, a constant weight increase may suggest that no new carbohydrate synthesis sites formed (i.e., no cells divide) over the period studied. Although differences in cell wall density were not monitored, no active cell division was observed for up to 12 days of protoplast culture. Other workers have drawn similar conclusions, although whether cell division occurs appears to be culture-dependent. Kirby (1980) and Strmen (1979) observed that the gymnosperm protoplasts derived from suspension cultures of Douglas-fir (needle) and Norway spruce, respectively, failed to divide after enzymatic treatment. In contrast, protoplasts from suspension cultures of carrots (Asamizu and Nishi, 1980) and Vinca rosea (Takeuchi and Komamine, 1978a) all divided within several days of culture. Within cultures, cell

division was reported to be dependent on illumination intensity (Shepard and Totten, 1977) and medium molarity (Pearce and Cocking, 1973). Callusing (Kirby and Cheng, 1979) and plant regeneration (Gamborg and Shyluk, 1976) is more commonly reported from protoplasts derived from intact tissues.

Special culture conditions are clearly necessary when large populations of protoplasts are generated. The concentrations of wall degrading enzymes, time, and incubation temperature are usually in excess of standard protoplast methods. For example, in order to conduct a large scale study of the molecular weight distribution of cellulose from the regenerated cell wall, Asamizu et al., (1977) reported that 5% cellulase and 1% pectinase were used to complete wall removal in carrot cultures. Takeuchi and Komamine (1978a), in order to enzymatically remove and then chemically characterize the regenerated wall of Vinca rosea cell suspensions, required 2% cellulase and 1% pectinase. The cultures in those studies were maintained at 30°C for 6 and 5 hours, respectively. In spite of comparable conditions used in this study, data in Tables II and III demonstrate that a chemically measurable cell wall remains on the protoplasts. No residual wall, however, could be detected on ultrathin sections in the TEM (see Horine and Ruesink, 1972). Nevertheless, SEM analysis revealed that small portions of wall material remain in some cases despite the fact that protoplasts are generated (see Fig. 7b).

A polymer containing mannose is synthesized as the initial polysaccharide in the wall and is probably responsible for the uptake of ^{14}C-glucose prior to 2 days of protoplast culture, since glucose can interconvert to mannose (Harran and Dickinson, 1978). In yeast protoplasts, mannan-protein deposition occurs in concert with the synthesis of β-(1,3)-glucan in the regenerating wall (Kopecka and Farkas, 1979). Mannan may be involved in the glycosylation and binding of the protein to the cell walls of the Douglas-fir system in an analogous fashion.

The composition of the regenerated cell wall includes glucose as its major carbohydrate with significant amounts of hemicellulosic components. Common hemicelluloses in most conifers are galactoglucomannan (0.1:1.0:3.0) and arabinogalactan (1:5) which may be similar in form to the constituents of the regenerated cell wall. If the regenerated wall hemicelluloses are similar, then a glucan will exist, since only a minor amount of glucose could be involved in the hemicellulosic fraction.

Several analyses of the regenerated wall were made to define the nature of the glucan. Analysis by x-ray diffraction techniques revealed that the regrown wall failed to exhibit a crystalline pattern characteristic of cellulose; Herth and Meyer (1977) experienced similar results. Their x-ray diffractograms of regenerated walls of tobacco revealed the absence of any crystallinity. In contrast, their negatively-stained cell surfaces showed massive arrays of microfibrils, and severe alkali extractions induced only a weak crystalline pattern characteristic of Cellulose II. In this regard Talmadge and coworkers report (1973) that primary wall microfibrils exhibit a highly crystalline core. In addition, microfibril crystallization occurs due to enzymatic induced alignment of glucose chains at the plasmalemma surface (Brown and Montezinos, 1976). Therefore, any observation that microfibrils are present in the regenerated wall would be complemented with a positive crystallographic analysis.

The intact plant cell wall has cellulose as its major component. In both natural and suspension systems, cells are able to synthesize β-(1,3)- and β-(1,4)-linked glucans (Brett, 1978; Van Der Woude et al., 1974; Bauer et al., 1973; Talmadge et al., 1973; Clark and Stone, 1963), although the former is found naturally only in specialized or wounded plant parts.

Researchers suggest that cultured plant cells may exhibit aberrant wall structures. Chanzy and coworkers (1979) analyzed a suspension culture of rose cell walls

by both electron and x-ray diffraction. Their study revealed the strong presence of Cellulose IV and the unexpected absence of Cellulose I. In the regenerated wall of rose protoplasts Pearce and Cocking (1973) reported that fibrillar synthesis was observed to be dependent on medium molarity after protoplast formation.

Acetolysis of the regenerated walls of Douglas-fir protoplasts revealed the absence of cellulose. These results confirmed the lack of any fibrillar material present on the cell membrane. Therefore, no significant amounts of a β-(1,4)-glucan appear to be present. These findings are in contrast to the analysis of Douglas-fir suspension cell walls (Burke et al., 1974). By methylation analysis Burke and coworkers demonstrated that 22% of the cell wall was cellulose, while the (1,3)-glucan was not detected.

In the present study other wall components were exclusively in either the regenerated or suspension cell wall of Douglas-fir. Although lignin was observed in the suspension cell wall, its presence went undetected after enzyme treatment. Talmadge et al., (1973) suggest that if suspension cells model a primary wall, then they should be devoid of lignin. However, others (Fukuda, 1978; Whitmore, 1978; Barnoid, 1965) demonstrated the presence of lignin in cultured tissues of poplar, slash pine, and sequoia. The inability to detect lignin after 6 days culture may also be related to the suggestion that lignin synthesis increases with age of culture (Fukuda, 1978).

After wall-degrading enzyme removal the Douglas-fir protoplast culture exhibited ultraviolet fluorescence upon reaction with aniline blue after 6 days of protoplast culture. Some cell surfaces were characterized by numerous "bubblelike" structures substantially smaller than the "blebs" found on the surface of Douglas-fir suspension cells (Parham and Kaustinen, 1976). The cells with surface "bubbles" were likely those with substantial wall material (i.e., regenerated wall) which

fluoresced as callose. Since callose is widely synthesized in response to wounding, it is reasonable that protoplasts have experienced a structural attack by the action of the wall-degrading enzymes and/or removal of the original cell wall. "Protoplasts are after all, injured cells which have to overcome many obstacles on the way to becoming healed" (Galston, 1978). The cells with surface "bubbles" then were those which attempted to repel the "attack."

The suggestion that callose may be a dominant polymer in wall regeneration is not new. Vinca rosea protoplasts incorporated the majority of glucose into the β-(1,3)-linked polymer (Takeuchi and Komamine, 1978a). Protoplasts generated by plasmolysis in Elodea were induced to produce callose (Prat and Roland, 1971) while Herth and Meyer (1977) implied that the enzymatically generated protoplasts of tobacco contained callose in the regrown wall. Strmen (1979) demonstrated that Norway spruce protoplasts contained enzyme systems to synthesize either β-(1,3)- or β-(1,4)-linked glucans. Callose may possibly assemble in fibrillar form in higher plants (Marchessault, 1979) as reported in yeast (Kreger and Kopecka, 1978) during cell wall regeneration. However, other evidence suggests that the presence of callose in the regenerated wall is not universal. Horine and Ruesink (1972) found that the regenerated walls of Convolvulus were devoid of callose.

Pectin was identified in both suspension and regenerated cell walls of Douglas-fir cultures. Takeuchi and Komamine (1978a) reported pectin synthesis and deposition in Vinca rosea protoplasts. They detected lower but yet significant yields (<10%) when compared with suspension cells. However, Hanke and Northcote (1974) reported soybean protoplast walls devoid of pectin, but later Boffey and Northcote (1975) observed that the rate of pectin synthesis in tobacco leaf protoplasts was similar before and after plasmolysis.

The presence of protein in the regenerated wall was detected by Herth and Meyer (1977) and Willison (1976), although composition was never reported. Figure 5 illustrates some important features of the regenerating cell wall. There was a net increase in cell wall protein over the period studied. This plot of amino acids probably indicates that deposition is a simple process only involving a few proteins. In this case the role of the protein(s) in the regenerating wall seems not to be similar to extensin (Lamport, 1978), but rather is probably associated with a wounding response.

The results in the present study provide insight into cell wall regeneration by protoplasts from suspension cells. With few exceptions (Davey and Mathias, 1979; Williamson et al., 1977) the bulk of freeze-etch studies on protoplasts have dealt with cells isolated from intact tissues. In general, such cells respond by renewed wall synthesis within 24 hours of isolation and consequently may differ significantly from suspension cells, since the latter are dedifferentiated through culturing. Robenek and Peveling (1977) and Pearce and coworkers (1974) report that vivid cytological differences exist between protoplasts from cultured and intact sources. The egg cell, a naturally occurring protoplast, perhaps should be compared with protoplasts arising from each population to note the similarities and differences.

In conclusion, cell wall regeneration by Douglas-fir protoplasts originating from cell suspensions appears not to be a model system for studying wall biogenesis. Rather, wall regeneration examined here seems to be largely a response to wounding and a protein, probably also related to wounding, is deposited prior to polysaccharide synthesis. These two aspects of wall regeneration are undoubtedly related, although the manner of action is not understood.

References

Asamizu, T., Nishi, A. Regenerated cell wall of carrot protoplasts isolated from suspension-cultured cells. Physiol. Plant. 48:207-12(1980).

Asamizu, T., Tanaka, K., Takebe, I., Nishi, A. Change in molecular size of cellulose during regeneration of cell wall on carrot protoplasts. Physiol. Plant. 40:215-18(1977).

Atalla, R. H., Whitmore, R. E. The influence of elevated temperatures on structure in the isolation of native cellulose. J. Polymer Sci. (Polymer Letters) 16:601-5(1978).

Barnoud, F. Lignins and Cell Wall Polysaccharides in Plant Tissues Cultured in vitro. In: Proceedings of an International Conference on Plant Tissue Culture, p. 157-69, White, P. R., Grove, A. R., eds., Berkeley: McCutchan 1965.

Bauer, W. D., Talmadge, K. W., Keegstra, K., Albersheim, P. The structure of plant cell walls. II. The hemicellulose of the walls of suspension-cultured sycamore cells. Plant Physiol. 51:174-87(1973).

Boffey, S. A., Northcote, D. H. Pectin synthesis during the wall regeneration of plasmolysed tobacco leaf cells. Biochem. J. 150:433-40(1975).

Borchardt, L. G., Piper, C. V. A gas chromatographic method for carbohydrates as alditol-acetates. Tappi 53:257-60(1970).

Braun, G. α-Cellobiose octaacetate. In: Organic Synthesis: Collective Vol. II. p. 124-6, Blatt, A. H., ed., New York, Wiley 1943.

Brenner, M., Niederwieser, A., Pataki, G. Amino Acids and Derivatives. In: Thin-layer Chromatography: A Laboratory Handbook, p. 391-432, Stahl, E., ed., Berlin, Heidelberg, New York, Springer 1965.

Brett, C. T. Synthesis of β-(1,3)-glucan from extracellular uridine diphosphate glucose as a wound response in suspension-cultured soybean cells. Plant Physiol. 62:377-82(1978).

Brown, R. M., Montezinos, D. Cellulose microfibrils: visualization of biosynthetic and orienting complexes in association with the plasma membrane. Proc. Nat. Acad. Sci. (U.S.A.) 70:143-7(1976).

Burgess, J., Fleming, E. N. Ultrastructural observations of cell wall regenerating tobacco protoplasts. Planta 146:203-10(1979).

Burgess, J., Linstead, P. J. Structure and association of wall fibrils produced by regenerating tobacco protoplasts. Planta 146:203-10(1979).

Burke, D., Kaufman, P., McNeil, M., Albersheim, P. The structure of plant cell walls. VI. A survey of the walls of suspension-cultured monocots. Plant Physiol. 54:109-15(1974).

Chanzy, H., Imada, K., Mollard, A., Vuong, R., Barnoud, F. Crystallographic aspects of subelementary cellulose fibrils occurring in the wall of rose cells cultured in vitro. Protoplasma 100:303-16(1979).

Clarke, A. E., Stone, B. A. Chemistry and biochemistry of β-(1,3)-glucans. Rev. Pure Appl. Chem. 13:134-56(1963).

Cocking, E. C. Plant cell protoplasts. Isolation and development. Ann. Rev. Plant Physiol. 23:29-50(1972).

Cocking, E. C. A method for the isolation of plant protoplasts and vacuoles. Nature 187:962-3(1960).

Currier, H. B., Strugger, S. Aniline blue and fluorescence microscopy of callose in bulb scales Allium cepa L. Protoplasma 45:522-59(1956).

