CORRELATIVE STUDIES ON CELL WALL ENZYMES AND GROWTH

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ABSTRACT

CORRELATIVE STUDIES ON CELL WALL
ENZYMES AND GROWTH

By

Allen Ketcik Murray

A comparison has been made of the growth rate of etiolated pea stem tissue with the level of certain enzymatic activities of cell walls isolated from the tissue. If cell wall hydrolytic enzymes are involved in extension growth, one would expect a correlation between hydrolytic activity of the cell walls and the growth rate of the tissue from which the walls were prepared.

Sections were taken from 0 to 5 mm, 6 to 10 mm, and 11 to 15 mm below the apical hook of 3 and 1/2 day old seedlings. The first region is rapidly elongating, the second is elongating only slightly, and the third has essentially stopped elongating; the relative growth rates being respectively 100:15:2. Cell walls were prepared from the sections by grinding the tissue sections in glycerol with glass beads as well as by an aqueous isolation method. The activities of α-galactosidase, β-glucosidase, acid phosphatase, and β-galactosidase were

investigated using the p-nitrophenyl esters as substrates. The β-glucosidase activity is considered to be the most meaningful of the activities studied, for the purposes of this work, since most of it was associated with the cell wall. The relative activities, expressed as specific activity (units of activity/mg of wall), from the 0 to 5 mm, 6 to 10 mm, and 11 to 15 mm sections were 100, 24, and 23 respectively for the glycerol prepared walls and 100, 42, and 23 respectively for the aqueous cell wall preparations. Thus there is a reasonably good correlation between the growth rate of the sections and the specific activity of the hydrolytic enzymes associated with cell walls from those sections.

The relative number of cells in each of the stem regions studied was determined by counting cells in logitudinal and cross sections prepared from the stem regions. The relative number of cells was found to be 100, 35, and 26 respectively for the 0 to 5 mm, 6 to 10 mm, and 11 to 15 mm regions. When the total amount of enzyme activity per section was divided by the relative number of cells in that section, the activity per cell was found to be a constant. Thus, for the physiological ages of tissue studied in this work, the total amount of activity per cell does not change despite the large changes in growth rates and cell size.

The cell wall β-glucosidase activity is inhibited by a soluble, heat-stable substance, which was not identified.

Isolated cell walls were found to incorporate a small amount of radioactivity when p-nitrophenyl-β-D-($U^{14}C$)-glucopyranoside was used as the substrate. This radioactivity was not removed by washings sufficiently thorough to remove the radioactivity from zero time or boiled wall controls. The radioactivity was removed from the walls by extraction with 0.5% ammonium oxalate-oxalic acid at 90° for 24 hours. The labeled compounds removed from the cell walls by this procedure were not identified.

The present work shows that there is a correlation between the specific activity of β-glucosidase associated with isolated cell walls and the growth rate of the tissue from which the cell walls were isolated. In addition, the amount of cell wall β-glucosidase activity per cell is constant and is independent of cell size.

CORRELATIVE STUDIES ON CELL WALL ENZYMES AND GROWTH

By

Allen Ketcik Murray

A THESIS

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To my parents.

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Acid Phosphatase: Orthophosphoric monoester phosphohydrolase (3.1.3.2)

Ascorbate Oxidase: L-ascorbate:oxygen oxidoreductase (1.10.3.3)

ATPase: ATP phosphohydrolase (3.6.1.3)

α-Glucosidase: α-D-glucoside glucohydrolase (3.2.1.20)

β-Glucosidase: β-D-glucoside glucohydrolase (3.2.1.21)

α-Galactosidase: α-D-galactoside galactohydrolase (3.2.1.22)

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Inorganic Pyrophosphatase: pyrophosphate phosphohydrolase (3.6.1.1)

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5'-Nucleotidase: 5'-ribonucleotide phosphohydrolase (3.1.3.5)

Pectinase: poly-α-1,4-galacturonide glycanohydrolase (3.2.1.15)

Pectin Methylesterase: pectin pectyl-hydrolase (3.1.1.11)

UDPG Pyrophosphorylase: UTP:α-D-glucose-1-phosphate uridylyltransferase (2.7.7.9)

LIST OF ABBREVIATIONS

Acetyl-D-glucosyl bromide: tetra-0-acetyl-α-D-glucopyranosyl bromide

ATP: adenosine-5'-triphosphate

BSTFA: Bis(trimethylsilyl)trifluoracetamide

CB: cellobiose

CPM: counts per minute

CTP: cytidine-5'-triphosphate

DNA: deoxyribonucleic acid

2,4 -D: 2,4-dichlorophenoxyacetic acid

EDTA: ethylenediamine tetraacetic acid

GLC: gas liquid chromatography

GTP: guanosine-5'-triphosphate

IAA: indole-3-acetic acid

MES: 2-(N-morpholeno)ethane sulfonic acid

p-nitrophenyl glucose: p-nitrophenyl-β-D-glucopyranoside

pNP-β-D-gluc: p-nitrophenyl-β-D-glucopyranoside

p-nitropheny-β-D-glucose tetraacetate: p-nitrophenyl-tetra-0-acetyl-β-D-glucopyranoside

pNP: p-nitrophenol

PAN: peroxyacetyl nitrile

RNA: ribonucleic acid

TLC: thin layer chromatography

TMS: trimethylsilyl

TMS-p-nitrophenyl-β-D-glucose: p-nitrophenyl-tetra-0-trimethylsilyl-β-D-glucopyranoside

Tris: tris(hydroxymethyl)aminoethane

UTP: uridine-5'-triphosphate

INTRODUCTION

The purpose of this literature review is to provide background for the experimental work. There are many good reviews of cell wall structure (1, 75, 63, 77, 82), and cell wall biosynthesis and formation (63, 2, 70). In addition, cell wall metabolism has been reviewed (50) as have the effects of auxin on cell wall metabolism (74) as well as cell wall extension (13) and growth (24).

Of major concern to this work is the primary cell wall--the wall that is present during extension of the cell. The primary cell walls of higher plants contain cellulosic microfibrils embedded in a matrix of non-cellulosic polysaccharides, pectic substances, and lesser amounts of protein. The major part of the primary cell wall is made up of non-cellulosic polysaccharides, the hemi-celluloses. The major monosaccharide constituents obtained by hydrolysis of the cell wall polysaccharides are D-glucose, D-mannose, D-galactose, D-xylose, L-arabanose, D-glucuronic acid, D-galacturonic acid, L-rhamnose, L-fucose, and sometimes L-galactose (2). A typical chemical composition of isolated cell walls follows: cellulose 27.4%, pectin 8.4%, lignin 5.4%, protein 5.1%, phosphorous 0.07%, sulfur 0.06%, nitrogen 0.8%,

ash 1.3%, with the remainder being made up presumably of hemicelluloses (44). In addition to small amounts of protein and lipid, cell wall preparations contain small amounts of RNA, first observed by Kivilaan, et al. (44), and have even been reported to contain ribosomes (38).

Phethean et al. (71) found highly purified cell walls from three sources to contain RNA, which was released by alkaline hydrolysis or by ribonuclease. The base composition of this cell wall RNA is identical to that of ribosomal RNA. The cell wall RNA is 7% of the total RNA of the tissue and only 0.2% of the cell wall RNA reportedly can be accounted for as being due to bacterial contamination. These workers deliberately contaminated their cell wall preparation with RNA and found no increase in the amount of cell wall RNA after reisolation of the cell walls.

Subsequent work by Jervis and Hallaway (38) showed ribosomes to be released from highly purified cell walls of barley shoots by passing the cell wall suspension through a French press. The sedimentation coefficients and base composition of the cell wall ribosomes are identical to those of cytoplasmic ribosomes. The authors suggest the ribosomes isolated from cell walls were originally incorporated into the wall structure during the formation of primary cell wall either as free ribosomes or attached to elements of endoplasmic reticulum. No

implication is made that the ribosomes in the cell wall serve any function.

Cell Extension

Because the experimental work involves the comparison of walls with widely differing growth rates during cell extension, it is appropriate to look at the phenomenon of cell extension. From its origin to its maturity, a plant stem cell may elongate by a factor of 10 to 50 fold. According to Lockhart (53), during extension the thickness of the cell wall remains approximately constant and the density of the wall does not change significantly. From this, one may thus conclude that the elongation of stem or root cells results in a great deformation of the cell wall and at the same time requires a great deal of new cell wall material (53). This may well be the case in a normal healthy happily growing stem or root, but it has been shown that the elongation of excised sections of Avena coleoptile (7) and pea epicotyl sections (56) is not quantitatively dependent on the incorporation of new cell wall material. However, the deposition of new cell wall material, when studied during cell elongation, has been shown to occur uniformly over the entire length of the cell in Avena coleoptile sections (81). This is certainly an area which deserves further attention, if these reports are in fact, contradictory.

The ultrastructure of the cell walls of growing cells was thoroughly reviewed by Roelofsen (76) as was entire phenomenon of cell wall extension by Cleland (13). A high rate of cell elongation is confined to a relatively narrow region of stems and roots. It is generally accepted that this elongation takes place via an increase in the plasticity of the cell wall while a relatively high turgor pressure is maintained in the cell (29). Cell expansion takes place as a result of the cell wall yielding to the pressure (53). The fact that the cellulose microfibrils of the wall are movable with respect to each other has been taken to mean that they do not independently limit extension growth (1). A study of the microfibrillar orientation during growth of excised pea epicotyl sections has recently been published by Veen (91, 92).

Almost all of the work on cell extension has utilized the plant growth hormone indoleacetic acid, which may be unfortunate since IAA does many more things than to stimulate cell extension and further, it has not been shown that IAA is the endogenous auxin in plant tissue. In my view, it is unfortunate that most workers have complicated their studies on cell extension with the use of the hormone since our understanding of hormonal function is far from complete. In retrospect, simple decapitation as a means of lowering endogenous hormone levels may be preferable to supplying exogenous IAA.

Optimum elongation growth of excised pea epicotyl sections occurs in the presence of 0.1 mg/l IAA and 2% sucrose (91). Under these conditions there is a continuous deposition of transverse cellulose microfibrils before, during, and for a while, after longitudinal growth. The microfibrils deposited before the longitudinal growth are turned from a transverse to an oblique direction during growth and these microfibrils, which are turned oblique during growth, remain parallel to each other (91). This is not in agreement with the multinet-growth-theory of Roelofsen (75). Microfibrils deposited during growth are also turned, more or less, in an oblique direction depending on how much wall deformation takes place after their deposition. During longitudinal growth, the transverse microfibrils become obliquely orientated, which means that the transverse layer must, in fact, be a flat spiral. If apposition of new transverse cellulose microfibrils occurs at the inside of the cell wall, then the oblique layer which is observed with polarization optical techniques must be situated at the outside of the cell wall and adjacent to the middle lamella (91). It is interesting to note that the mechanical stretching of cell walls whose plasticity has been increased by the application of a growth substance to the tissue results in less reorientation of the cellulose microfibrils along the direction of

stretching than does the stretching of cell walls from tissue which was not treated with a growth substance (7).

Under the influence of high IAA (10 mg/l) and high sucrose concentration (8%) the orientation of newly deposited cellulose microfibrils is changed from transverse to longitudinal to the cell axis and as a result longitudinal growth is prevented and lateral growth or swelling takes place (92). That is to say, following elongation the microfibrils are orientated oblique to the longitudinal cell axis but in the case of the lateral swelling response the microfibrils are found to be oriented parallel to the longitudinal cell axis.