Davey, M. R., Mathias, R. J. Close-packing of plasma membrane particles during wall regeneration by isolated higher plant protoplasts. Fact or artifact? Protoplasma 100:85-99(1979).

Elbein, A. D., Forsee, W. T. Studies on the Biosynthesis of Cellulose. In: Biogenesis of Plant Cell Wall Polysaccharides, p. 259-96, Loewus, F., ed. New York, London, Academic 1973.

Eschrich, W., Currier, H. B. Identification of callose by its diachrome and fluorochrome reactions. Stain Tech. 39:303-7(1964).

Fahn, A. Plant Anatomy. Oxford, Pergamon 1974.

Flowers, H. M., Batra, K. K., Kemp, J., Hassid, W. Z. Biosynthesis of insoluble glucans from uridine-diphosphate-D-glucose with enzyme preparations from Phaseolus aureus and Lupinus albus. Plant Physiol. 43:1703-9(1968).

Fukuda, T. Studies on tissue cultures of tree cambium. VII. Sugar composition of cell wall polysaccharides of suspension-cultured poplar cells. J. Japanese Wood Res. Soc. 24:677-82(1978).

Galston, A. W. The Use of Protoplasts in Plant Propagation and Improvement. In: Propagation of Higher Plants through Tissue Culture. A Bridge Between Research and Application, p. 200-12, Hughes, K. W., et al., ed., Springfield, VA, U.S. Department of Energy 1978.

Gamborg, O. L., Shyluk, J. P. Tissue culture, protoplasts, and morphogenesis in flax. Bot. Gaz. 137:301-6(1976).

Gee, M., Reeve, R. M., McCready, R. M. Reaction of hydroxylamine with pectinic acids. Chemical studies and histochemical estimation of the degree of esterification of pectic substances in fruit. Agric. Food Chem. 7:34-8(1959).

Green, J. W. Preparation of Cellobiose Octaacetate as a Diagnostic Test for Cellulose. In: Methods in Carbohydrate Chemistry. Vol. III. Cellulose, p. 70-2, Whistler, R. L., ed., New York, London, Academic 1963.

Grout, B. W. W. Cellulose microfibril deposition at the plasmalemma surface of regenerating tobacco mesophyll protoplasts: A deep-etch study. Planta 123:275-82(1975).

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

Harada, T. Production, properties, and application of curdlan. A.C.S. Symp. Series 45:263-85(1977).

Harran, S., Dickinson, D. B. Metabolism of myo-inositol and growth in various sugars of suspension-cultured tobacco cells. Planta 141:77-82(1978).

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

Horine, R. K., Ruesink, A. W. Cell wall regeneration around protoplasts isolated from Convolvulus tissue culture. Plant Physiol. 50:438-45(1972).

Kirby, E. G. Personal communication, 1980.

Kirby, E. G., Cheng, T. Y. Colony formation from protoplasts derived from Douglas-fir cotyledons. Plant Sci. Letter 14:145-54(1979).

Klein, A. S., Delmer, D. P. Characterization of the regenerating wall of soybean protoplasts. Plant Physiol. 63 (Suppl.): 51(1979).

Kopecka, M., Farkas, V. RNA synthesis and the formation of the cell wall. Effect of lomofungin on the regenerating protoplasts of Saccharomyces cerevisiae. J. Gen. Microbiol. 110:453-63(1979).

Kreger, D. R., Kopecka, M. Nature of the nets produced by protoplasts of Schizosaccharomyces pombe during the first stage of wall regeneration in liquid medium. J. Gen. Microbiol. 108:269-74(1978).

Lamport, D. T. A. Cell Wall Carbohydrates in Relation to Structure and Function. In: Frontiers of Plant Tissue and Cell Culture, p. 235-44, Thorpe, T. A., ed., Calgary, Alberta, Univ. of Calgary 1978.

Lamport, D. T. A. The protein component of primary cell walls. Adv. Bot. Res. 2:151-218(1965).

Marchant, H. J., Hines, E. R. A role of microtubules and cell-wall deposition in elongation of regenerating protoplasts of Mougeotia. Planta 146:41-8(1979).

Marchessault, R. H. Structure and Mechanical Properties of Cell Wall Systems. In: Structure and Biochemistry of Natural Biological Systems, Proc. of the Third Philip Morris Science Symp., p. 35-49, Walk, E. M., ed., Richmond, V. A., Philip Morris 1979.

McCready, R. M., Reeve, R. M. Test for pectin based on reactions of hydroxamic acids with ferric ion. Agric. Food Chem. 3:260-2(1955).

Meyer, Y., Herth, W. Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division, in protoplasts of Nicotiana tabacum L. cultured in vitro. Planta 142:253-62(1978).

Murashige, T., Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-97(1962).

Parham, R. A., Kaustinen, H. M. Organization and Surface Features of Douglas-fir Cells in Culture. In: Scanning Electron Microscopy. Vol. VII, p. 470-6, Johari, O., Becker, R. P., eds., Chicago, IL, IIT Research Institute 1976.

Pearce, R. S., Withers, L. A., Willison, J. H. M. Bodies of wall-like materials ("Wall-Bodies") produced intracellularly by cultured isolated protoplasts and plasmolyzed cells of higher plant protoplasts. Protoplasma 82:223-36(1974).

Pearce, R. S., Cocking, E. C. Behavior in culture of isolated protoplasts from "Paul's Scarlet" rose suspension culture cells. Protoplasma 77:165-80(1973).

Prat, R., Roland, J.-C. Biologie vegetale - Etude ultrastructurale des premiers stades de neoformation d'une enveloppe par les protoplastes vegetaux separes mecaniquement de leur paroi. C.R. Acad. Sc. Paris, Ser. D, 273D, 165-8(1971).

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

Robenek, H., Peveling, E. Ultrastructure of the cell wall regeneration of isolated protoplasts of Skimmia japonica Thunb. Planta 136:135-45(1977).

Robinson, K. W. Unpublished work, 1979.

Ruesink, A. W. Protoplasts of plant cells. Methods in Enzymology 23(A):197-209 (1971).

Seligman, A. M., Hanker, J. S., Wasserkrug, H., Dmochowski, H., Katzoff, L. Histochemical demonstration of some oxydized macromolecules with thiocarbohydrazide (TCH) or thiosemicarbazide (TSC) and osmium tetroxide. J. Histochem. Cytochem. 13:629-36 (1965).

Shepard, J. F., Totten, R. E. Mesophyll cell protoplast of potato. Isolation, proliferation, and plant regeneration. Plant Physiol. 60:313-16(1977).

Smeltzer, R. H., Johnson, M. A. Evidence for the presence of cyclic adenosine 3',5'-monophosphate and its metabolism in loblolly pine (Pinus taeda L.) callus. Can. J. Forest Res. 7:68-75(1977).

Strmen, J. Biosynthesis of polysaccharides in tissue cultures and protoplasts of Norway spruce. Lesnictvi 25:177-82(1979).

Takeuchi, Y., Komamine, A. Changes in the composition of cell wall polysaccharides of suspension-cultured Vinca rosea during culture. Physiol. Plant. 42:21-8(1978a).

Takeuchi, Y., Komamine, A. Composition of the cell wall formed by protoplasts isolated from cell suspension cultures of Vinca rosea. Planta 140:227-32(1978b).

Talmadge, K. W., Keegstra, K., Bauer, W. D., Albersheim, P. The structure of plant cell walls. I. The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccharides. Plant Physiol. 51:158-73(1973).

Tsutsui, K., Kumon, H., Ichikawa, H., Tawara, J. Preparative method for suspended biological materials for SEM by using a polycationic substance layer. J. Electron Microsc. 25:163-4(1976).

Updegraff, D. M. Semi-micro determination of cellulose in biological materials. Anal. Biochem. 32:420-4(1969).

Van Der Woude, W. J., Lembi, C. A., Morre, D. J., Kindinger, J. I., Ordin, L. β-Glucan synthetases of plasma membrane and Golgi apparatus from onion stems. Plant Physiol. 54:333-40(1974).

Whitmore, F. W. Lignin-carbohydrate complex formed in isolated cell walls of callus. Phytochem. 17:421-5(1978).

Williamson, F. A., Fowke, L. C., Weber, G., Constabel, F., Gamborg, O. Microfibril deposition on cultured protoplasts of Vicia hajastana. Protoplasma 91:213-19(1977).

Willison, J. H. M. Synthesis of Cell Walls by Higher Plant Protoplasts. In: Microbial and Plant Protoplasts, p. 283-97, Peberdy, J. F. et al., ed., London, New York, San Francisco, Academic 1976.

Willison, J. H. M., Cocking, E. C. Microfibril synthesis at the surfaces of isolated tobacco mesophyll protoplasts, a freeze-etch study. Protoplasma 84:147-59 (1975).

Willison, J. H. M., Cocking E. C. The production of microfibrils at the surface of isolated tomato-fruit protoplasts. Protoplasma 75:397-403(1972).

Yllner, S., Enstron, B. Studies of the adsorption of xylan on cellulose fibres during the sulphite cook. Part 1. Svensk Papperstid. 59:229-32(1956).

STUDY OF THE REGENERATING CELL WALLS OF DOUGLAS-FIR PROTOPLASTS

ARTICLE II. CELL WALL GLYCOPROTEIN CHARACTERIZATION

Abstract

A glycoprotein was isolated from the regenerating cell walls of Douglas-fir protoplasts by two different techniques and partially characterized. One extraction method using sodium borohydride effectively removed protein from the wall material and prevented the hydrolysis of polysaccharides while severely degrading the protein. A second and milder extraction method conducted at lowered temperatures with a mild alkaline solution removed limited amounts of wall glycoprotein with little degradation. The glycoprotein mainly contains glycine, alanine, glutamic acid, serine, glucose, galactose, and mannose, yielding a single peak in the ultracentrifuge with an estimated molecular weight of 282,000 but resolvable by isoelectric focusing into two species with isoelectric points of 4.2 and 4.4. Although hydroxyproline can be found in the cell wall hydrolyzates of suspension cells, the regenerating wall on protoplasts lacks this amino acid.

Introduction

Over the past 20 years several investigations have confirmed the presence of proteins in the cell walls of higher plants, ending a debate which lasted almost a century (Lamport, 1965). Two types of glycoproteins have been found in cell walls. Those abundant in the rare amino acid, hydroxyproline, were named extensin (Lamport, 1963) and have been investigated extensively in carrot (Sadava and Chrispeels, 1978), excised Avena coleoptiles (Cleland, 1968; Fujii, 1978), and sycamore suspension (Lamport, 1978) systems. The other glycoprotein was reported to be hydroxyproline-poor (Brown and Kimmins, 1979a) and synthesized largely in response to mechanical wounding. It was a high molecular weight substance rich in alanine, glycine and glutamic acid (Brown and Kimmins, 1978) and deposited in the cell walls

of bean leaves and other intact leaf tissues after multiple surface lesions were made.

It was considered that cell wall regeneration on plant protoplasts might provide a unique opportunity to explain some fundamental aspects of cell wall biogenesis (Willison, 1976), especially with regard to the role of wall proteins (Lamport, 1978). In the present study the protein deposited in the regenerating cell walls of Douglas-fir protoplasts was partially characterized and then compared with the two types of cell wall glycoproteins described above. The protein found in the cell walls of Douglas-fir suspension cells from which the protoplasts were generated was examined for comparative purposes.

Materials and Methods

Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] regenerating cell walls were isolated from both needle- and hypocotyl-derived protoplasts originating from suspension cultures as already described (Robinson, Thesis, Part I). The protoplast medium was enriched with ^{14}C-L-proline and factors necessary for proline hydroxylation (Sadava and Chrispeels, 1971). The recovered cell walls represented growth for 0 to 6 days after the cell wall-degrading enzymes were removed.

Protein Extractions

Two methods of alkali extraction were used separately to remove and isolate the cell wall-bound protein material. The first approach was purported to limit the degradation of carbohydrate through $NaBH_4$ reduction. The protein extracted is called the wall elimination product (WEP) (Lamport, 1979). The second technique involved a milder alkali extraction (MAE) aimed at limited degradation of nascent protein (Brown, 1979).

Wall Elimination Product (WEP)

The WEP preparation was made by mixing a 1 g composite of cell walls (representing 0 to 6 days cell wall regeneration) at 45°C with 50 mL DMSO, 40 mL of 25% ethanol, and 10 mL of 2N NaOH in which 3.8 g of $NaBH_4$ was dissolved. After 5 hours the mixture was filtered through a coarse sintered glass funnel, the filtrate discarded, and the cake washed with 100 mL of 95% ethanol. The air-dried cake was then washed with 150 mL of distilled water. The resulting filtrate was neutralized with acetic acid, evaporated at 40°C to 50 mL, mixed with 200 mL of 95% ethanol, and left standing overnight in a graduated cylinder. The supernatant was drawn off, leaving a precipitate. The precipitate was further isolated by centrifugation and then allowed to air-dry.

The ethanol-precipitated WEP was fractionated by gel permeation chromatography. A Sephadex G-75 column (1.2 x 35 cm) with a 150 mm hydrostatic head, 6.3 mL/hour flow rate, and a void volume of 11.7 mL were used. The excluded volume was determined with equine ferritin. WEP (2 mg/mL) was added and eluted at room temperature in water. Column elution was followed at 280 nm with ISCO Models 659 scanner and UA-5 absorbance monitor. Twenty-minute fractions were collected and freeze-dried.

Several fractions were chosen for further analysis based upon their position on the chromatogram (see Fig. 1). Fraction numbers 7, 12, 22, and 28 were separately acid hydrolyzed as described earlier (Robinson, Thesis, Part I) for amino acid analysis. Radioactive fractions collected from the amino acid analyzer were counted in a scintillation counter.

Other fractions (numbers 10, 14, 18, and 23) were isoelectrically focused to establish the character of the WEP extracted protein. An LKB 2117 Multiphor electro-focusing apparatus at a constant power of 25 W was employed using LKB PAG plate gels having an ampholyte range of pH 3.5 to 9.5. Parallel samples of each

fraction were run to determine if the WEP was a glycoprotein. After duplicate samples were focused on the gel, they were separated and stained with either Coomassie Blue or periodic acid-Schiff's reagent (Kitpatany and Zebrowski, 1973) for the respective detection of protein or carbohydrate.

Mild Alkali Extraction (MAE)

The second technique patterned after Brown and Kimmins (1978) was used to remove cell wall-bound proteins with only moderate degradation. Varying amounts of freeze-dried regenerating cell walls (from 25 to 300 mg) from cell cultures of 0 to 6 days regeneration were mixed with 10 mL of 1N NaOH and carefully maintained at 2°C with occasional stirring. The solution was neutralized after 30 minutes with glacial acetic acid. The cell wall residue was sedimented and the supernatant collected and dialyzed against distilled water. The dialysis was performed over 24 hours with 3 exchanges of water to remove contaminating salts or low molecular weight carbohydrates. The dialyzate was freeze-dried after mixing it with pyridine to make a 10% solution.

After dissolution in 0.02M NaOH-glycine buffer at pH 9.0, the cell wall extract was eluted with the same buffer from a Sephadex G-200 column (2.5 x 50 cm) with a 150 mm hydrostatic head and 9.2 mL/hour flow rate or from a G-75 column described above. The void volume was determined with Blue Dextran 2000. The MAE protein was found to be totally excluded by the G-200 column and was collected, dialyzed, and freeze-dried as before. Samples from 2 to 6 days cell wall regeneration were separately evaluated for their isoelectric points and homogeneity. Samples from days 0 through 6 were analyzed for their carbohydrate and amino acid content as described earlier (Robinson, Thesis, Part I). Molecular weight of a sample from day 6 was estimated by employing a Beckman Model E analytical ultracentrifuge for determinations of the sedimentation and diffusion constants.

^{14}C-Proline in Cell Walls of Douglas-fir Cultured Cells

To determine if a protein rich in proline or hydroxy-proline was deposited in the regenerating cell wall, ^{14}C-proline was added to the medium at 0.1 μCi/mL at zero regeneration time. After 6 days the cell walls were assessed for the source of their radioactivity after acid hydrolysis. Fractions were collected from an amino acid analyzer for scintillation counting. Cell suspensions cultured in both the suspension and protoplast media (Robinson, Thesis, Part I) were similarly given ^{14}C-proline and the amino acid composition of cell walls analyzed after 6 days. Appropriate fractions were collected for scintillation counting of the proline and hydroxyproline peaks.

Results

Two extraction schemes used on regenerating cell wall samples provided insight into the nature of the deposited protein. For clarity the analytical results for both extracts are presented concurrently.

Chromatography of the two cell wall extracts produced quite different results. A chromatogram of the WEP preparation from a G-75 column is shown in Fig. 1 with the void volume marked by the exclusion of ferritin. The most striking feature of Fig. 1 is the demonstration of extract heterogeneity. The MAE yielded much less material for chromatography, since only nascent glycoproteins are expected to be removed under the mild extraction conditions (Brown, 1979). Chromatography on a G-200 column resulted in only an excluded fraction. Due to the faster flow rate, a G-75 column was used in subsequent runs and the excluded fraction similarly collected.

Isoelectric focusing of proteins from both extraction procedures provided valuable data on the cell wall regeneration and extraction processes. Four WEP fractions indicated in Fig. 1 were isoelectrically focused as shown in Fig. 2. All

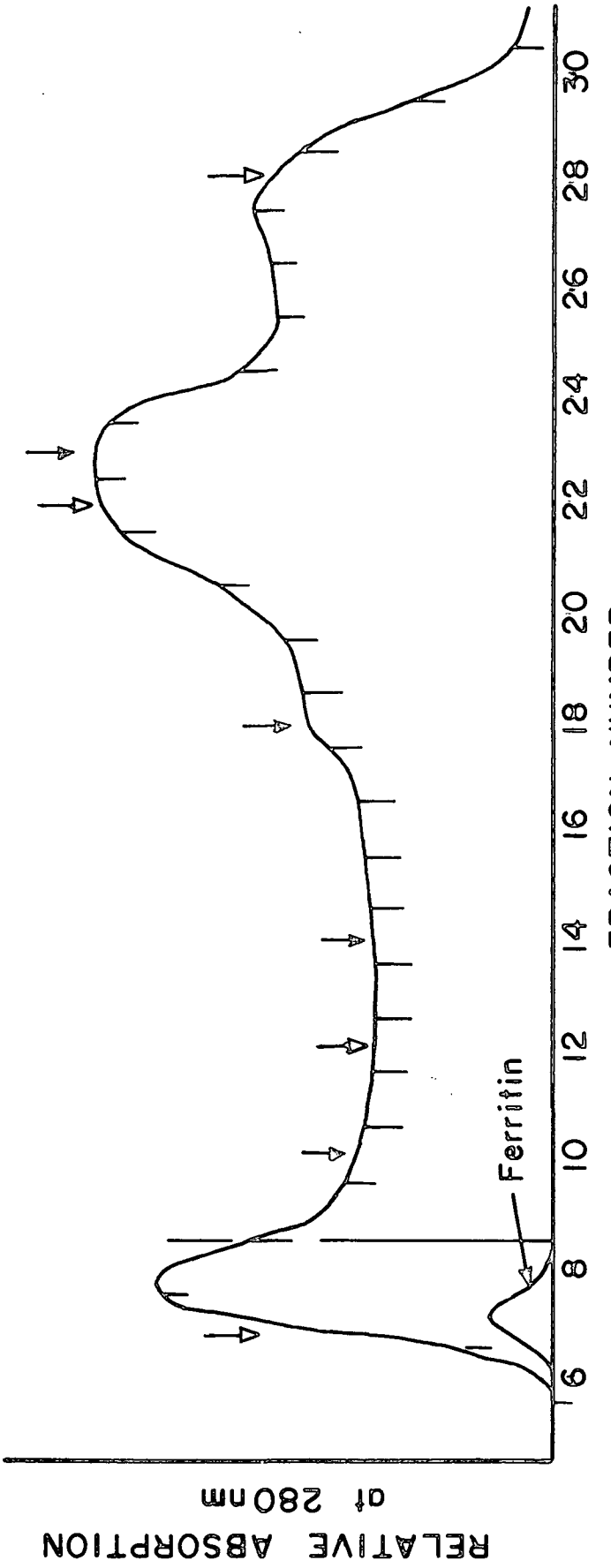


Figure 1. G-75 chromatogram of the WEP extract from a regenerating cell wall composite as it absorbed at 280 nm. The ∇ positions represent the fractions used for amino acid analysis (Table I) while + represent those used in isoelectric focusing (Fig. 2).

samples were readily soluble and migrated to isoelectric pH's between 3.0 and 5.0. Figure 2 shows that sample heterogeneity increases with decreasing molecular weight. It is also important to note that the centroid of the focused fractions is about 4.3. In addition, equivalent bands were observed to react with both carbohydrate and protein stains, demonstrating that the WEP fragments were glycoprotein.

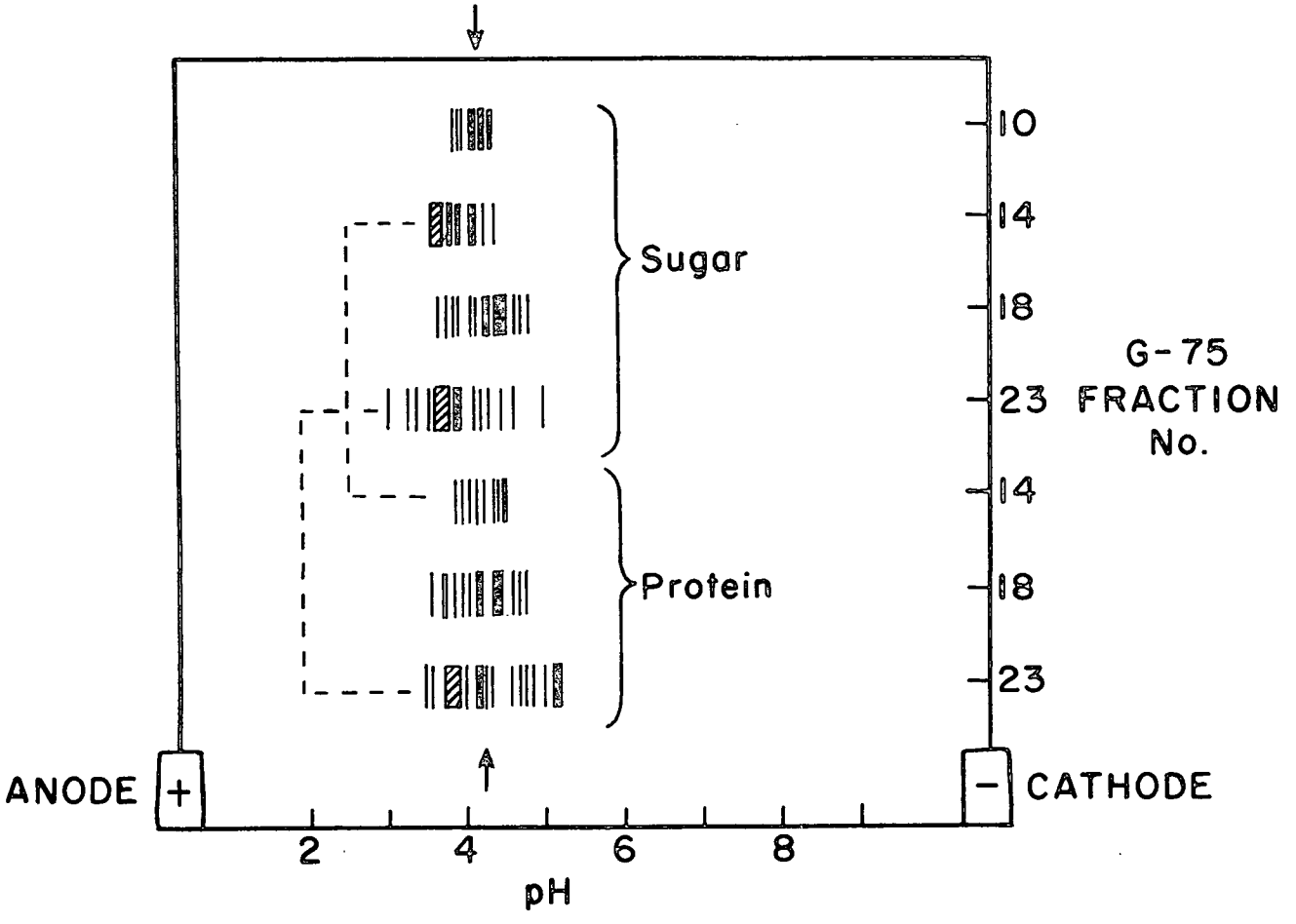


Figure 2. Electrofocused pattern of WEP extracted cell wall protein. The four WEP fractions are shown to be heterogenous and centered about pH 4.3 (see arrow). The complementary carbohydrate and protein staining indicates that the wall material extracted was a glycoprotein.