IAA induces elongation growth in excised coleoptile sections from Avena and other grasses which can be inhibited by Ca^{++} or Sr^{++} (15). In the case of the IAA stimulated elongation of excised barley tissue, sections treated with the same concentration of IAA (1 mg/l) were homogenized and a soluble enzyme preparation was isolated. The enzymatic activity from IAA treated sections liberated 50% more reducing sugars from barley hemicellulose and sclerotan (a fungal glucan from Sclerotinia libertiana which contains β-1,3 and β-1,6 linkages in a ratio of 4:1) but it liberated the same amount of reducing sugars from starch as controls (86). Treatment with 2,4-D (1 mg/l), which promotes elongation of excised pea epicotyl sections, caused a 22% increase in reducing sugar released from pea

hemicellulose by a crude soluble enzyme preparation from the treated sections. There was no effect on reducing sugars released from carboxymethyl cellulose or starch (86).

It has been reported that the elongation response to IAA in excised oat coleoptile sections can be duplicated by incubating the sections in a solution of a fungal β-1,3-glucanase for one hour (60). The enzyme was "slightly" contaminated with pectin methylesterase and cellulase. This treatment also resulted in an increase in cell wall extensibility as measured by stress-strain analysis of the treated sections after killing in boiling methanol. However, this response is less than striking and attempts to repeat it have been unsuccessful (12, 79). The failure to repeat this work may well be due to the failure to use an enzyme with the proper substrate specificity (59).

More recent work by Masuda et al. (59) on the β-1,3-glucanase induced elongation of oat coleoptiles has shown that only exo-β-1,3-glucanase induces elongation. There was no effect when an endo-β-1,3-glucanase was used. The exo-enzyme enhanced both elongation and the extensibility of the cell wall, and its effect was not additive to that of IAA. The endo-glucanase had no effect on the elongation and did not interact with the exo-enzyme. The authors suggest that the mechanism of cell wall loosening

and consequently that of cell elongation, caused by auxin, involve endwise splitting, but not random splitting, of cell wall polysaccharides which contain β-1,3-glucan or β-1,3-glucoside linkages, such as hemicelluloses.

Another effect of IAA or β-1,3-glucanase on excised coleoptile sections is an increased loss of ^{14}C label from the prelabeled hemicellulose fraction of the cell wall as growth increases (93). IAA also increased incorporation of ^{14}C-glucose into the pectic fraction of the cell wall and β-1,3-glucanase caused elongation of excised pea stem sections and oat coleoptile sections. It is hypothesized that the enzymatic degradation of hemicellulosic polysaccharides, the major component of the cell wall matrix, causes an increase in cell wall extensibility resulting in elongation. Treatment of Avena coleoptile sections with β-1,3-glucanase or β-1,3-glucanase and pectic methyl-esterase resulted in an increased elastic extensibility (58).

However, excised Avena coleoptile sections treated with a fraction of a fungal filtrate rich in a cellulase did not have an increased elongation rate although the turgor pressure remained constant and the wall extensibility was increased (79). IAA did give an increased elongation rate and an increase in wall extensibility greater than the cellulase treatment. These experiments were done with intact sections and with sections with the

epidermis removed. It could be that in this case the wrong enzyme was used and that is why this appears to contradict the β-1,3-glucanase experiments. However, this does indicate that the cellulose microfibrils themselves do not limit extension growth.

I think it can safely be concluded that gross changes in the cell wall are involved during elongation growth of the plant cell. It is inconceivable that these changes can come about by any means other than enzymatic modification of the cell wall polysaccharides. Consequently, the evidence for enzymatic activity associated with the cell wall will be examined further.

Turnover of Cell Wall Components

If there are enzymes associated with the cell wall *in vivo* that are capable of modifying the wall polysaccharides, as well as enzymes capable of synthesizing wall polysaccharides, then it is not unreasonable to suspect that there be a turnover of cell wall components during growth. There are several reports of turnover of cell wall constituents (39, 55, 56, 61).

It has been proposed that extensive breakdown of pre-existing cell wall be regarded as an essential feature of plasticization in enlarging cells, rather than the synthesis of new cell wall (56). Tissue sections of pea epicotyl were incubated on ^{14}C-glucose and the increase

in weight of the cell wall, as ethanol-ether insoluble dry weight, was less than that calculated from the amount of ^{14}C incorporated. This result only occured in apical (elongating) sections and not in basal (non-elongating) sections. Sections which were incubated on water alone and increased in length showed a loss in dry weight of the cell wall (56).

Further evidence for turnover of wall constituents as related to growth rate has been shown by Matchett and Nance (61). Their work is possibly the closest to what might occur in an intact plant since they used decapitated pea stems with the cotyledons attached. In this work, tissues were preincubated with ^{14}C-glucose and then subjected to treatment. IAA caused increased growth, as measured by increase in fresh weight, and it increased the loss of ^{14}C labeled constituents from noncellulosic cell wall polysaccharides as well. Incubation in galactose (0.1 M), which inhibits growth, inhibited the loss of ^{14}C labeled constituents from cell wall polysaccharides. However, these treatments had no effect on pea stem slices. Matchett and Nance attribute the lack of demonstrable turnover in pea stem slices as being, due to the lack of dilution of the ^{14}C-glucose by sugars from the cotyledons. This would result in more ^{14}C being incorporated initially and as a result, during turnover, more may have been reincorporated since the tissue was starved

for glucose at the beginning of the experiment. This explanation may be true, but from my own experience, the extension growth of excised pea stem sections is not uniform throughout the section so turnover may not be seen. Another problem may be that the only parameter of growth measured was an increase in fresh weight. Roleofsen (76) doubts that metabolic turnover of cell wall substances was what Matchett and Nance demonstrated. He feels that ". . . this still does not prove the occurence of true breakdown, since it was not demonstrated that the turnover could not be ascribed to mere exchange of monomers between the cell-wall substances and their precursors for synthesis, catalyzed, of course, by the synthesizing enzymes present in the wall." I find it hard to differentiate between turnover and the exchange of the constituents of an insoluble polysaccharide.

In the case of the growth of root hairs, a lowering of the atmospheric oxygen concentration will stop the growth of the root hair. A few minutes after stopping the growth in this manner, the root hair bursts at its tip and the protoplasm is exuded. It has been suggested that this bursting is a result of wall softening enzymes being less sensitive to the lack of oxygen than the enzymes synthesizing new microfibrils needed for the growth of the surface of the softened wall (24). Ca^{++} does in part reverse this anaerobically induced bursting in

germinating pollen tubes (61). It appears that treatments which increase wall plasticity, also stimulate cell wall degradation.

A detailed study of cell wall turnover in pea stem sections has been reported by Maclachlan and Duda (55). Turnover was defined by ^{14}C-sucrose incorporation exceeding the polysaccharide increment. In apical sections of pea epicotyl turnover occurred in the pectin (EDTA soluble) and dilute HCl soluble fractions of which most likely components are hexosans. In basal sections turnover occurred only in the EDTA soluble fraction, probably pectic acid and hexosans. The only cell wall components suspected of a metabolism specific to growing tissue are the hexose-containing polysaccharides. The only components that decreased in concentration or turned over specifically in the apical sections were glucose and galactose containing polysaccharides, mainly soluble in hot dilute HCl. It was suggested that the decrease in glucose in the dilute HCl fraction when grown on water may have been due to the conversion of amorphous to crystalline cellulose. Cellulose was the only wall fraction which increased in weight during elongation of sections on water. The authors suggest that the lack of turnover in basal sections is due to the presence of secondary wall and fewer sites for cellulose synthesis.

Katz and Ordin (39) observed significant turnover of galactose, xylose, arabinose, and glucose in the cell walls of Avena coleoptile sections following incubation on a solution of ^{14}C-glucose. Coleoptile sections were incubated on ^{14}C-glucose for three hours and ^{14}C was incorporated into the cell wall. After transfer to unlabeled medium, ^{14}C was lost almost immediately from the water soluble fractions, but continued to increase in the water insoluble fractions. The amount of ^{14}C in cellulose did not change. Cell walls were isolated and the various wall fractions were hydrolyzed and the constituent sugars eluted from chromatograms and amounts determined by ^{14}C content and colorimetrically. By comparing the net increase of wall components, as measured by a weight increase, with the increase calculated from the ^{14}C content, it was found that the weight increase was less than the ^{14}C calculated increase. This is interpreted to indicate turnover. There was turnover in the hexoses of the dilute acid soluble fractions, but no turnover was detected in cellulose or the alkali soluble fractions.

Baker and Ray (3) could not observe turnover of cell wall material in pea epicotyl sections with incubations of up to 24 hours. This could be accounted for by growth only from the ends of the sections or some other reason. They did observe gross cell wall synthesis in

the presence of IAA even when elongation was prevented by the presence of Ca^{++}.

The breakdown of cell walls has been observed during a period of starvation in celery collenchyma cells. Presumably the sugars from the cell walls were utilized by the cells as metabolic substrates. One peculiarity about this observation is that there was no decrease in cell wall glucose, but there was a decrease in galactose, arabinose, mannose and xylose (94).

The evidence of cell wall turnover has been criticized by Ray (74) for several reasons. He feels that the evidence involving more ^{14}C incorporation than can be accounted for by weight increase in wall material is too dependent on total recovery and the accuracy of chemical or gravimetric determinations. In the case of decrease in labeled cell wall material on incubation in unlabeled medium during net cell wall synthesis Ray feels the turnover is not great enough to be significant. Another point he makes is that cell wall preparations are not free of starch and that the turnover may be due to starch turnover. Ray's strongest objection is that turnover studies have not dealt with the individual types of macromolecules that comprise the wall. I must say that this last point is a good criticism of almost all of the work on cell wall polysaccharides. Our definitions for the various cell wall fractions are only operational

definitions and may have very little to do with the actual polysaccharide structure of the cell wall. It just happens that polysaccharide chemistry has not progressed to the stage where cell wall polysaccharides can be isolated without a great deal of degradation and consequently a lot of cross contamination of the different fractions.

My personal objections to the studies on cell wall turnover and cell wall extension is that these studies are usually done with excised sections. The tissue is damaged in the case of excised sections as well as that of decapitated seedlings, but the damage is probably less severe in the case of decapitated seedlings since nutrients are still available from the cotyledons. In my own attempts to use excised pea epicotyl sections elongation of incubated sections did not resemble the elongation of those same sections that would occur if they were part of an intact seedling. The apparent elongation involved extension from the ends of the sections and this extended tissue was translucent. Growth was certainly less than intact tissue and was not uniform throughout the sections.

Although there are deficiencies in the studies on the turnover of cell wall components during growth I think there is enough evidence, especially when the wall extension and cell wall enzyme information is included, to say that there probably is turnover of some non-cellulosic cell wall polysaccharides during growth. I

certainly feel the case is strong enough to merit further study. The main hurdle lies in designing experiments that will give more meaningful results.

Enzymatic Activities Associated With Cell Walls

This next section will present the evidence for enzymatic activities being associated with cell walls of higher plants. This review will only cover enzymatic proteins and will not cover the hydroxy proline rich cell wall protein "extensin" which is hypothesized by Lamport (49, 50) to play a structural role. Except for the mention of one fungal wall enzyme, no attempt has been made to review the bacterial or fungal wall enzyme literature (25, 72). The presence of enzymes localized in the plant cell wall was first suggested by Dippel (21) and later by others as discussed by Kivilaan *et al*. (45). Only the more recent literature will be discussed in this review.