The MAE protein was also electrofocused to determine sample homogeneity as a function of cell wall regeneration time. Figure 3 shows the isoelectric positions of cell wall extracts from day 2 through 6. The samples, although partially

soluble, were all resolved into bands at pH 4.2 and 4.4 and sedimented as a homogeneous material. Although the MAE glycoprotein was not homogeneous by isoelectric focusing (Fig. 3), it behaved as a single peak in the ultracentrifuge. Therefore, from determined S and D values and an assumed partial specific volume of 0.74 (Smith, 1970), the molecular weight of the two components sedimenting as one was estimated at 282,000.

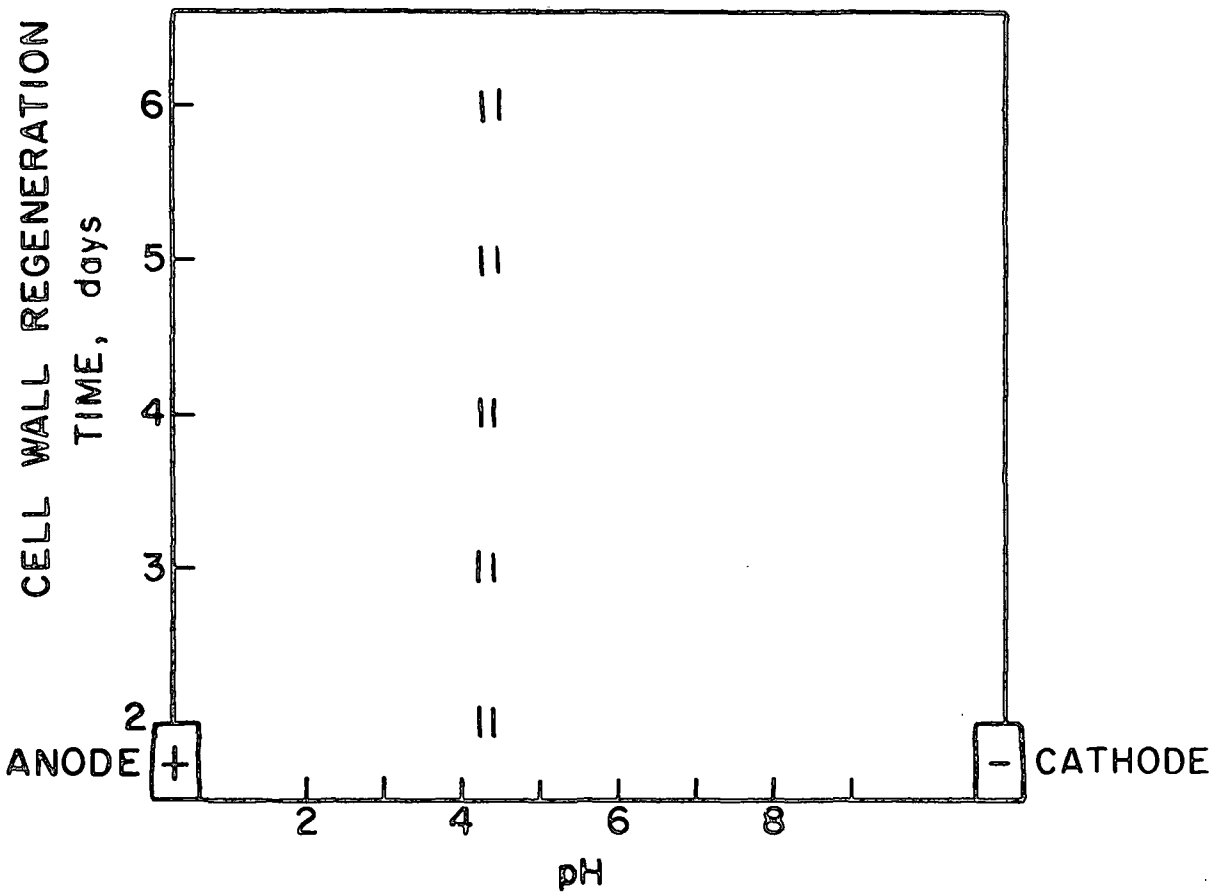


Figure 3. Isoelectric focusing of MAE. The extract isoelectric focused in two bands from 2 to 6 days regeneration time, suggesting that no more than two proteins are deposited over that period.

Amino acid compositions from the acid hydrolyzates of proteins from both MAE and WEP extractions are displayed in Table I. MAE proteins as a function of regeneration time and WEP proteins of fractions from Fig. 1 are compared with a wound-induced cell wall glycoprotein of Phaseolus vulgaris (Brown and Kimmins, 1978).

TABLE I

The molar percentages of amino acids in acid hydrolyzed cell wall glycoproteins extracted from the regenerating cell wall compared with a wound-induced glycoprotein from bean cell walls.

		Douglas-fir Regenerated Cell Walls								
	Wounded Bean	Regeneration Time[c], days					G-75 Fraction Number[d]			
Amino Acid[a]	Cell Walls[b]	0	1	2	3	6	7	12	22	28
Glycine	11	15	18	20	16	14	5	6	5	11
Alanine	11	16	13	9	16	13	7	7	7	7
Aspartic acid	7	11	11	10	13	12	12	13	15	13
Serine	7	10	11	13	9	8	5	4	4	11
Glutamic acid	10	6	7	8	11	9	13	15	13	20
Leucine	7	7	8	8	6	7	10	10	10	5
Threonine	5	7	5	5	6	7	4	4	4	3
Lysine	7	6	5	6	6	6	5	4	3	6
Proline	7	5	3	2	4	5	5	4	6	2
Arginine	4	4	4	4	3	4	6	5	4	2
Valine	6	4	3	3	2	5	7	7	6	0
Phenylalanine	4	3	3	4	2	3	7	6	5	1
Isoleucine	5	2	2	3	2	3	6	5	4	2
Tyrosine	4	2	3	3	2	2	4	3	7	0
Histidine	1	2	2	2	1	1	2	2	2	6
Methionine	1	4	4	4	3	4	0	1	1	0
Hydroxyproline	2	0	trace	0	trace	trace	1	2	3	0

[a]Neither cysteine nor cystine were detected.
[b]Brown Kimmins (1978).
[c]MAE proteins.
[d]WEP proteins.

The carbohydrate portion of the MAE glycoprotein is shown in Fig. 4. The dominant sugar was glucose. The data in this determination suffered only in an absolute sense from the handling and weighing of minute amounts (200 to 630 μg) of sample, although the general relationship can be appreciated.

Radioactive proline was fed to suspension cells and protoplasts under various conditions that subjected the cells to increasing osmotic and mechanical stress. Cell wall acid hydrolyzates were made, and only the proline and hydroxyproline peaks were collected from the amino acid analyzer for counting in a scintillation counter. The results are displayed in Table II and demonstrate that as the medium molarity of the suspension cultures was increased a reversal in the ratios of hydroxproline to proline was observed. Protoplasts which were subjected to both the high molarity conditions and the enzymatic removal of the cell wall exclusively yielded the radioisotope as proline.

Discussion

In the past 15 years the major obstacle in the characterization of cell wall proteins has been extraction of the material intact. Since the proteins extensively studied to date have been glycoproteins, additional linkages obviously compound the problem. Of the two extraction approaches used in this study, the WEP procedure appears to degrade the protein structure while the MAE removes the newly deposited and possibly less highly glycosylated or cross-linked protein (Brown, 1979). Figures 1 and 2 show the extent of the WEP degradation. Over 95% of the material is included on G-75. In contrast, the MAE protein only exhibits marginal solubility in water, suggesting that it is less highly glycosylated than the WEP. Less overall degradation of the protein appears to occur as indicated by its high apparent molecular weight.

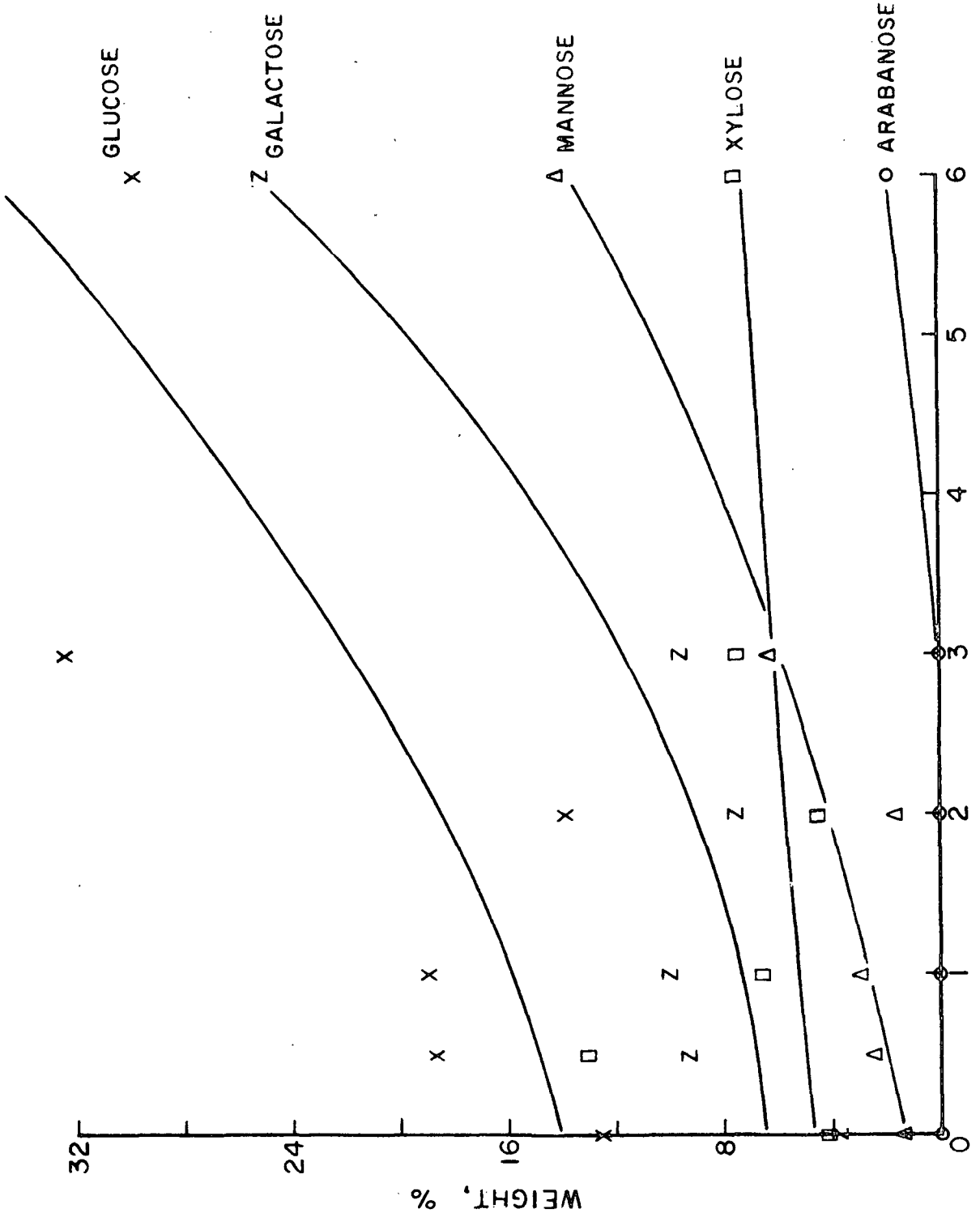


Figure 4. Carbohydrate analysis of the MAE glycoprotein over 6 days wall regeneration time. Glucose is the dominant sugar, although parallel increases over the period studied are seen with galactose and mannose. These increases suggest additional protein glycosylation with time.

TABLE II

The recovery of ^{14}C-proline from the cell walls of suspension cells and protoplasts after 6 days uptake. Increasing the osmotic stress in suspension cultures results in a decrease of isotope incorporation as hydroxyproline. The high molarity medium and enzymatic removal of the cell wall results in the exclusive appearance of proline.

Cell Type and Condition	% Relative Radioactivity	
	Proline	Hydroxyproline
Suspension cells, normal medium	43	57
Suspension cells, high molarity medium	65	35
Protoplast regenerated wall	100	0

Isoelectric focusing experiments revealed that the WEP fragments focused in a pH range centered about pH 4.3 which is similar to the less degraded protein of the MAE. The high resolving power of isoelectric focusing taken together with the single peak seen in the ultracentrifuge indicate that the two components of the MAE seen in Fig. 3 must be of very similar size.