Bryan and Newcomb (8) reported that pectin methylesterase is associated with the cell wall and cell surface of the callus of tobacco pith cultures and that the enzymatic activity increases in response to IAA, which causes an increase in cell enlargement as measured by an increase in fresh weight. Later, Glasziou (27) reported the pectin methylesterase of *Avena* coleoptiles to be localized in the free space, which includes the cell wall.

The problems involved in assigning an enzyme's location to the cell wall are discussed by Jansen, Jang, and Bonner (37). These authors showed that aqueous suspensions of Avena coleoptile cell walls are able to bind up to 200 times more added pectin esterase than is endogenous to the cell walls. This is true also for walls that have been previously treated with pectin esterase to remove pectic methyl ester groups. The binding is apparently ionic as was shown by the binding of other enzymes and their removal by salt.

Enzymatic activities associated with purified cell walls isolated in glycerol from corn coleoptiles are ATPase, invertase, UDPG pyrophosphorylase, inorganic pyrophosphatase, and α-glycerolphosphatase (45). The adequacy of the purification procedures in removing cytoplasmic enzymes was shown by deliberate contamination of the walls and then re-isolating the walls. Thus, nonspecific adsorption of cytoplasmic enzymes was not appreciable in this work unless, of course, there had been saturation of the cell wall with contaminating enzymes during the initial homogenization. Hall and Ordin (30) have countered that UDPG pyrophosphorylase is largely a soluble enzyme in Avena coleoptiles. They did find that the specific activity was slightly higher in the cell walls than in the soluble fraction but, that the walls only had 3% of the total activity. It is very difficult

to prove that an enzyme is associated with a cell wall *in vivo* when the isolated cell wall has only a small portion of the total activity in the tissue. Again, though, when the same enzyme is observed in walls using different methods of isolation and different tissues, one finds that a strong case can be made.

The fact that enzymes external to the plasmalemma may be involved in growth is shown by the application of β-1,4-glucanase or pectinase to 1 hour old pollen tubes. The result is an increase in growth rate. The stimulatory effects of β-1,4-glucanase and pectinase are additive and boiled enzymes have no effect on growth rate. In addition, replacing the growth medium 1 hour after beginning germination stops pollen tube growth, but this growth can be restored by adding a mixture of cellulase and pectinase to the medium (78). Knox and Heslop-Harrison (47) have shown the walls of pollen grains to contain acid phosphatase, ribonuclease, esterase, and amylase.

Corn coleoptile cell walls will autolyze *in vitro* (43, 51). Presumbably this enzymatic reaction also occurs *in vivo* so it may be a portion of the process which occurs during extension growth. The autolytic activity solubilizes only one polysaccharide, a lichenan-type polymer composed of 1→3 and 1→4 linked glucosyl units, and a small amount of free glucose (43). This is good evidence that at least one oligosaccharide can be excised by an enzymatic

activity associated with highly purified cell wall preparation, and strengthens the possibility that a glucan may be involved in extension growth.

Katz and Ordin (40) have shown that a cell wall preparation from Avena coleoptiles contains enzymes capable of hydrolyzing cell wall polysaccharides. It appears that one enzyme breaks down polysaccharides to oligosaccharides, and is sensitive to PAN, while another enzyme breaks down the oligosaccharides to monosaccharides and is not sensitive to PAN. Their data support the hypothesis that IAA increases growth in part by increasing the specific activity of the cell wall degrading enzymes in vivo. Sections incubated with glucose have a slightly higher cell wall hydrolyzing activity while sections incubated with IAA have a greatly increased activity. On the other hand, sections incubated with mannitol or IAA + mannitol have a cell wall hydrolyzing activity equal to the control, freshly cut sections.

Heyn (33) reported a β-1,3-glucanase activity to be associated with Avena coleoptile cell walls. This activity is increased by treatment with 0.8% digitonin. In addition, these walls have a β-1,4-glucanase (as measured by decreasing viscosity of carboxymethyl cellulose), β-glucosidase (cellobiase), and β-1,6-glucosidase activity (pustulan substrate). The β-1,4-glucanase activity was mostly an exo-glucanase with very little endo-glucanase activity present. In a more recent paper, Heyn (34) reports

dextranase activity associated with cell walls of Avena coleoptiles. This activity is measured using a dextran with 95% α-1,6 linkages and supposedly is sensitive to auxin. The author did not specify which auxin was used or its concentration, since sections of tissue were dipped in a powder of the hormone.

About 70% of the total ascorbate oxidase activity in maize root tips has been found to be associated with the cell wall (62). This wall bound ascorbate oxidase activity is highest in segments taken from the rapidly elongating region of the root and then decreases in the more mature segments; however, if expressed on a per cell basis, the activity is continually increasing as the cell goes from a young elongating cell to a mature non-elongating cell.

A detailed comparison of the cell wall associated ascorbate oxidase and the soluble enzyme has shown that although they have the same pH optimum and Km, the cell wall enzyme has a higher energy of activation and is less sensitive to inhibition by 8-hydroxyquinoline, azide, and diethyldithiocarbamate (31). The cell wall enzyme can not be released by 0.15 M ionic concentration, 1.5 M sucrose, or 5% deoxycholate, but incubation of the walls with cellulase does release some of the activity. Up to 50% of the ascorbate oxidase is accessible to ascorbate in the medium bathing barley roots, which leads one to

believe the enzyme may be located in the cell wall *in vivo*. In this study, 70% of the ascorbate oxidase of cabbage leaves was found to be associated with the cell wall, while in barley root tips 80-100% of the enzyme was associated with the cell wall fraction (31).

Histochemical studies have shown that the peroxidase of juvenile onion root cells is localized in the cytoplasm, but that in mature cells the peroxidase is associated with the cell walls (19). The data do not support the theory that peroxidase may be involved in *in situ* cell wall synthesis, but that conjugation with the cell wall may be incidental and only indirectly related to the natural redox function of the enzyme.

Lai and Thompson (48) have found 5'nucleotidase and glucose-6-phosphatase to be associated with highly purified cell walls from *Phaseolus vulgaris* cotyledons. The cell walls had specific activities enriched by 11-14 fold for the 5'-nucleotidase and 2-5 fold for the glucose-6-phosphatase although the total enzymatic activities of the wall fractions were only 7-8% and 2-3% of the homogenate respectively. In this work the cell wall fraction was enriched 3-8 fold in cellulose, but there was some starch contamination. The phospholipid content of the wall preparation was 1% of that of the homogenate and went up to 4-10% when the authors corrected for the cell wall yield of only 10-25%. These workers did not preclude

the possibility that the enzymes may be bound to membranes which are in turn embedded in the wall matrix. Their cell wall fraction was also enriched in ATPase activity.

The reported cases of β-glucosidase activity associated with cell walls are of particular importance to the experimental work I will present later.

Horikoshi and Ikeda (35) have found a considerable amount of bound β-glucosidase in the conidia coats of dormant and germinating conidia of Aspergillus oryzae. The ratio of bound to soluble enzyme increases as germination proceeds. The bound form is not removed by detergents, EDTA, urea, salts up to 1.0 M, or by β-1,3-glucanase, but sonication is somewhat effective in releasing the activity. The physical and chemical properties of the bound and soluble enzymes are identical.

Chkanikov et al. (10) have found the β-glucosidase of several higher plant tissues to be associated with the cell wall. They found no β-glucosidase activity associated with chloroplasts, mitochondria, or ribosomes and only a very small amount of activity in the soluble fraction. The specific activity of the β-glucosidase in the cell walls (on a per mg protein basis) exceeded that of the supernatant. The enzyme did not wash off with salt or buffer solutions, but these workers may have used ionic concentrations that were too low since they only used up to 0.1 M. They suggest that β-glucosidase participates

in the synthesis of cell wall material and/or the transport of β-glucosides. It is interesting to note that the β-glucosidase activity in the cell walls of oat blades and of corn coleoptiles exceeded that of the homogenate from which the walls were isolated. The authors offer no explanation for this observation, but I have found the same thing with pea epicotyl sections. The authors ruled out the presence of an inhibitor as being responsible for low activity in the supernatant on the basis that the β-glucosidase activity was not altered after being salted out of the supernatant with ammonium sulfate or separated from low molecular weight substances on R-30 biogel. As will be discussed more fully later, in pea epicotyl sections there is a substance in the soluble fraction which is inhibitory to the cell wall β-glucosidase.

Nevins (67), in looking at glycosidases associated with bean hypocotyl growth, has found that α-glucosidase, β-galactosidase, α-galactosidase, and β-xylosidase activities all decline as a function of increasing tissue age during the first 13 days of the life of the seedling of Phaseolus vulgaris L. var. Red Kidney. On the other hand, β-glucosidase activity increased rapidly from three days after imbibition to a maximum activity at 5 days and then declined to 1/3 maximum activity by the 7th day. The β-glucosidase activity peak immediately preceeded the logarithmic phase of hypocotyl growth. The β-glucosidase

activity was strongly associated with the cell walls and was removed with 0.5 M sodium citrate for assay as a soluble activity. Whole hypocotyls were used for this work.

Datko and Maclachlan (18) have shown the β-glucosidase, as measured by hydrolysis of p-nitrophenyl-β-D-glucose, and expressed on a per cent fresh weight, protein, or DNA content basis, to be concentrated in the meristematic region of the plumule and hook of pea seedlings. β-glucosidase was almost absent from the adjacent growing and maturing regions of the third internode of 8 day old seedlings. Other glycosidase activities had no specific localization. They concluded that β-glucosidase was useful to the cells during or shortly after cytokinesis, but that none of the glycosidase activities studied were needed for elongation or expansion. They found the β-glucosidase to be mostly wall bound but that most of it comes off upon treatment with 0.5 M NaCl. In subsequent work, Maclachlan et al. (54) showed that although the highest β-glucosidase activity was in the plumule and hook, there was a four fold decrease in activity between the 0-10 mm and 10-20 mm regions below the hook. They suggest that β-glucosidase is selectively inactivated in expanding cells and not replaced during maturation. Most of the activities they studied fell by 10 fold during the development from the plumule to the epicotyl 10-30 mm

below. Calculated on the basis of DNA content (per cell), the activities increased two fold during growth and maturation, which the authors interpreted to mean that all of the enzymes must be slowly synthesized during growth--except for the β-glucosidase activity. There may be some problem using DNA content as a measure of relative cell number, since in my own work DNA content did not agree with cell counts.

Keegstra and Albersheim (41), using suspension cultures of sycamore cells, have shown that β-glucosidase, α-galactosidase, and one of two β-galactosidase activities are associated with the cell surface. These enzymatic activities can, to a limited extent, degrade isolated sycamore cell walls. The β-glucosidase activity and one β-galactosidase activity, which is probably not localized at the cell surface, increase as the cells go through a period of growth and then decrease as cell growth ceases. Evidence for the association with the cell surface is that the activities are not removed by a 0.1 M buffer wash, but they are removed, although not quantitatively, by a subsequent wash with 0.5 M buffer. These treatments do not damage the integrity of the cell membrane or the cells in general.

I think the evidence of the papers cited makes a substantial case for the association of β-glucosidase with the cell wall, particularly the work of Keegstra and

Albersheim (41). In addition, from the work of Nevins and others, there is reason to believe that the β-glucosidase is associated with growth. For these reasons the experimental work presented was started.

Glucosyl Hydrolases and Transferases

After a correlation was found between growth rate and β-glucosidase activity, the possibility of glucosyl transferase activity was investigated. From an energetic point of view, it would seem more reasonable for the cell wall associated β-glucosidase to actually be functioning as a β-glucosyl transferase *in vivo*, since both activities appear to be catalyzed by the same enzyme.

It is well known that, under the proper conditions, the same glycosidase can transfer the glycosyl residue of the substrate to any of several acceptor alcohols--ROH (69). In fact, hydrolysis is a special case in which water--HOH--serves as the acceptor. Most often, aliphatic alcohols or sugars present in suitable concentrations serve as acceptors. The extent to which an enzyme favors hydrolysis or formation of new glycosides depends on the specificity of the individual enzyme for its substrate and the acceptors present, as well as other experimental conditions. The configuration at the anomeric carbon atom of the newly produced glycoside is the same as in the original substrate, so there is no inversion of

configuration taking place at C-1 during these transfer reactions.

There are numerous reports of the formation of various oligosaccharides from cellobiose in β-glucosidase systems (5, 17, 23, 36, 46, 57). The study of the transferase activity of β-glucosidase from almond emulsin by Courtois and Leclerc (16) is of particular interest. They observed the transfer of the β-glucose of the β-glucosides of phenols to a wide range of acceptor compounds with a primary or secondary alcohol group. There was no transfer to compounds with tertiary alcohol groups. Using methanol as the acceptor, the influence of temperature, pH, length of reaction time, and donor and acceptor concentrations make it appear as if the same enzyme can either hydrolyze the donor to glucose and the phenolic compound or transfer the glucose of the donor to the acceptor alcohol.

In a study by Suzuki (85) using p-nitrophenyl-β-D-glucose as substrate and β-glucosidases from several plant sources, it was shown that in the absence of acceptor alcohols or sugars, the reaction products are not always only p-nitrophenol and glucose. He reported the formation of p-nitrophenyl-β-gentiobioside, p-nitrophenyl-β-cellobioside, possibly other p-nitrophenyl-β-biosides or higher β-oligosides, cellobiose, gentiobiose, and other oligosaccharides. The products vary with incubation time. Suzuki

considers the reaction to be that of transfer of a β-glucosyl group from one p-nitrophenyl-β-glucoside to any one of the hydroxyl groups of the glucosyl moiety of another, resulting in p-nitrophenyl-β-oligosides and the hydrolysis of these resulting in heterosides giving the aglycone and β-oligosides. It is important to note that the extent of transglucosylation of β-glucosidases between donor molecules varies greatly from one enzyme source to another.

In conclusion, I think it would be far easier task to assign a function to the cell wall associated β-glucosidase if it also had transferase capabilities. A transferase activity could easily be associated with cell wall synthesis or possibly with the changes involved in the non-cellulosic portion of the wall during elongation growth. A transferase activity might also account for turnover of cell wall constituents. It must be kept in mind that the transferase capability of hydrolytic enzymes varies with the assay conditions so an enzyme could have transferase activity <u>in vivo</u> without it being observed <u>in vitro</u>, and vice versa.

PURPOSE OF THE EXPERIMENTAL WORK

Initially, I asked the question: Is there a correlation between the rate of elongation growth of a tissue and the β-glucosidase activity of the cell walls isolated from the tissue? This question is based on the premise that there is breakdown or turnover of existing cell wall during growth as new cell wall material is synthesized. I was interested in determining the specific activity of β-glucosidase both on a per weight wall and on a protein basis. This would give some idea of the enzymatic activity of the cell wall. The cell walls would be isolated from tissues with widely differing rates of elongation growth. As an extension of this question, I wished to determine β-glucosidase activity per cell. This information would indicate whether the observed decrease in specific activity of the glycosidase was due simply to increased amount of cell wall. It is plausible that each cell starts out with the same amount of enzyme and the observed decrease in specific activity could be a consequence of that same amount of enzyme being spread out over more cell wall substance as the cell grows.

Finally, I wished to know whether cell wall β-glucosidase was capable of transglucosylation from the

artificial substrate to an endogenous acceptor in the cell wall preparation. This was the reason for the experiments using p-nitrophenyl-β-D-glucose with the glucosyl moiety ^{14}C labeled.

MATERIALS AND METHODS

Tissue

Pea seed, Pisum sativum var. Alaska 28-57 W. R., obtained from Farm Bureau Services, Lansing, Michigan, was used for all experiments. The seeds were surface sterilized in 1% sodium hypochlorite for 15 minutes, then soaked in running tap water for 18 hours before planting. Seeds were germinated on Kimpak Germination Paper (Seedburo Equipment Co., Chicago, Illinois) in plastic trays, covered with an inverted tray during the first 24 hours. Then the covering tray was inverted to allow room for seedling growth. The trays were kept in a dark room at 25° and 85% relative humidity. Seedlings were harvested 84 hours after planting.

Growth rate of the seedlings was determined by marking the seedlings with ink marks at 2.5 mm intervals and then, at a later time, measuring the distance between the marks with a millimeter ruler.

Sequential epicotyl sections were taken from 0-5 mm, 6-10 mm, and 11-15 mm starting just below the forming apical hook. These 5 mm sections were either dropped into beakers on dry ice immediately upon cutting in the case of samples for cell wall preparation by the non-aqueous

method, or, in all other cases, weighed out in 0.5 g lots and then frozen on dry ice as soon as 0.5 g was obtained. The tissue was stored at -80° until used.

Cell Wall Preparation

Cell walls were isolated by the method of Kivilaan et al. (44) as is shown in the flow sheet in Figure 1. Tissue sections, glass beads (200 μ diameter, purchased from Minnesota Mining and Manufacturing Co., St. Paul, Minnesota), and glycerol (redistilled in vacuo), in the ratio of 6 g: 11 g: 68 g respectively for a typical preparation, were placed in the cup of a Servall omnimixer and homogenized. Homogenization was for 5 minutes at top speed (16,000 RPM) followed by 10 minutes at very slow speed, to facilitate cooling, and again at top speed for another five minutes. The cup of the omnimixer was immersed in an ice bath during this procedure.

The homogenate was allowed to stand at 0° for about twenty minutes to allow the bulk of the beads to settle out. The suspension was then decanted onto a filter bed of the 200 μ glass beads (1 cm deep) on top of a layer of Miracloth. The Buchner funnel was surrounded by an ice jacket. The homogenate was filtered with the aid of suction from a filter pump and the surface of the filter bed was periodically scraped to prevent a reduction in the effective pore size as cell wall material accumulated

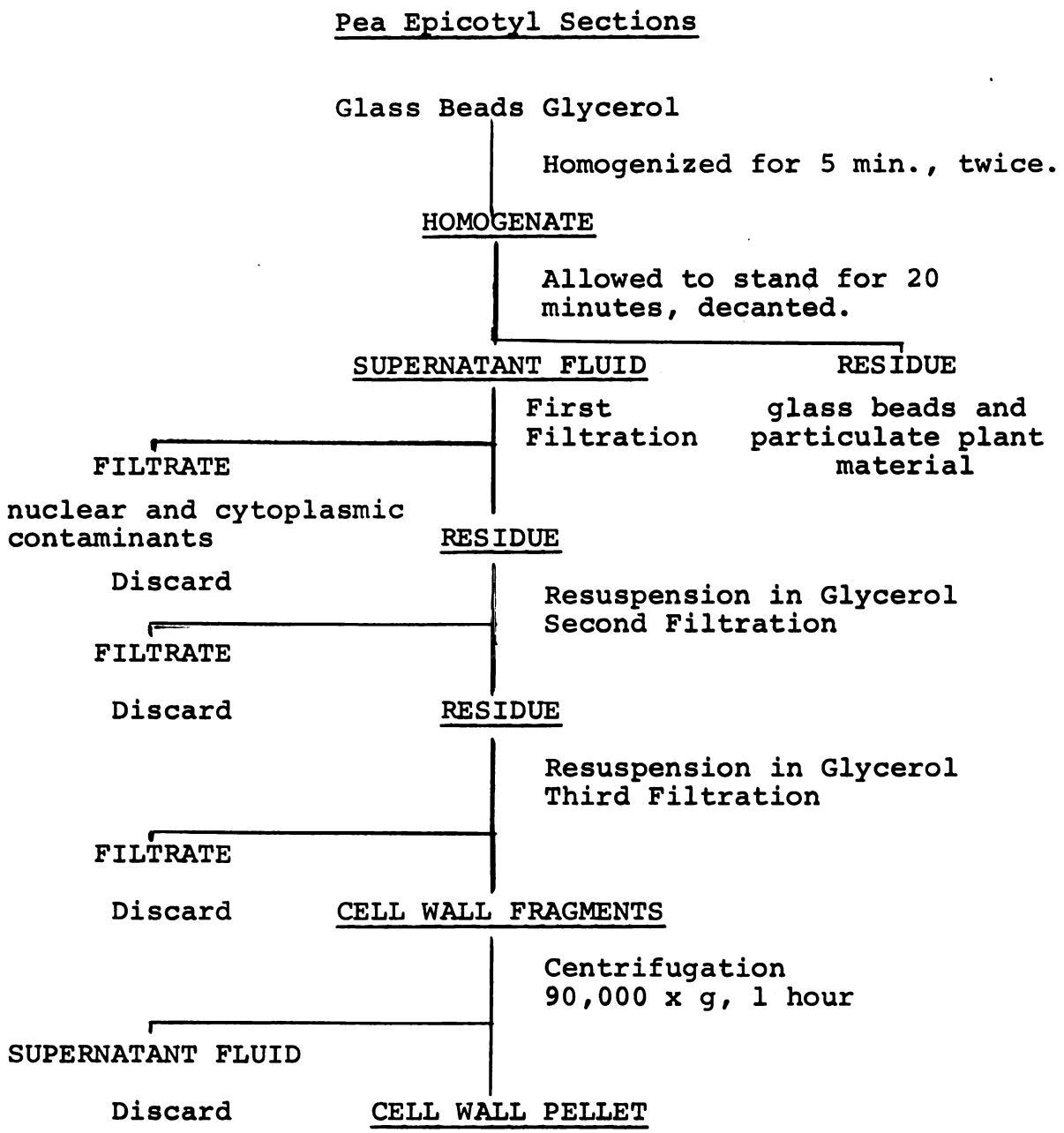


Figure 1. Isolation of cell wall from pea epicotyl sections.

on the filter. The filtrate was discarded and the bead mat with the cell residue scraped into a beaker and resuspended in fresh cold glycerol. The beads were allowed to settle from suspension again. The procedure of filtration, resuspension, and sedimentation was repeated two more times. After the third filtration the wall material was again suspended in the usual manner and after the beads had settled out, the suspension was centrifuged at 90,000 x g for 1 hour in a Spinco model L ultracentrifuge, in a SW 25.1 swinging bucket rotor. The glycerol was discarded and the pellets were twice resuspended in absolute ethanol and the residue collected by filtration under suction. Next, the residue was washed once with acetone and once with ether. All solvents were at -20° or below. The wall material was dried in an evacuated dessicator over P_2O_5, $CaCl_2$, and paraffin at 4° for 24-48 hours. The resultant white powder was weighed and stored in the freezer in a sealed jar over $CaSO_4$.

A variation in the procedure employed water rather than solvents to remove glycerol from the walls. In place of the solvent wash, the pellet was suspended in cold water and centrifuged to resediment the wall. This procedure was repeated twice for a total of three water washes. The cell wall material was then collected by filtration and dried over P_2O_5 <u>in vacuo</u> and handled as previously described.