Amino acid analyses showed that the two wall extracts differed significantly in their content of hydroxyproline. The more severe conditions of the WEP procedure probably resulted in a contribution from residual suspension cell walls remaining after protoplast formation. Since the immediate precursor of hydroxyproline is proline (Lamport, 1963), the results in Table II confirm the absence of hydroxyproline synthesis under the conditions and during the period of wall regeneration studied. This finding suggests that extensin was not deposited during wall regeneration in this investigation. Other evidence to support this view is seen in the vastly different amino acid analysis (Lamport, 1969), carbohydrate content (Miller, et al., 1972), and isoelectric point (Brysk and Chrispeels, 1972) of extensin compared with the glycoprotein extracted in this present study. This result is perhaps surprising

since the synthesis of extensin has been related to mechanical (Sadava and Chrispeels, 1978) or fungal (Esquerré-Tugayé et al., 1979) wounding, although it is also apparently related to the state of cellular development (Steward et al., 1974; Roberts and Northcote, 1972).

Another cell wall protein gaining prominence in recent years is a hydroxyproline-poor, wound-induced glycoprotein (Brown and Kimmins, 1979a). This has significant similarities to the MAE glycoprotein extracted from the regenerating cell walls of Douglas-fir protoplasts. The amino acid composition of the wall protein of Phaseolus vulgaris (Brown and Kimmins, 1978) was compared with the similarly extracted material (see columns 2 through 6, Table I) from the regenerating wall. In general, the amino acid compositions are ordered in parallel within experimental error, although subtle differences do exist. The carbohydrate portions of the bean and Douglas-fir glycoproteins have all of the same sugars (Brown et al., 1975) although Brown and Kimmins (1978) found that the absolute quantities were quite variable. The bean glycoprotein exhibited an isoelectric point of 3.63 (Brown and Kimmins, 1979a) and a molecular weight of 520,000 (Brown and Kimmins, 1979b). While the bean glycoprotein isoelectric point differed from that deposited in the regenerating Douglas-fir cell wall, the variance may be an acceptable species difference. The bean glycoprotein molecular weight is a little less than twice the 282,000 reported in the present study, making it tempting, but for the assumed partial specific volume, to speculate that the bands in Fig. 3 are two nearly equal parts of one original glycoprotein. In addition a final similarity evidenced is that both the bean and Douglas-fir glycoprotein appear to be synthesized and deposited in response to wounding or stress, and neither was catabolized over the period studied.

Brown and Kimmins have not speculated on the exact role which their wound-induced cell wall glycoprotein appears to play. Both their mechanical disruption of the cell wall and the enzymatic attack used to generate protoplasts in this study resulted in the deposition of a protein. The protein's rapid synthesis upon wounding in both bean and Douglas-fir strongly suggests a role in extracellular repair.

The presence of a cell wall wound-induced glycoprotein rich in glycine, alanine, and glutamic acid has been further demonstrated in fava beans, corn, tobacco, and rhubarb (Brown, 1979). The extensin-related work, however, has emphasized hydroxyproline and a few associated amino acids (e.g.: serine, lysine, valine, tyrosine, and Tryptophan) (Lamport, 1969) and ignored the presence of other cell wall amino acids which were not part of the model (Lamport, 1970, 1965). In green algae (Thompson and Preston, 1967) and in both woody gymnosperms (Scurfield and Nicholls, 1976) and angiosperms (Lüdke and Liefländer, 1970), cell wall proteins rich in many of the wound-generated amino acids in Table I have been found, suggesting that the occurrence of this protein may be widespread throughout the plant kingdom.

References

Brown, R. G. Personal communications, 1979.

Brown, R. G., Kimmins, W. C. Linkage analysis of hydroxyproline-poor glycoprotein from Phaseolus vulgaris. Plant Physiol. 65:557-61(1979).

Brown, R. G., Kimmins, W. C. Alkaline degradation of hydroxyproline-poor and hydroxyproline-rich glycoproteins from Phaseolus vulgaris. Plant Physiol. 63 (Suppl.), 32(1979b).

Brown, R. G., Kimmins, W. C. Protein-polysaccharide linkages in glycoproteins from Phaseolus vulgaris. Phytochem. 17:29-33(1978).

Brown, R. G., Kimmins, W. C., Lindberg, B. Structural studies of glycoproteins from Phaseolus vulgaris. Acta Chem. Scand. B. 29:843-52(1975).

Brysk, M. M., Chrispeels, M. J. Isolation and partial characterization of a hydroxyproline-rich cell wall glycoprotein and its cytoplasmic precursor. Biochim. Biophys. Acta 257:421-32(1972).

Cleland, R. Hydroxyproline formation and its relation to auxin-induced cell elongation in the Avena coleoptile. Plant Physiol. 43:1625-30(1968).

Esquerre-Tugaye, M. T., Lafitte, C., Mazau, D., Toppan, A., Touze, A. Cell surfaces in plant-microorganism interactions. II. Evidence for the accumulation of hydroxyproline-rich glycoproteins in the cell walls of diseased plants as a defense mechanism. Plant Physiol. 64:320-6(1979).

Fujii, T. Effects of IAA on oxygen-sensitive growth and on hydroxyproline protein level in cell wall. Plant Cell Physiol. 19:927-33(1978).

Kapitany, R. A., Zebrowski, E. J. A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal. Biochem. 56:361-9(1973).

Lamport, D. T. A. Personal communications, 1979.

Lamport, D. T. A. Cell Wall Carbohydrates in Relation to Structure and Function. In: Frontiers of Plant Tissue Culture, Proceedings of the 4th International Congress of Plant Tissue Culture, p. 235-44, Thorpe, T. A., ed., Calgary, Alberta: Univ. of Calgary 1978.

Lamport, D. T. A. Cell wall metabolism. Ann. Rev. Plant Physiol. 21:235-70(1970).

Lamport, D. T. A. The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzyme degradation of primary cell walls. Biochem. 8:1155-63(1969).

Lamport, D. T. A. The protein component of primary cell wall. Adv. Bot. Res. 2:151-218(1965).

Lamport, D. T. A. Oxygen fixation into hydroxproline of plant cell wall protein. J. Biol. Chem. 238:1438-40(1963).

Lüdtke, V. M., Lieflander, M. Quantitative Bestimmung der Aminosauren im Wandprotein von pflanzlichen Faserzellen. Holzforschung 24:33-7(1970).

Miller, D. H., Lamport, D. T. A., Miller, M. Hydroxyproline heterooligosaccharides in Chlamydomonas. Science 176:918-20(1972).

Roberts, K., Northcote, D. H. Hydroxyproline: Observations on its chemical and autoradiographical localization in plant cell wall proteins. Planta 107:43-51(1972).

Robinson, K. W. Study of the regenerated cell walls of Douglas-fir protoplasts. I. Carbohydrate and protein metabolism in the cell wall.

Sadava, D., Chrispeels, M. J. Synthesis and secretion of cell wall glycoprotein in carrot root disks. In: Biochemistry of Wounded Tissue, p. 85-102, Kahl, G., ed., Berlin, New York: Walter de Gruyter 1978.

Sadava, D., Chrispeels, M. J. Hydroxyproline biosynthesis in plant cells. Peptidyl proline hydroxylase from carrot disks. Biochim. Biophys. Acta 227:278-87(1971).

Scurfield, V. M., Nicholls, P. W. Amino-acid composition of wood proteins. J. Exp. Bot. 21:857-68(1970).

Smith, M. H. Molecular weight of proteins and some other materials. In: CRC Handbook of Biochemistry. Selected data for molecular biology, p. C3, Sober, H. A., ed., Cleveland, OH, The Chemical Rubber Co. 1970.

Steward, F. C., Israel, H. W., Salpeter, M. M. The labeling of cultured cells of Acer with (^{14}C) proline and its significance. J. Cell. Biol. 60:695-701(1974).

Thompson, E. W., Preston, R. D. Proteins in the cell wall of some green algae. Nature 213:684-5(1967).

Willison, J. H. M. Synthesis of cell walls by higher plant protoplasts. In: Microbial and Plant Protoplasts, p. 283-97, Peberdy, J. F., et al., eds., London, New York, San Francisco, Academic 1976.

CONCLUSIONS

This research showed that the regenerating cell wall material on the membrane surface of Douglas-fir protoplasts, derived from suspension cells, differed in composition from a normal cell wall. Subsequent to enzymatic wall removal neither the reappearance of cellulose nor mitosis was observed for these protoplasts over a six-day period in culture.

The uptake of proline and glucose in the regenerating wall was monitored and revealed that a protein component was formed prior to carbohydrate deposition. The protein was characterized as a high molecular weight glycoprotein rich in glycine, alanine, glucose, and galactose and shown to be similar to a wound-induced cell wall glycoprotein of Phaseolus vulgaris (17). The carbohydrate deposited in the regenerating cell wall was rich in glucose, although mannose was the first sugar to be incorporated after enzyme removal. Both x-ray diffraction analysis and polysaccharide acetolysis failed to demonstrate the presence of cellulose in the regenerating cell wall. However, histochemical analysis revealed the probable presence of callose, a β-(1,3)-linked glucan, usually also synthesized and deposited in response to wounding.

SUGGESTIONS FOR FUTURE WORK

The research largely involved quantitatively and semiquantitatively describing the processes involved in the regeneration of the cell wall from Douglas-fir protoplasts. These results combined with others (18) suggest that wound-related stress results in the generation of a wall-like structure. Further experimentation, though, is required to more completely describe the overall response of the cells to enzyme treatment. For instance, protoplasts directly derived from Douglas-fir explants should be isolated and used to determine whether the general woundlike response observed in this present study is due to the use of cultured cells or is a fundamental reaction to enzymatic cell wall removal. In addition, future studies should extend beyond the six-day period of wall regeneration used in the present work in order to further monitor the process.

More information is also needed on the nature of the glycosidic linkages of both the general wall polysaccharides and glycoprotein carbohydrates deposited during cell wall regeneration. Employing well-characterized techniques such as methylation analysis (19) at various wall regeneration times may provide a clearer understanding of the time-course development of sugar linkages.

Autoradiographical techniques may provide insight into the spatial placement of the cell wall protein. For instance, ^{14}C-proline incorporation in the wall may be found to specifically locate in proximity to some cellular organelle or wall structure which could be useful in ascertaining the protein's role.

GLOSSARY

Annealing - High temperature treatment to facilitate molecular alignment in solids.

Callose - A β-(1,3)-linked glucan synthesized in higher plants. Although it can be found in specialized plant parts, it is usually identified with wounding.

Cellobiose - A disaccharide of D-glucose units linked with a β-(1,4)-glycosidic bond; the repeating unit of cellulose.

Cellulysin - A fungal enzyme preparation from Trichoderma viride principally exhibiting cellulase activity along with some hemicellulase, protease, and amylase action.

Curdlan - A linear β-(1,3)-glucan synthesized by bacterial cultures.

Laminaribiose - A disaccharide of D-glucose units linked with a β-(1,3)-glycosidic bond and the repeating unit of callose.

Macerase - A crude enzyme preparation of Rhizopus sp. having pectinase as its principal activity.

Protoplasts - Cells which lack a cell wall. As used in this study it applies to plant cells which have had the cell wall removed through the action of fungal enzymes.

Regenerated cell wall - The material found external to the plasma membrane of cultured protoplasts after removal of enzymes used to degrade the cell wall.

Suspension cells - Cells in a liquid medium.

ABBREVIATIONS

Ci - Curie

DMSO - Dimethyl sulfoxide

GLC - Gas-liquid chromatography

GPC - Gel permeation chromatography

IAA - Indole acetic acid

MAE - Mild alkali extraction

M-S - Murashige-Skoog (medium)

NAA - Naphthalene acetic acid

PAS - Periodic acid - Schiff's reagent

SEM - Scanning electron microscopy

TEM - Transmission electron microscopy

TLC - Thin-layer chromatography

(U-^{14}C) - Uniformly distributed ^{14}C

WEP - Wall elimination product

ACKNOWLEDGMENTS

The author acknowledges the assistance of others whose help and support has made this thesis possible. I especially appreciate the insight and encouragement of Dr. Morris A. Johnson, my major advisor, and my Thesis Advisory Committee consisting of Doctors D. J. Durzan, R. A. Parham, and L. L. Winton. Thanks to Dr. Russell L. Steere of the U.S. Department of Agriculture, Beltsville, MD, for his abundant assistance during the initial direction of this thesis involving freeze-etch technology.

The expert technical assistance of L. G. Borchardt, J. A. Carlson, R. P. Feirer, H. M. Kaustinen, and S. A. Verhagen was invaluable in quantitatively describing processes elucidated in this thesis. The financial support of The Institute of Paper Chemistry is also much appreciated.