Aqueous Cell Wall Preparation

Another method employed in the preparation of a cell wall fraction was the more conventional aqueous method, shown in Figure 2. In this procedure 0.5 g of frozen tissue sections were ground in a 50 ml conical glass homogenizer (Kontes Duall, size E), with 3.0 ml of buffer solution until no bits of tissue were visible. The homogenate was transfered to a conical centrifuge tube (10 ml) along with two 1 ml washings of the homogenizer and the volume of the homogenate was recorded. The homogenate was then mixed on a Vortex mixer and centrifuged for 10 min at 4 x g (max.). All procedures were carried out at 0-4°. The supernatant fluid was removed and the pellet resuspended in 4.0 ml volume and centrifuged again for 10 min at 4 x g. Following the second centrifugation the pellet was again resuspended and centrifuged at 440 x g for 5 minutes. The pellet from this centrifugation was then suspended for assays and will be referred to as the 4 x g "wall" pellet. The two supernatant solutions from the 4 x g centrifugations were combined and centrifuged at 130 x g for 10 minutes. The pellet from this 130 x g centrifugation is referred to as the 130 x g pellet and the supernatant fluid was centrifuged at 10,000 x g for 20 minutes. The supernatant solution following centrifugation at 10,000 x g was assayed

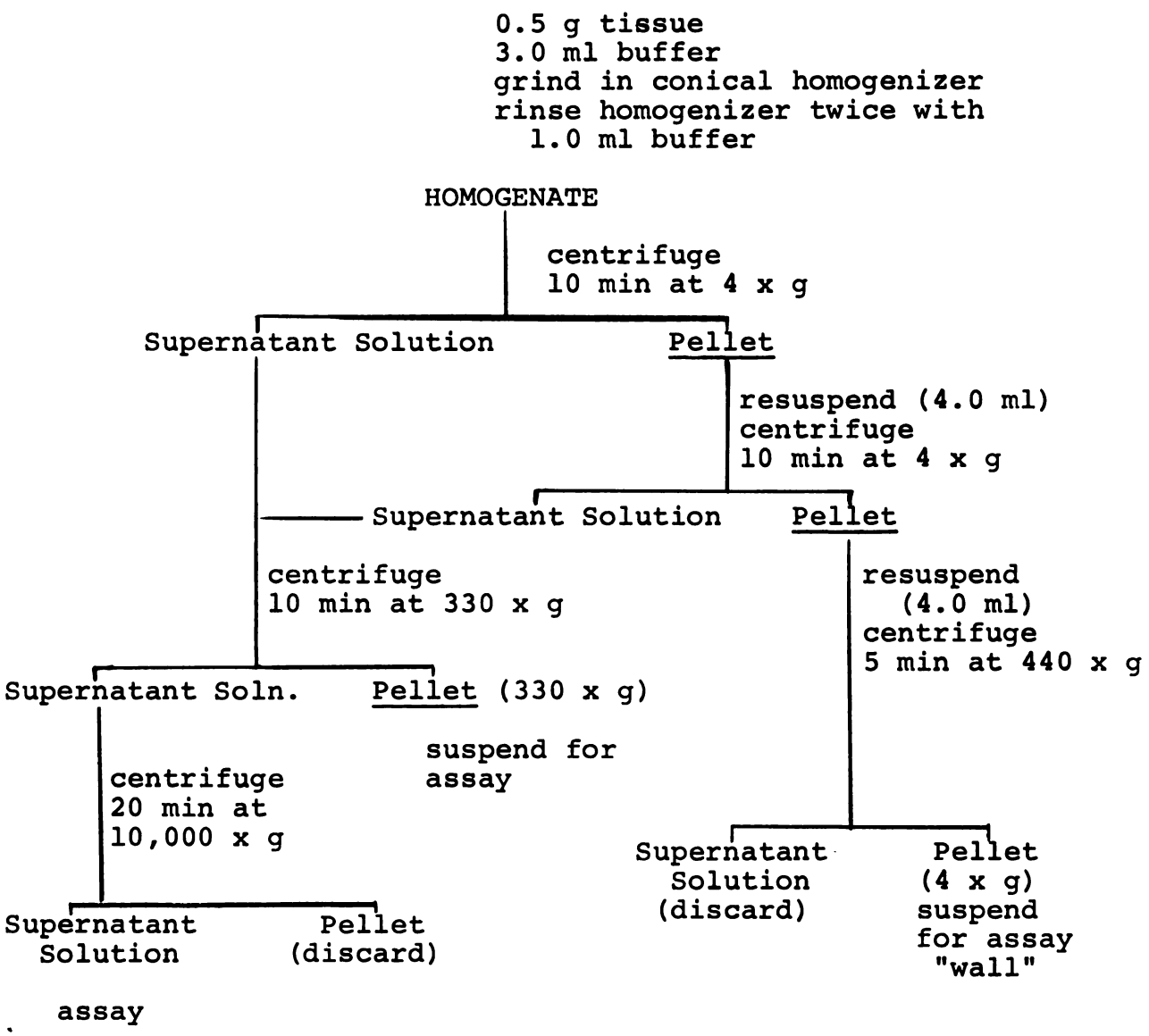


Figure 2. Procedure for the isolation of cell wall and other fractions after grinding in buffer.

for the activities and was called the soluble fraction; the pellet was discarded.

Fixation of Tissue for Cell Counts

Tissue sections were fixed in Formalin, Acetic Acid, and 70% Ethanol (1:1:18) for 24 hours and then transferred to 70% ethanol. The sections were dehydrated, stained with Safranin-O and embedded in Paraplast for sectioning. Sections 8 microns thick were cut and mounted on slides for counting. The number of cells in a row was counted along the length of the longitudinal sections, exclusive of the stele and vascular bundles, and multiplied by the number of cells in diameter of the corresponding cross section, exclusive of the stele and vascular bundles. The product was used as a parameter of the relative cell number of the sections.

Protein Content of Cell Wall Preparations

Samples of cell wall material were hydrolyzed in 6 N HCl for 18 hours in sealed tubes. Following hydrolysis the samples were evaporated to dryness until there was no longer a detectable acid smell. The residue was then taken up in 0.1 N HCl to dissolve amino acids, neutralized with 0.1 N NaOH and amino acids determined with ninhydrin (11). To a 0.5 ml sample, 1.5 ml of ninhydrin solution was added and the mixture boiled for 20 minutes.

After cooling, 8 ml of 50% n-propanol was blown in and the tube was mixed immediately. After 10 minutes the absorbance was read at 570 nm (OD_{570}) in either a Beckman DU Spectrophotometer with a Gilford attachment or in a Gilford 240 spectrophotometer. Glycine was used as a standard and values were calculated on the basis of nmoles of α-amino acid. An average molecular weight of 100 was used for conversion to per cent protein.

DNA Content

For determination of the DNA content of sections, 0.5 g of tissue was homogenized in 5.0 ml of 80% ethanol and centrifuged in a clinical centrifuge. The pellet was extracted two more times with 5.0 ml of 80% ethanol and centrifuged. The residue from the ethanol extractions was twice extracted with 3.0 ml of 0.5 N perchloric acid at 70° for 15 minutes. The DNA content of the perchloric acid extracts was determined by the diphenylamine reaction according to the method of Burton (9). To 1.0 ml of the perchloric acid extract, 2.0 ml of diphenylamine reagent was added and the color was allowed to develop for 17 hours at room temperature. Following color development the optical density was read at 600 nm. Salmon sperm DNA (Calbiochem, Los Angeles, California) was used as a standard.

β-Glucosidase Assay

A weighed aliquot (usually 20 to 30 mg) of cell wall material was suspended in 0.5 M MES buffer (pKa 6.15 @20°, ΔpKa/°C = -0.011) (28) at pH 5.8 and allowed to hydrate for at least four hours before being assayed. Cell Wall suspensions were pipeted with an Eppendorf Micropipette (Brinkman Instruments Inc., Westbury, New York). The reaction mixture contained 0.5 mg cell wall and 1 umole of p-nitrophenyl-β-D-glucopyranoside in a total volume of 1.0 ml of 0.5 M MES buffer at pH 5.8. Tubes were incubated at 37° and the reaction was stopped by adding 0.4 ml of 0.2 M Na_2CO_3 which also converted liberated p-nitrophenol to the yellow sodium salt. The mixture was centrifuged to pellet the cell wall material. In the case of the crude homogenate or soluble fractions, the reaction was first stopped by addition of an equal volume of 5% TCA and centrifuged to precipitate soluble protein prior to the addition of the Na_2CO_3. Controls in all experiments were boiled for two minutes prior to the addition of substrate. Optical density was read at 400 nm, which is the absorption maximum of the compound, to determine free p-nitrophenol. A molar extinction coefficient of 1.832×10^4 was determined using recrystalized p-nitrophenol and this value was used for all calculations.

Other Glycosidase and Acid Phosphatase Assays

Other glycosidases were assayed in the same manner as β-glucosidase except that the appropriate p-nitrophenyl substrate was used. In the case of aqueous cell wall preparations, all isolation procedures were carried out at the pH optimum of the enzyme being assayed. All p-nitrophenyl substrates were obtained from Pierce Chemical Co., Rockford, Illinois.

Reducing Sugar Determinations

Reducing sugar determinations were done according to the procedure of Nelson (65) as modified by Somogyi (83) and described by Samen et al. (80), but on a smaller scale. To 0.1 ml of sample, containing 10 to 40 µg of glucose or its equivalent, 0.2 ml of Somogyi sugar reagent was added and the mixture heated in a boiling water bath for 10 minutes. After cooling, 0.2 ml of Nelson arsenomolybdate chromogenic reagent was added and the tube shaken to remove CO_2; 2.0 ml of water was added and the optical density was read at 540 nm. A solution of β-D-glucose was used for the standard curve and reducing sugar was expressed as glucose equivalents.

Synthesis of p-Nitrophenyl-β-d-($U^{14}C$)-Glucopyranoside

p-Nitrophenyl-β-D-($U^{14}C$)-Glucopyranoside was synthesized by first preparing acetyl-D-glucosyl bromide from $U^{14}C$-α-D-glucose, then condensing it with the potassium salt of p-nitrophenol to give p-nitrophenyl-β-D-glucose tetraacetate which was then deacetylated to give the desired product.

Acetyl-D-glucosyl bromide was prepared according to the procedure of Bárczai-Martos and Körösy (4). For starting material, 250 μc of $U^{14}C$-glucose, in 0.25 ml of 25% ethanol (Biochemical and Nuclear Corporation, Burbank, California), was added to 100 mg of α-D-glucose (Mann Research Laboratories, New York, N. Y.). The ethanol was removed in a rotary flash evaporator and the remaining solution was lyophilized overnight. The 100 mg of ^{14}C-glucose was added gradually, over about thrity minutes, to 0.4 ml of acetic anhydride and 2.4 μl of perchloric acid in a 37° water bath. The tube was fitted with a drying tube and mixed occasionally over a period of about two hours or until all of the glucose was dissolved, to get complete acetylation. Next, the tube was cooled in an ice bath and 30 mg of amorphous phosphorous was added; then 180 mg of bromine was added gradually, while keeping the tube in the ice bath, followed by the addition of 36 μl of water. The tube was then stoppered and kept at

room temperature for two hours. The product was extracted by the addition of 0.3 ml of chloroform followed by 0.8 ml of ice water, all procedures being done in a 10 ml conical centrifuge tube. The chloroform layer excludes most of the phosphorous and the chloroform phase was filtered through glass wool in a small funnel made from glass tubing. The filtered chloroform was extracted two more times with an equal volume of ice water and the water was then extracted twice with 0.1 ml of chloroform before being discarded. Finally, the chloroform was extracted with about 1 ml of saturated sodium bicarbonate solution to bind the last traces of acid, which resulted in a pH of about 6. The yellow solution was then dried over anhydrous sodium sulfate with some sodium bicarbonate added. After a few minutes, 5 mg of charcoal (Norite) was added with slight agitation to decolorize the solution. Finally, after a half an hour, the solution was filtered again through a glass wool filter to remove the charcoal. The resulting pale yellow solution was evaporated under reduced pressure on a rotary evaporator in a water bath with a final temperature of 60°. The temperature was elevated very slowly to avoid bumping, which was a problem in the 10 ml conical centrifuge tube. The dried product was an oil with some crystal formation near the top where it was dried on the tube. The product was dissolved in 0.5 ml of ethyl ether and crystallized immediately on cooling,

but was allowed to crystallize overnight at 4° to obtain as high a yield as possible. The crystalline product was dried in vacuo over P_2O_5 for 24 hours and weighed. Yield was 85 mg and the melting point of the product was sharp at 86°. The acetobrome glucose was used without recrystalization.

p-Nitrophenyl-β-D-glucose tetraacetate was synthesized according to the method of Glaser and Wulwek (26), with some minor modifications. The potassium salt of p-nitrophenol was prepared by adding KOH in methanol to a solution of p-nitrophenol (Calbiochem Corp., Los Angeles, California) in methanol. The salt was precipitated out with petroleum ether. To the 85 mg of ^{14}C-acetyl-D-glucosyl bromide and 51.2 mg of the potassium p-nitrophenolate, 0.584 ml of acetone and 0.396 ml of water were added at 0°. Most of the acetobrome glucose went to the bottom as an oil. After 18 hours at 0° the stoppered tube was brought up to room temperature and kept at room temperature for 7.5 hours with occasional mixing. By the time the oil dissappeared, there were large slender crystals of product formed. The acetone was removed under reduced pressure and about 0.3 ml of chloroform was added. The product was partitioned into the chloroform phase which was removed and taken to dryness under reduced pressure. One ml of 95% ethanol was added and the product dissolved at slightly elevated temperature. All of a sudden the

product crystallized out, even before cooling to room temperature, and crystallization was allowed to proceed for two hours on ice. The crystals were centrifuged down and the mother liquor removed with a Pasteur pipet. The tube containing the product was dried overnight *in vacuo* over P_2O_5, $CaCl_2$, and paraffin. All of the preceeding operations were done in a 10 ml conical centrifuge tube. The yield was 25 mg of p-nitrophenyl-β-D-glucose tetra-acetate.

Deacetylation was accomplished according to the procedure of Tsou and Seligman (87). One ml of freshly distilled anhydrous methanol which had just been saturated with anhydrous ammonia, was added to the tube containing the tetraacetate. The mixture was kept at 0° for 18 hours and then taken to dryness on a rotary evaporator followed by drying *in vacuo* over P_2O_5 for a few hours. The final white product was removed and weighed. The yield was 3.5 mg and the melting point was 165-166°. There remained some oily yellow material in the tube following deacetylation so it was repeatedly dissolved in absolute methanol and taken to dryness three times. This procedure removed the yellow and more white crystalline product appeared; this product proved to be identical to that obtained on the first drying. This yield was 6.3 mg for a total yield of 9.8 mg of p-nitrophenyl-β-D-(U^{14}C)-glucopyranoside.

The identification of the product will be covered in the next section.

Identification of p-nitrophenyl-β-D-($U^{14}C$)-glucopyranoside

The melting points for both the p-nitrophenyl-β-D-glucose and its tetraacetate were in agreement with those reported at 165-166° and 175° respectively. In addition, purity of the product of the synthesis was checked by comparison of the tetraacetate and trimethylsilyl derivatives with the respective derivatives of the p-nitrophenyl-β-D-glucopyranoside purchased from Pierce Chemical Co. on gas-liquid-chromatography.

The p-nitrophenyl-β-D-glucose tetraacetate was prepared by heating 1 mg of p-nitrophenyl-β-D-glucose with 0.5 ml of acetic anhydride and 0.5 ml of pyridine at 50° for two hours; this mixture was then injected directly onto the column. In the case of my product, the tetraacetate intermediate was used directly, before deacetylation. The TMS p-nitrophenyl-β-D-glucose was prepared by heating 1 mg of p-nitrophenyl-β-D-glucose with 20 µl of Regisil (BSTFA) (Regis Chemical Co., Chicago, Illinois) and 20 µl of pyridine at 50° for two hours. This mixture was injected directly onto the column.

Gas liquid chromatography was done on an F and M Model 402 chromatograph with a hydrogen flame ion detector.

Figure 3. GLC Charts. The major peak, at 6 min., is the product. The small peak at 2.2 min. is caused by pyridine. The 2 small peaks at 3.2 and 3.8 min. are not identified.

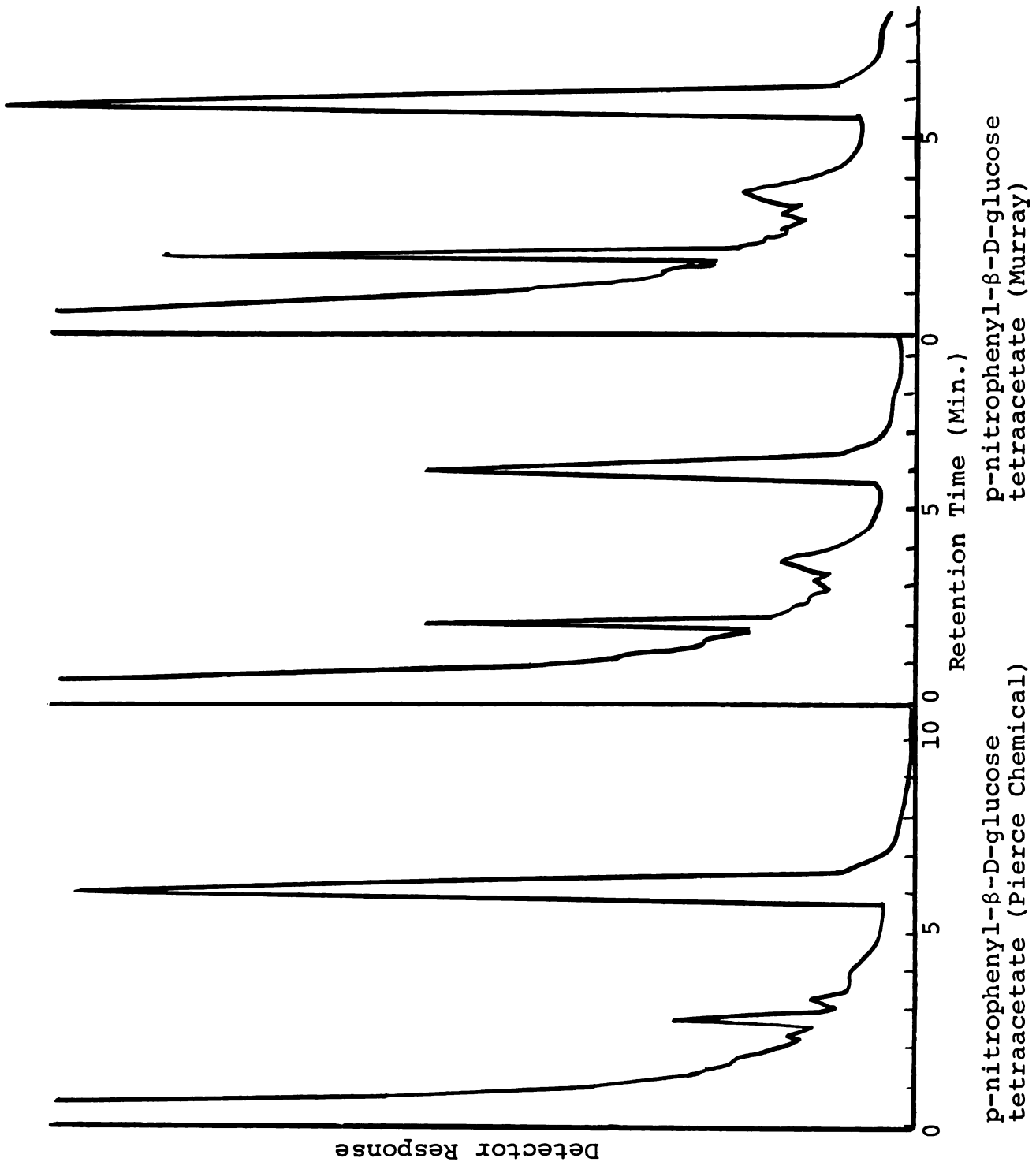
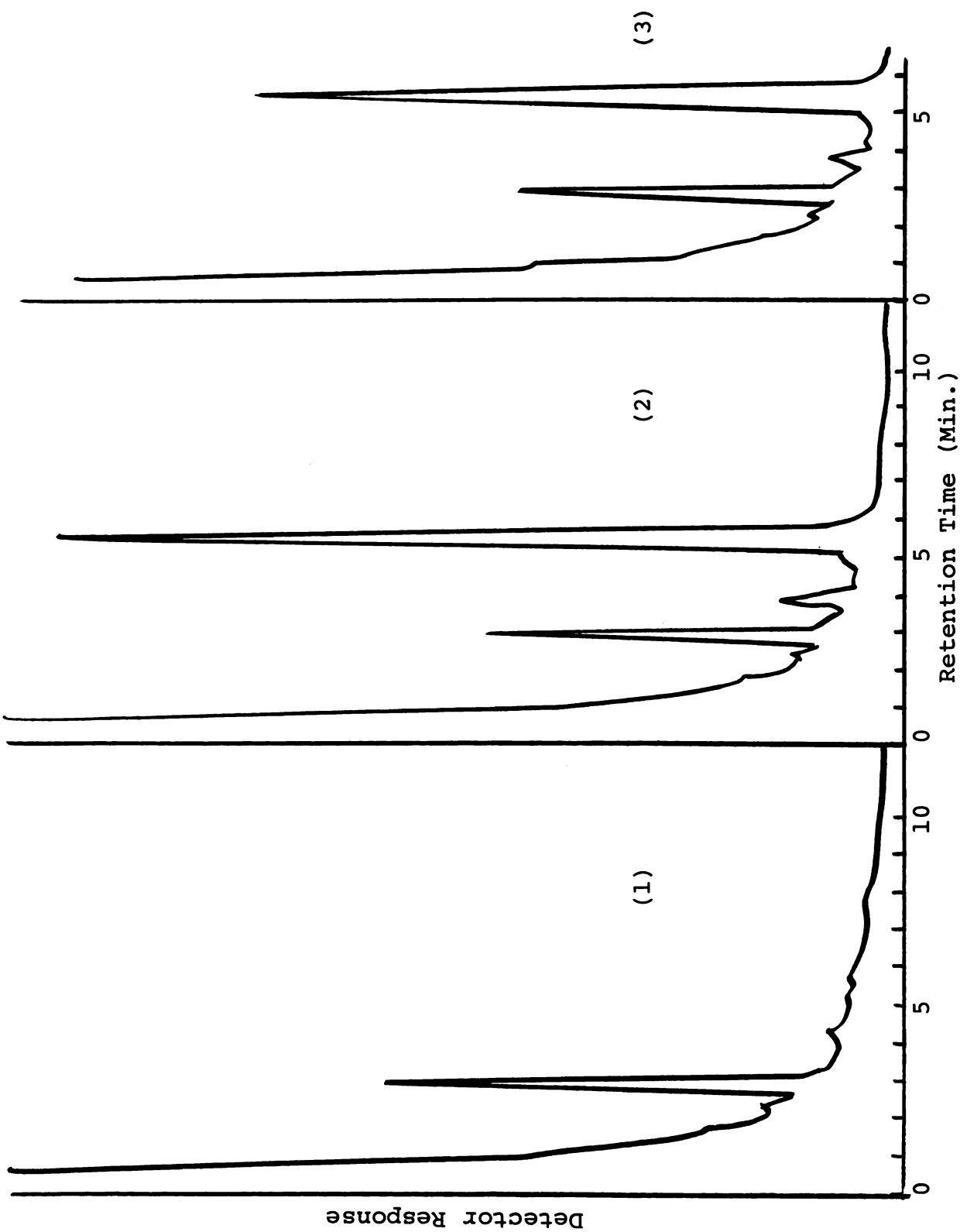

Detector Response
0
5
10 0
5
0
5
Retention Time (Min.)
p-nitrophenyl-β-D-glucose
tetraacetate (Pierce Chemical)
p-nitrophenyl-β-D-glucose
tetraacetate (Murray)

Figure 4. GLC Charts.
(1) Solvent Blank - pyridine:BSTFA (1:1)
(2) TMS-p-nitrophenyl-β-D-glucose (Murray)
(3) TMS-p-nitrophenyl-β-D-glucose (Pierce Chemical Co.)