Finally, I would like to thank my wife, Carol, who faithfully encouraged my academic efforts through constant love, edification, and prayer.

LITERATURE CITED

1. Preston, R. D. The physical biology of plant cell walls. London, Chapman and Hall, 1974, 491 p.

2. Delmer, D. P. The biosynthesis of cellulose and other plant cell polysaccharides. Recent Adv. Phytochem. 11:45-77(1977).

3. Willison, J. H. M. and Brown, J. M. An examination of the developing cotton fibers: Wall and plasmalemma. Protoplasma 92:21-41(1977).

4. Shore, G. and Maclachlan, G. A. The site of cellulose synthesis. J. Cell. Biol. 64:557-71(1975).

5. Heath, I. B. A unified hypothesis for the role of membrane bound enzyme complexes and microtubules in plant cell wall synthesis. J. Theor. Biol. 48: 445-9(1974).

6. Elbein, A. D. and Forsee, W. T. Studies on the Biosynthesis of Cellulose. In Biogenesis of Plant Cell Wall Polysaccharides. New York, Academic Press, Inc., 1973. p. 259.

7. Northcote, D. H. Chemistry of the plant cell wall. Ann. Rev. Plant Physiol. 23:113-132(1972).

8. Colvin, J. R. and Preston, R. D. The structure and biosynthesis of cellulose. CRC Critical Rev. in Macromolecular Sci. 1:47-81(1972).

9. Shafizadeh, F. and McGinnis, G. D. Morphology and biogenesis of cellulose and plant cell-walls. Adv. Carbohyd. Chem. 26:297-349(1971).

10. Preston, R. D. and Goodman, R. N. Structural aspects of cellulose microfibril biosynthesis. J. Royal Microsc. Soc. 88:513-27(1968).

11. Mueller, S. C., Brown, R. M., and Scott, T. K. Cellulose microfibrils: nascent stages of synthesis in a higher plant cell. Science 194:949-51(1976).

12. Willison, J. H. M. and Cocking, E. C. The production of microfibrils at the surface of isolated tomato-fruit protoplasts. Protoplasma 75:397-403(1972).

13. Robenek, H. and Peveling, E. Ultrastructure of cell wall regeneration of isolated protoplasts of Skimmia japonica Thunb. Planta 136:133-45(1977).

14. Grout, B. W. W. Cellulose microfibril deposition at the plasmalemma surface of regenerating tobacco mesophyll protoplasts: A deep-etch study. Planta 123: 275-82(1975).

15. Willison, J. H. M. Synthesis of Cell Wall by Higher Plant Protoplasts. In Microbial and Plant Protoplasts. New York, Academic Press, Inc., 1976. p. 283.

16. Robinson, K. W. Unpublished work, 1977.

17. Brown, R. G. and Kimmins, W. C. Protein-polysaccharide linkages in glycoproteins from Phaseolus vulgaris. Phytochem. 17:29-33(1978).

18. Takeuchi, Y. and Komamine, A. Composition of the cell wall formed by protoplasts isolated from cell suspension cultures of Vinca rosea. Planta 140:227-32(1978).

19. Bjorndal, H., Lindberg, B. and Svensson, S. Mass spectrometry of partially methylated alditol acetates. Carbohyd. Res. 5:433-40(1967).

20. Sadava, D. and Chrispeels, M. J. Hydroxyproline biosynthesis in plant cells. Peptidyl proline hydrolase from carrot disks. Biochim. Biophys. Acta 227:278-87(1971).

21. Davis, B. J. Disc electrophoresis - II. Method and application to human serum proteins. Annals N.Y. Acad. Sci. 121:404-27(1964).

22. Kapitany, R. A. and Zebrowski, E. J. A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal. Biochem. 56:361-9(1973).

23. Brewer, J. M. Electrophoresis. In Experimental Techniques in Biochemistry. p. 128. Englewood Cliffs, New Jersey, Prentice-Hall, 1974.

24. Chervenka, C. H. A manual of methods for the analytical ultracentrifuge, Palo Alto, CA, Beckman Instruments, 1969. 100 p.

25. Smith, M. H. Molecular Weight of Proteins and Some Other Materials. In CRC Handbook of Biochemistry. p. C3-C28. Cleveland, OH, The Chemical Rubber Co., 1970.

26. Beckman 119CL Instruction Manual. Section 4. Sample Preparation, p. 1. Palo Alto, CA, Beckman Instruments. 1977.

27. Farjanel, J., Cabocel, J. P., Rattner, A., and Frey, J. Factors altering optimum liquid scintillation counting of ^{3}H of ^{14}C amino acids. Int. J. Appl. Rad. and Isotopes 30:537-41(1979).

28. Braun, G. α-Cellobiose Octaacetate. In Organic synthesis. Vol. 2. p. 124. New York, John Wiley, 1943.

29. Harada, T. Production, properties, and application of curdlan. ACS Symp. Ser. 45:265-83(1977).

APPENDIX I

CULTURE MEDIA

Cell suspension and protoplast cultures were both grown on variations of the same medium. The specific formulations of those media are displayed in Table I shown on the next page.

TABLE I

Composition of suspension and protoplast culture media.

Component	Normal Suspension Cell Medium, mg/L	Protoplast Medium, mg/L
NH_4NO_3	825	82.5
KNO_3	950	95.0
$MgSO_4 \cdot 7H_2O$	185	18.5
KH_2PO_4	85	8.5
$CaCl_2 \cdot 2H_2O$	220	22.0
$MnSO_4 \cdot 4H_2O$	8.45[c]	1.12
$ZnSO_4 \cdot 7H_2O$	5.3[c]	0.43
H_3BO_4	3.1	0.31
KI	0.42	0.042
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.0125
$CoCl_2 \cdot 6H_2O$	0.0125	0.00125
$CuSO_4 \cdot 5H_2O$	0.0125	0.00125
$FeSO_4 \cdot 7H_2O$	13.9	1.39
$Na_2 \cdot EDTA \cdot 2H_2O$	18.6	1.86
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$[a]	--	500
L-Ascorbic acid[a]	--	180
Nicotinic acid	0.25	0.025
Thiamine · HCl	0.05	0.005
Pyridoxine · HCl	0.25	0.025
Myo-inositol	50	5.0
α-Naphthaleneacetic acid	2.0	0.2
Sucrose	15,000	5,000
Citric acid[a]	--	3,761
Na_2HPO_4	--	1,625
Mannitol	--	96,000
pH	5.8 ± 0.1[b]	5.8 ± 0.1

[a]Aids in proline hydroxylation (20).
[b]Adjusted with 1% KOH or 1N HCl before autoclaving.
[c]Concentrations of these salts taken from Brown, C., and R. Lawrence, For. Sci. 14:62-4(1968).

APPENDIX II

LIQUID SCINTILLATION COUNTING

Radioactivity counting was performed at ± 2% statistical error in a Beckman DPM-100 liquid scintillation counter using a full window (^{3}H + ^{14}C) and Cocktail D [10 g 2,5-diphenyloxazole (PPO) and 200 g naphthalene made up to 2 liters with Baker AR-grade dioxane]. Absolute cocktail scintillation efficiency based on the use of ^{14}C standards was 93.6%.

Curves were constructed to determine the quench characteristics of materials similar to those counted. A small amount (< 100,000 cpm) of ^{14}C-proline was added to a 10mL solution of Cocktail D. Quenching chemicals were incrementally added at small volumes. The resulting cpm and corresponding efficiencies were determined. The quenching which occurred upon addition of ferric chloride or a 1N lithium-citrate buffer (to which a few drops of methanol had been added) gave the efficiency characteristics seen in Fig. 1. Beyond corrections for quenching in Fig. 1, other adjustments from the detected cm were required for normal background. In addition, samples (2 mL) collected from the amino acid analyzer in lithium-citrate buffer required a further baseline correction.

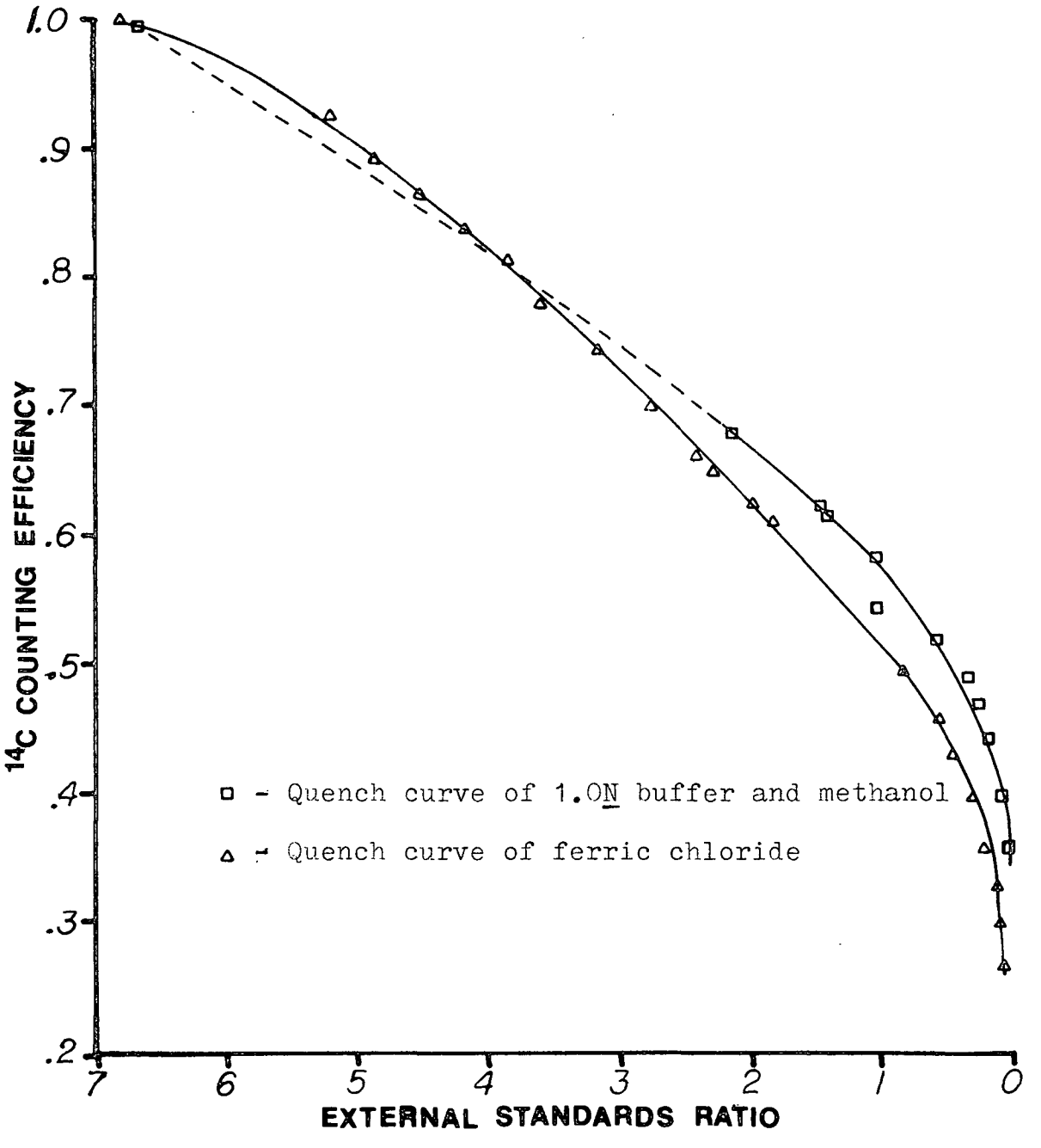


Figure 1. ^{14}C-Efficiency curves for various quenching agents in Cocktail D.

APPENDIX III

CONTROLS IN RADIOISOTOPE EXPERIMENTS

Controls were used to establish the relative importance of both cell wall surface sorption and cytological debris on the uptake of ^{14}C-proline and ^{14}C-glucose. The results from the control designed to assess the extent of surface adsorption of radioactivity onto the cell walls as shown in Table II were found to be significant and were used to correct the data presented earlier. For a batch of protoplasts boiled for 10 minutes immediately following the addition of either ^{14}C-proline or ^{14}C-glucose, adsorption processes were observed to effect an average uptake of 84 and 46 cpm, respectively. These average values were subtracted from the radioactivity detected in the cell wall samples from living tissue as an adjustment for this effect.

The results from the second control which was used to assess the contribution of cell debris suggest that this effect was not a significant factor in the cell wall uptake of radioactivity. The experiment was largely influenced by widespread fungal contamination which was first visually detected at two days. Samples representing up to and including 2 days exhibited on average radioactive uptake of 75 and 28 cpm for proline and glucose, respectively. These values are slightly less than the averages shown in Table II and likely reflect the influence of similar adsorption effects. Therefore, in summary, the effect of cell debris in cell wall radioactivity uptake is apparently minimal.

TABLE II

Radioactivity in cell wall control from the adsorptivity of ^{14}C-proline and ^{14}C-glucose.

Time, days	Sample: Proline A	Proline B	Glucose A	Glucose B
0	123	121	74	73
0.5	46	116	31	90
1	31	87	40	N.D.[a]
2	81	77	19	N.D.
3	40	174	37	41
4	50	133	16	19
5	12	132	68	N.D.
6	65	55	33	66
Average	84		46	
Standard deviation	45		25	

[a]N.D. - not determined.

APPENDIX V

DETERMINATION OF MOLECULAR WEIGHT

The sedimentation and diffusion coefficients were determined for a MAE glycoprotein representing 6 days cell wall regeneration. The determination was made by J. A. Carlson, and the log sheets are on file at The Institute of Paper Chemistry (Beckman Model E, Log Sheet No. 1901).

The sample was dissolved in a 0.02M NaOH-glycine buffer, pH 9, and any insoluble material was removed by preliminary centrifugation. At 25°C the sedimentation and diffusion of the sample was observed using schlieren optics. The plot from which the sedimentation coefficient was calculated is shown in Fig. 3.

Due to the low concentration of the sample in solution, the apparent diffusion coefficient, D_A, was determined using schlieren optics and employing the area height method (24). The calculation is shown in Eq. (1):

$$D_A = \frac{(A_{sch})^2}{\left(\frac{dc}{dr}\right)^2} \cdot \frac{1}{4\pi t} = \left(\frac{0.13}{0.25}\right)^2 \cdot \frac{1}{4\pi \cdot 780} = 2.8 \times 10^{-7} \qquad (1)$$

where D_A = apparent diffusion coefficient

A_{sch} = peak area by schlieren optics

c = concentration

r = radial distance

t = time

π = 3.14159

The sedimentation and diffusion constants were used to determine the molecular weight of the sample. The partial specific volume of the protein was estimated at 0.74, an average value for structural proteins (25). The calculations were made according to Eq. (2):

$$M = \frac{RTs}{D_A\,(1 - v\rho)} = \frac{(8.314 \times 10^{7})\,(2.98 \times 10^{2})\,(8.3 \times 10^{-13})}{(2.8 \times 10^{-7})\,(0.26)} = 282{,}470 \qquad (2)$$

where M = molecular weight,

R = universal gas constant,

T = absolute temperature,

s = sedimentation coefficient,

D_A = apparent diffusion coefficient,

$\bar{v}$ = partial specific volume,

ρ = solvent density.

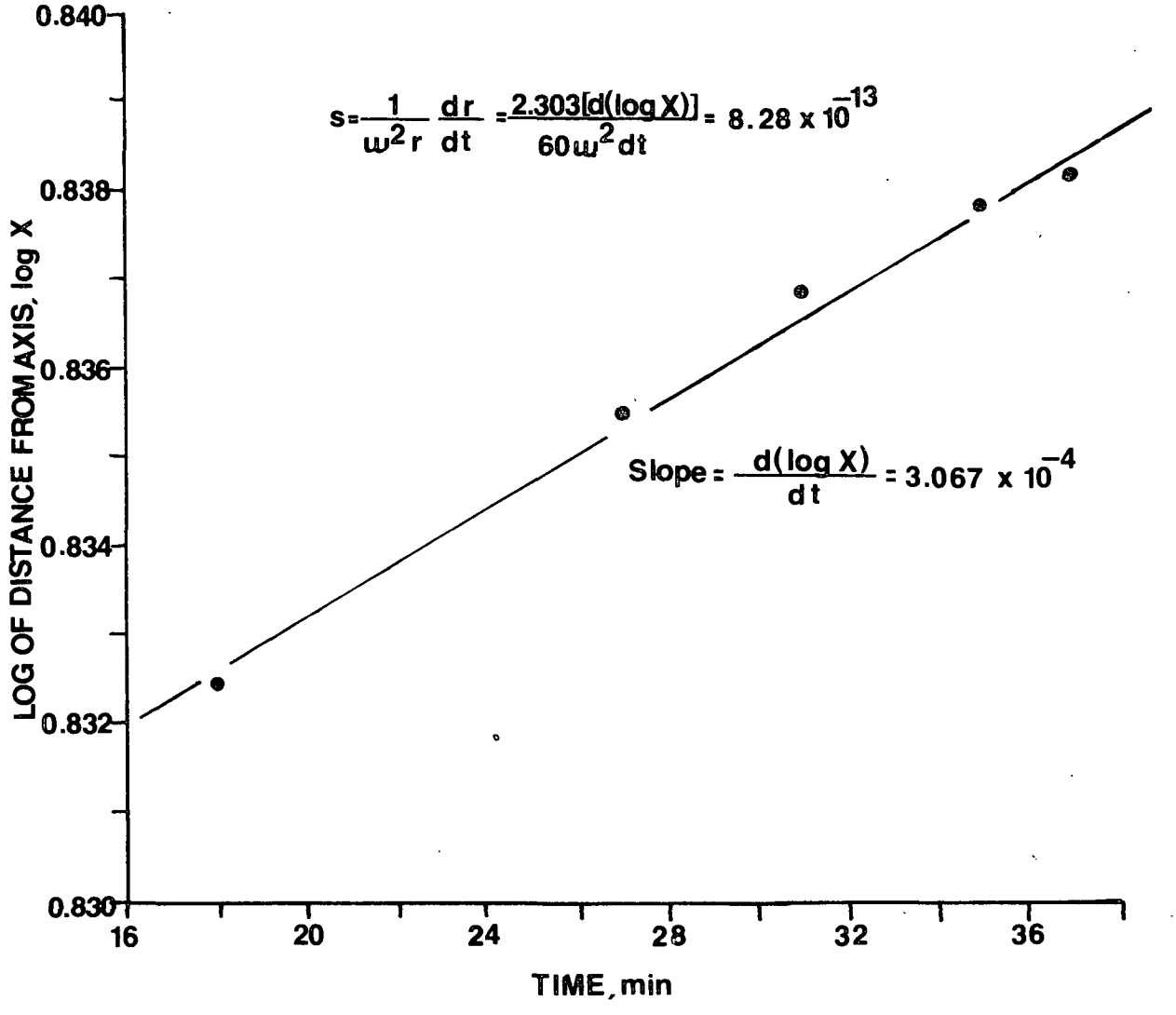


Figure 3. Sedimentation characteristics of MAE protein.

APPENDIX VI

PROTEIN HYDROLYSIS AND AMINO ACID ANALYSIS

Proteins from both regenerated cell wall samples and cell wall extractions were analyzed for amino acid composition. Sample size was usually less than 0.5 mg dry weight and required special considerations in experimental isolation.

Samples were hydrolyzed in 1 mL of double-distilled constant-boiling HCl to which 2-mercaptoethanol [at a ratio of 2000:1 (HCl:mercaptoethanol)] had been added. Samples were placed in hydrolysis tubes and evacuated by aspiration three times. Each time the tubes were purged with purified nitrogen gas while sitting in an acetone-solid CO_2 bath. The tubes were transferred to a 110 ± 0.5°C tube heater for 22 hours. If humins formed, they were removed by centrifugation. The hydrolyzate was transferred to a wide-mouth vial and dried *in vacuo* over NaOH pellets at 60°C (26). The sample was then submitted for amino acid analysis where it was dissolved in 400 µL of 0.15*N* lithium-citrate buffer, pH 2.2. A standard volume (300 µL) was injected onto the ion exchange column of a Beckman 119CL amino acid analyzer. Analyses were performed by R. P. Feirer, and the chromatograms are on file at The Institute of Paper Chemistry.

Recovery of the amino acids from protein hydrolyzates was tested and found to result in some yield losses where parallel sets of crystallized bovine serum albumin samples were acid hydrolyzed. One set was dried *in vacuo* over NaOH pellets as before, and the other was diluted with buffer to make a pH 2.2 solution. Any humin present was removed by centrifugation. A portion of the pH 2.2 solution was injected directly into the analyzer, while the dried sample was redissolved in pH 2.2 lithium-citrate buffer. The results of both approaches are displayed in Table III.

TABLE III

Yield losses for amino acid hydrolyzates of bovine serum albumin isolated by different techniques.

	% Yield	
	Redissolved Sample	Never-dried Sample[a]
	53.9	96.4
	50.0	95.1
		103.0
		108.0
Average	52.5	100.6
Standard deviation	2.4	6.0

[a]Analyses were performed by R. P. Feirer.

Only the method involving redissolution of the hydrolyzate resulted in significantly lower yields and some variability in amino acid composition. Both of these anomalies can probably be accounted for in the irreversible adsorption of the amino acids on the surfaces of the glass vial in which the samples were dried (27). Farjanel and coworkers (27) demonstrated that up to 60% of ^{14}C-hydroxyproline is not desorbed after once being dried in a vial. They also observed that some amino acids differentially compete for the adsorption sites, resulting in composition variability.

For the present study, no attempt has been made to modify the data presented earlier to reflect the yield losses shown in Table III. The aim of this discussion is to point out some possible incongruities in the data previously presented and to suggest some limitations of a commonly used method.

APPENDIX VII

LIGHT MICROSCOPY STUDY

Suspension cells and protoplasts with regenerating cell walls were embedded and sectioned for UV fluorescence microscopy. Shown in Fig. 4 is a cross section of a population of newly formed protoplasts stained with Toluidine Blue O. In the cell suspension sample, the cell wall region autofluoresced when irradiated as shown in Fig. 5, suggesting the presence of lignin. The regenerated cell wall sample, however, failed to fluoresce under similar conditions.

Both cell populations were then reacted with 0.01% aniline blue in water. Only the regenerated wall group fluoresced, indicating the probable presence of callose; an example is shown in Fig. 6.

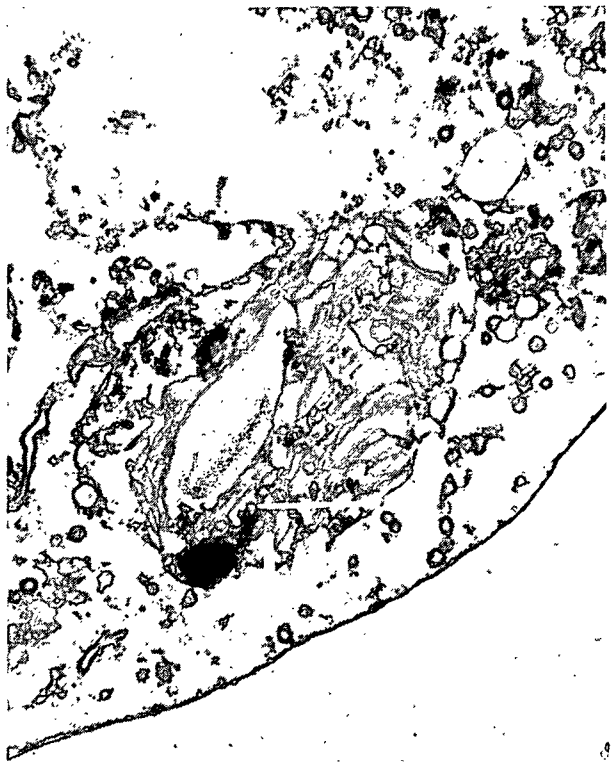


Figure 4. Cross section of protoplast population stained to enhance cell contents.