The small peak at 3.0 minutes is caused by pyridine.
The small peak at 3.9 minutes is not identified.


(1)
(2)
(3)
0
5
10
0
5
10
0
5
Retention Time (Min.)
Detector Response

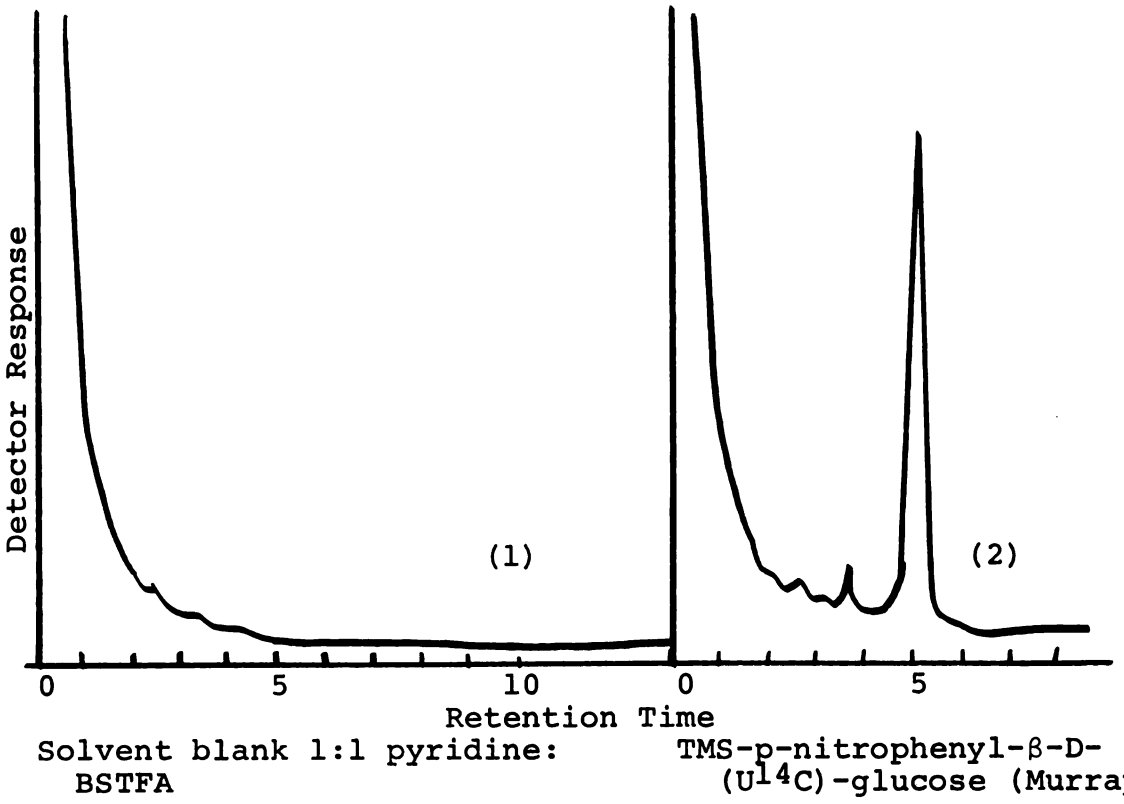


Figure 5. GLC Charts. The small peak at 3.7 minutes is not identified.

The column dimensions were 3 mm x 4 ft. with a 3% SE-30 liquid phase on Supelcoport (100/120 mesh) support (Supelco, Inc., Bellefonte, Pennsylvania). Nitrogen was used as carrier gas at a flow rate of 75 ml/min. Samples were chromatographed isothermally at a temperature of 225°. The retention times were identical for the respective derivatives of the product of my synthesis and that obtained from Pierce Chemical Co. The retention times were 6.0 minutes and 5.4 minutes respectively for the p-nitrophenyl-β-D-glucose tetraacetate and the

TMS-p-nitrophenyl-β-D-glucose. The chart tracings for these derivatives are shown in Figures 3, 4, and 5.

Assay for Cell Wall Labeling Activity

This assay was performed in the same manner as the β-glucosidase assay with the exception that p-nitrophenyl-β-D-(U^{14}C)-glucopyranoside (specific activity 0.1 μc/μmole) was used as the substrate. After the reaction was stopped in the usual manner, the cell wall material was suspended in about 4 ml of water and mixed well on a Vortex mixer and centrifuged. This washing procedure was done three times, after which the wall material was dried overnight *in vacuo* over P_2O_5. Following drying, the wall material was weighed on a Cahn Electrobalance and dispensed into scintillation vials for counting. The controls used were both a zero time incubation as well as cell wall material that had been boiled for two minutes prior to the addition of substrate. In addition, ^{14}C-glucose was incubated with the 4 x g pellet to see if any radioactivity was bound. In all cases the washing procedure was sufficiently rigorous to remove all radioactivity from the control cell wall material as well as from the 4 x g pellet which had been incubated with ^{14}C-glucose.

Scintillation Counting

All samples were placed in scintillation vials along with 15 ml of scintillation fluid. Vials were counted in a Packard Tri-Carb scintillation counter.

Lipid Extractions

Lipid extractions were done by combining the method of Lennarz and Talamo (52) with that of Sutherland and Norval (84). Following incubation the cell wall material was first extracted at room temperature with 1 ml of chloroform:methanol (2:1, v/v) for 2 hours (4 x g pellet) or 14 hours in the case of purified walls. This was followed by a second extraction for 5 minutes at 60° with 1 ml of chloroform:methanol (2:1) and partitioned against 0.5 ml of 0.9% sodium chloride. The extracts were combined, taken to dryness, and taken up in a suitable volume of chloroform:methanol for counting purposes. The 4 x g pellet extract, that was added to a reaction mixture, was taken up in toluene.

Preparation of ^{14}C-Labeled Cell Wall Material

The labeled cell wall material was prepared in four identical incubation tubes (10 ml conical centrifuge tubes). Each tube contained 4.5 ml of cell wall suspension (3 mg/ml) in 0.05 M MES, pH 5.8, and 0.5 ml of 20 mM

p-nitrophenyl-β-D-(U^{14}C)-glucopyranoside (specific activity 0.3 μc/μmole) in 0.05 M MES at pH 5.8. The reaction mixture was incubated for four hours at 37°. Following incubation, the tubes were centrifuged in a clinical centrifuge to sediment the cell wall material. An aliquot of the supernatant of the reaction mixture was removed for determination of p-nitrophenol liberated and the rest was stored in a freezer. The cell wall material was washed thoroughly twice with water by suspending in 5 ml of water, mixing thoroughly on a Vortex mixer, and then centrifuging. The wall material was stored overnight in a refrigerator at 4° and washed three more times the next morning in the same manner. An aliquot of each wash was counted in the scintillation counter. The wash water from the fourth and fifth washes was essentially free of label as is shown in Table 1. The cell wall material was then dried to constant weight over P_2O_5 *in vacuo* (24 hours) and aliquots were counted.

Table 1. Radioactivity in washings of ^{14}C-labeled cell walls.

Description	Volume	Aliquot Counted	CPM
Supernatant of Reaction Mixture	20.0 ml	10 μl	19850
First Water Wash	16.6 ml	20 μl	3562
Second Water Wash	25.0 ml	20 μl	368*
Third Water Wash	19.0 ml	20 μl	78*
Fourth Water Wash	18.2 ml	20 μl	6*
Fifth Water Wash	23.6 ml	20 μl	0*

*Counted for 100 minutes.

Fractionation of ^{14}C-Labeled Cell Wall Material

It was my intention to attempt to successively extract the pectin, hemicellulose, and cellulose fractions from the wall to determine in which fraction the incorporated radioactivity occurred. Since, however, all of the label was extracted in the first step (that designed to remove pectin), the procedure was not followed beyond this point.

The labeled cell walls were fractionated according to the procedure of Dever et al. (20) to determine which wall fraction was labeled as a result of the β-glucosyl transferase activity. The pectin fraction was removed by extracting twice with 0.5% ammonium oxalate--oxalic acid (1:1, 0.25% each) at 90° for 24 hours. Aliquots of the extracts were taken for counting. The extracts were made up to 80% ethanol with slow stirring and then allowed to stand overnight at -10° to allow the pectin fraction to precipitate. The pectin fraction was then collected by centrifugation and an aliquot of the 80% ethanol supernatant was taken for counting to see if perhaps any of the label was still in the ethanol. The second pectin fraction was collected in the same manner as the first and combined with the first fraction. The combined pectin fractions were washed with 80% ethanol. Since 90% of the label was recovered in the oxalate extract, further

fractionation was not done. The extraction of the labeled material is diagramed in Figure 6.

Identification of ^{14}C-Labeled Product

The 80% ethanol supernatant from the precipitation of the pectin fraction was the starting material for the characterization of the labeled product. The oxalate was removed by precipitation with calcium hydroxide, the fraction was titrated to pH 8 in several small batches and the precipitate washed with 80% ethanol. An aliquot of the combined 80% ethanol supernatant and wash was used for counting to be sure none of the label had been lost. The 80% ethanol phase was taken to dryness and then made up to a know volume with water and an aliquot counted. Another aliquot was used for a uv spectrum in a Cary 15 recording spectrophotometer, since the presence of phenolic compounds was suspected due to the brownish color.