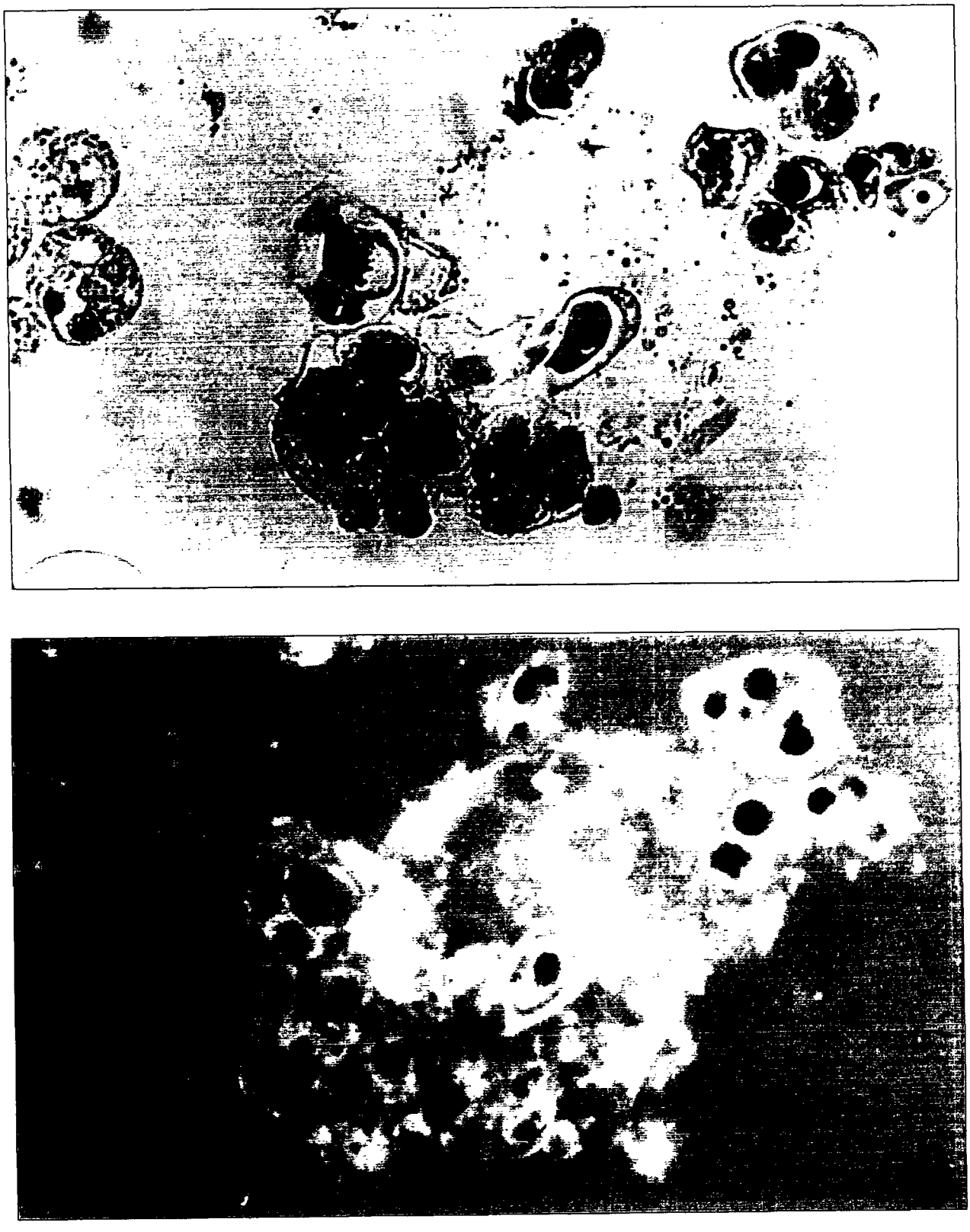


Figure 5. Light micrographs of cross sections of Douglas-fir suspension cells. Suspension cells are shown under visible light (top) and UV fluorescence (bottom) suggesting the presence of lignin (320X).

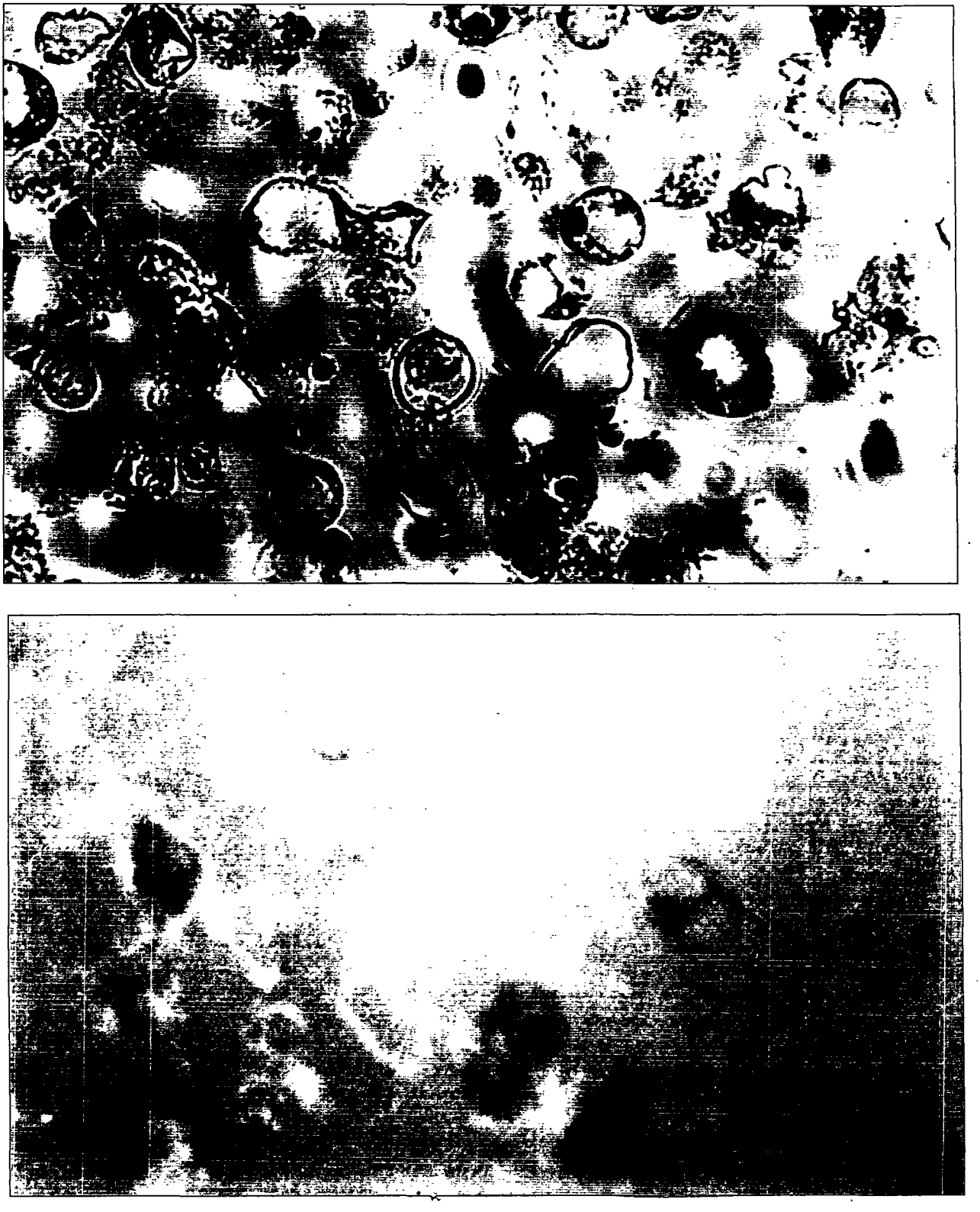


Figure 6. Cross sections of Douglas-fir protoplasts after 6 days cell wall regeneration. Upon reaction with aniline blue, a light (top) and UV photomicrograph (bottom) indicate the probable presence of callose in the regenerating cell wall region (320X). Debris was caused by fixation procedures.

APPENDIX VIII

POLYSACCHARIDE ACETOLYSIS ANALYSIS

The regenerated cell wall was evaluated for the presence of cellulose by a modification of Braun's method (28). Octaacetyl-α-cellobiose octaacetate is the stable and dominant acetolysis product of cellulose but was not detected in GLC chromatograms of a regenerated cell wall acetolyzate. The minor hydrolysis product of cellulose or of any other glucose-containing polysaccharide, glucose pentaacetate, was found in abundance.

Three samples were used as controls to monitor the validity of the procedure. They included anhydrous D-glucose, curdlan (29), and filter paper. The last two samples were used to demonstrate the results expected from callose and cellulose, respectively. All samples including a composite regenerated cell wall sample (representing 0 to 5 days) were separately reacted with acetic anhydride and sulfuric acid according to Braun (28) for 6 days. After the reactions were quenched in cold water, the samples were extracted three times with chloroform. The chloroform was evaporated and samples submitted to the Analytical Department of The Institute of Paper Chemistry where the samples were subjected to gas-liquid chromatography on a Packard Model 417 system using inositol hexaacetate as an internal standard. The chromatograms of the samples are displayed in Fig. 7.

As expected, the filter paper acetolyzate yielded predominantly octaacetyl-α-cellobiose and small quantities of glucose pentaacetate. The curdlan yielded a majority of the acetylated glucose monomer while exhibiting an appreciable concentration of the disaccharide, laminaribiose, which was similar in coelution time with cellobiose octaacetate. Therefore, the results of the acetolysis experiment confirmed that the method was indeed sensitive for the diagnostic detection of cellulose and that none was present in the regenerated cell wall.

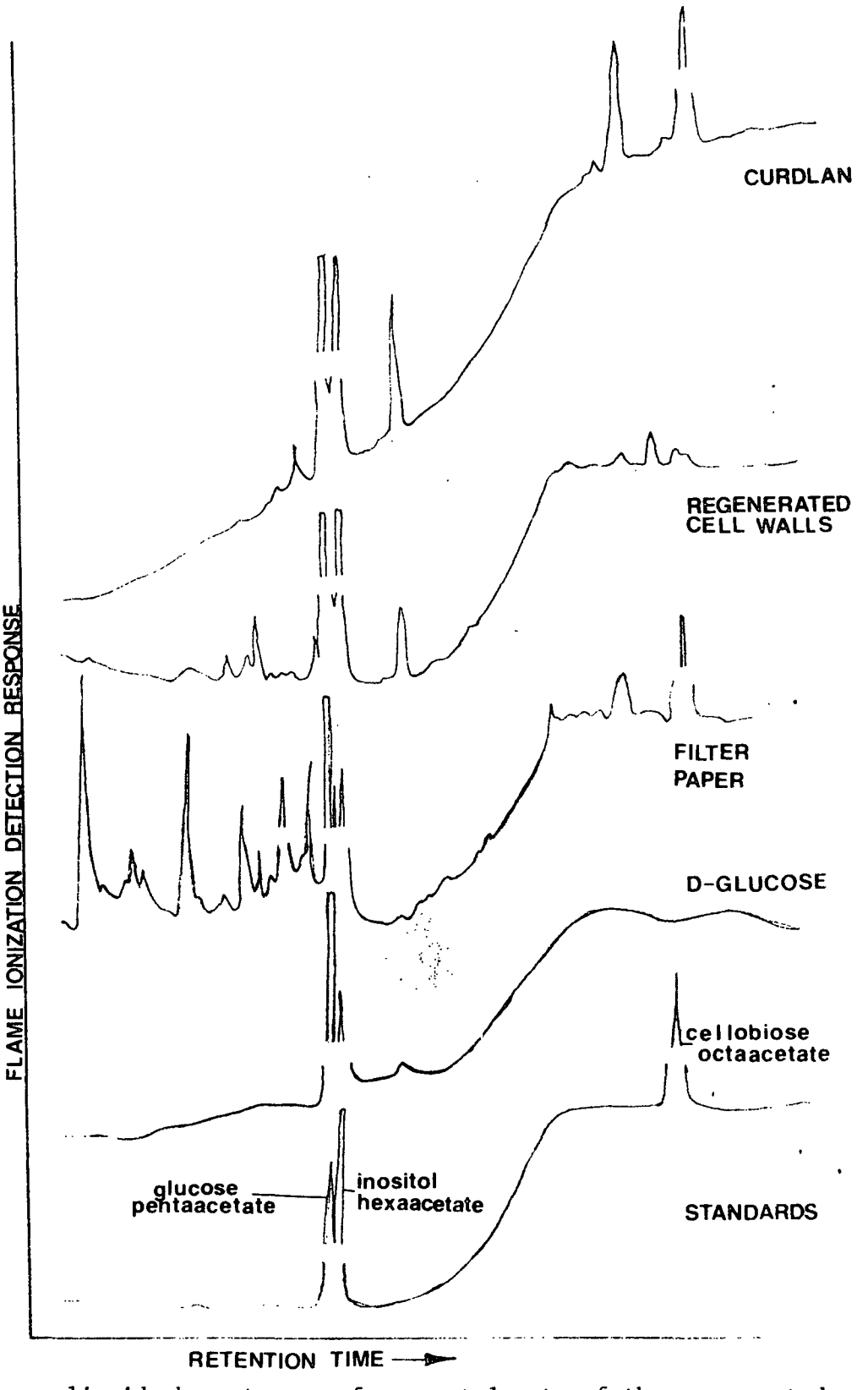


Figure 7. A gas-liquid chromatogram of an acetolyzate of the regenerated cell wall compared to acetolyzates of known samples. The regenerated wall material was shown to be devoid of cellulose through the absence of cellobiose octaacetate.