The 10 ml sample from above was centrifuged to remove some particulate matter and then taken to dryness in a 10 ml conical centrifuge tube in a stepwise manner. The sample was then taken up in 0.3 ml of water and, after mixing, centrifuged in a clinical centrifuge to remove a small amount of yellowish precipitate. The supernatant solution was quite brownish in color. An aliquot of the solution was used for counting and the rest was loaded onto a Sephadex G-15 column (89 cm x 1.5

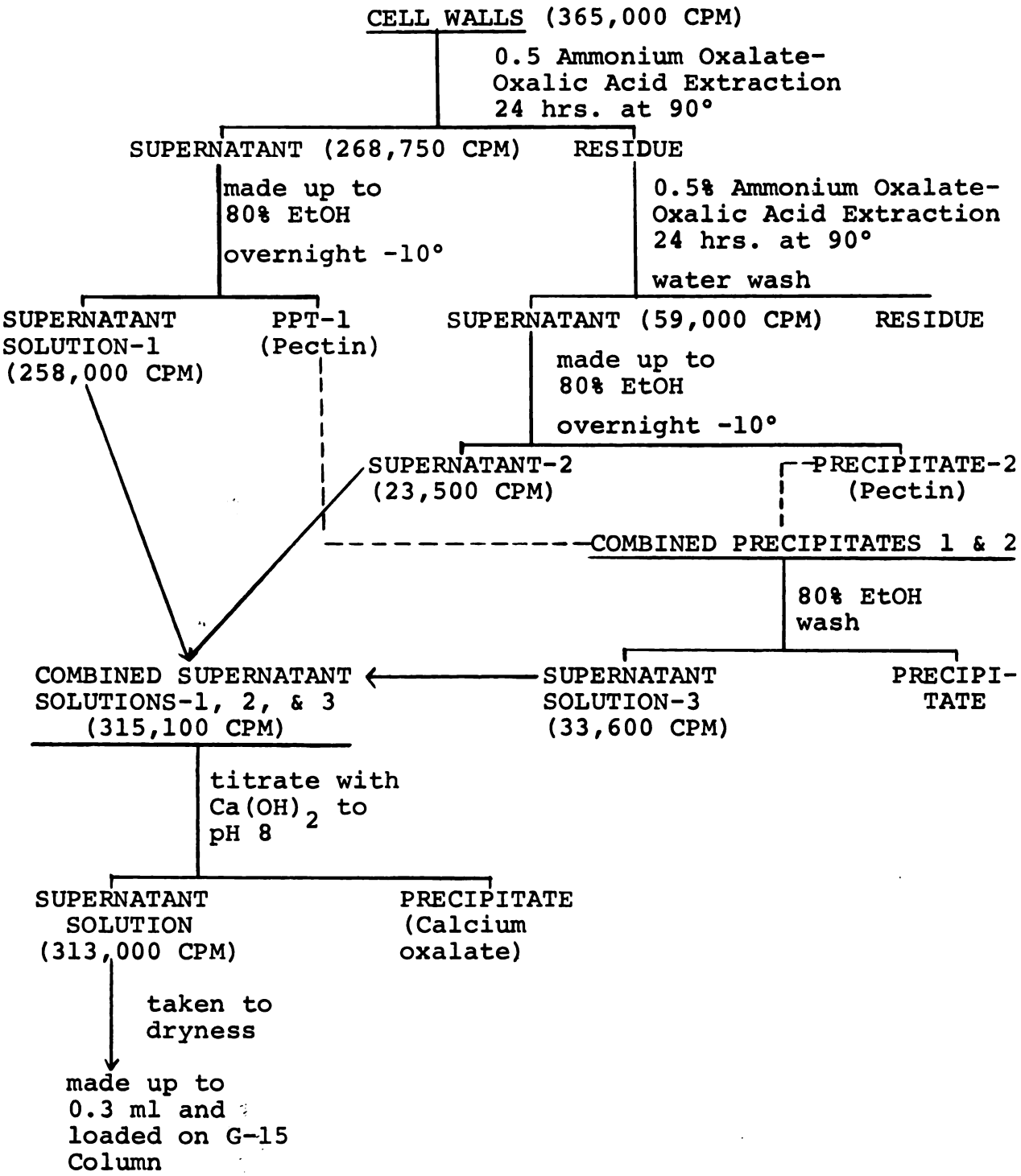


Figure 6. Extraction of ^{14}C-labeled material from cell walls.

cm), eluted with glass distilled water (8.7 ml/hr) pumped by a Buchler pump and 32 drop (1.25 ml) fractions were collected. The column was monitored for radioactivity, by counting aliquots of each fraction, as well as for reducing sugars.

An aliquot of the radioactive peak fraction was chromatographed on a thin layer chromatogram (Silica gel on aluminum plate) using n-butanol:acetic acid; ethyl ether:water (9:6:3:1) as the developing solvent system. Reducing sugars were detected by spraying the dried plate with 0.5% potassium permanganate in 1 N sodium hydroxide. Spots on the plate were marked and regions of the plate were marked. The silica gel was scraped off the plate and counted in scintillation vials. An autoradiogram of the thin layer plate was made by exposing it to Kodak No-Screen X-Ray film for 21 days and developing.

Materials and Methods: Reagents Used

Ninhydrin Solution: (11)

400 mg reagent grade $SnCl_2 \cdot 2\ H_2$) in
250 ml of 0.2 M Citrate Buffer, pH 5 added to
250 ml methyl cellosolve containing
10 g ninhydrin (dissolved)
Solution was stored in the freezer.

Somogyi Sugar Reagent: (80)

Dissolve 24 g of anhydrous sodium carbonate and 12 g of Rochelle salt in about 250 ml of boiled, cooled distilled water. To this solution is added 4.0 g of copper (II) sulfate pentahydrate dissolved in 40 ml of boiled distilled water.

After mixing, 16 g of sodium hydrogen carbonate is added, and, when dissolved, the solution is poured into a 1 liter graduated cylinder. Next, 180 g of anhydrous sodium sulfate are dissolved in about 500 ml of hot water and the solution is boiled to expel air. After cooling, this solution is added to the cylinder and the combined mixture is diluted to the same mark.

Nelson Arsenomolybdate Chromogenic Reagent (80)

Dissolve 25 g of ammonium molybdate in 450 ml of distilled water, adding 21 ml of conc. sulfuric acid, mixing, and then adding 3 g of disodium hydrogen (monobasic sodium) ortho-arsenate heptahydrate ($Na_2HAsO_4 \cdot 7\ H_2O$) dissolved in 25 ml of water. This solution is placed in an incubator at 37° for 24-48 hours before use and stored in brown bottles.

Diphenylamine Reagent (9)

1.5 g diphenylamine
100 ml acetic acid
1.5 ml conc. H_2SO_4
on day of use 0.1 ml aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent.
Stored in the dark.

Scintillation Fluid

To make 1 liter of solution:
234 ml ethanol
384 ml dioxan
384 ml toluene
40 g napthalene
5 g PPO (2,5-diphenyloxazole)
0.2 g Dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene)

RESULTS

Enzyme activity as a function of pH is shown for the cell wall β-glucosidase in the data of Figures 7 and 8. The optimum is between pH 5.5 and 6.0 and thus all β-glucosidase assays were conducted at pH 5.8 in 0.05 M MES buffer. It can also be seen that the slight activity observed at pH 8.0 is almost completely inhibited by Tris as has been observed for glucosidases with transferase activity (66). These pH experiments were done in the early stages of this research and the assay involved the incubation of 5 mg of cell wall in 1 ml of 3 mM substrate without preincubation to hydrate the walls.

The fact that β-glucosidase activity is linear with respect to incubation time is shown in Figures 9 and 10. The experiment in Figure 9 was also one of the earlier experiments and was done with 5 mg of wall incubated in 1 ml of 3 mM substrate. The slight decrease of activity for the longer time (Figure 9) can be explained by depletion of substrate. Figure 10 demonstrates that the activity is linear up to 4 hours if substrate is not depleted. The variation between wall preparations is shown in Figure 10. It is important to note that the tissue sections from which the cell walls used in Figure 10

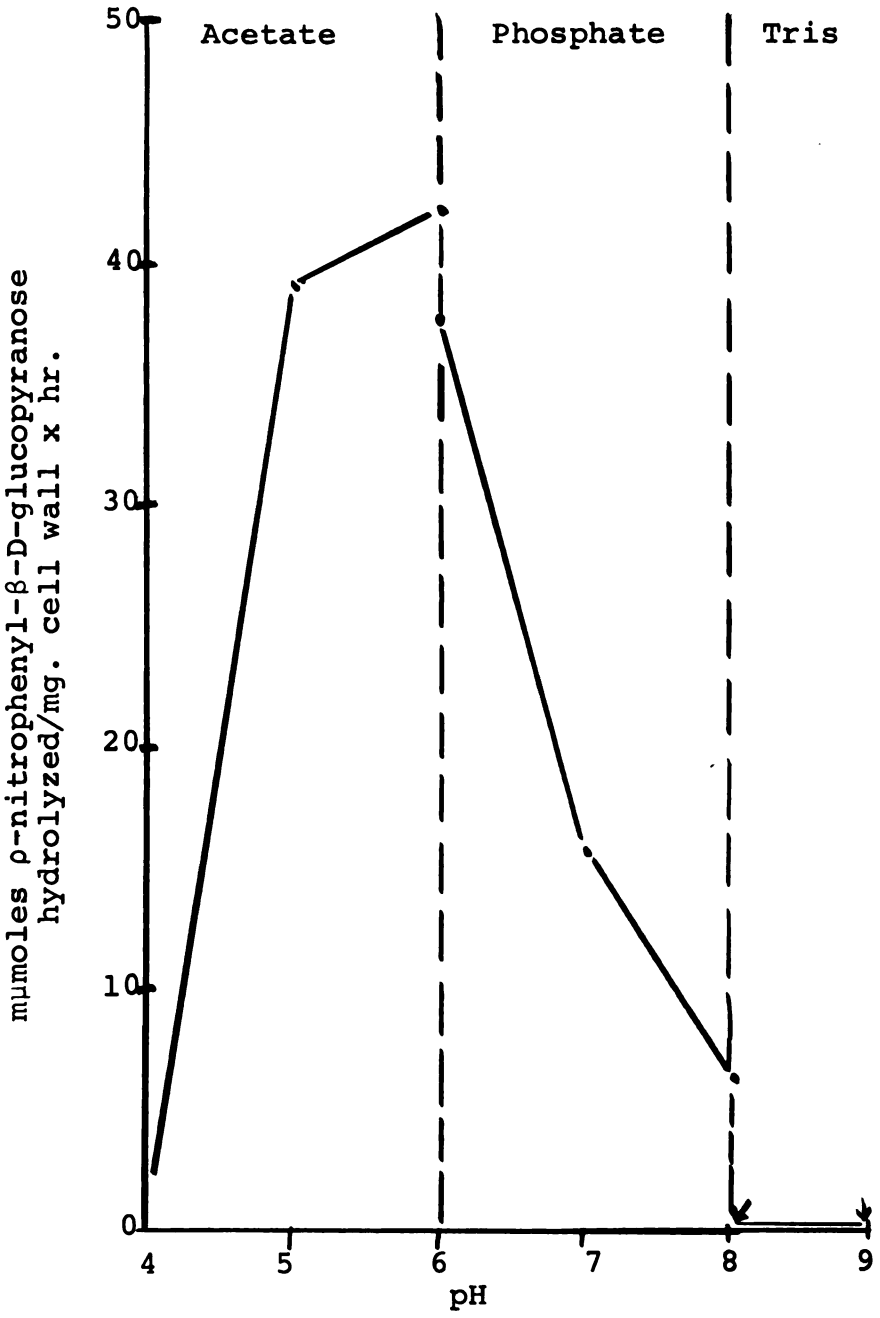


Figure 7. pH Optimum of cell wall β-glucosidase activity.

Substrate Conc.: 3mM
Incubation Time: 45 min.
Wall Preparation II.
Buffer Concentrations: 0.05 M

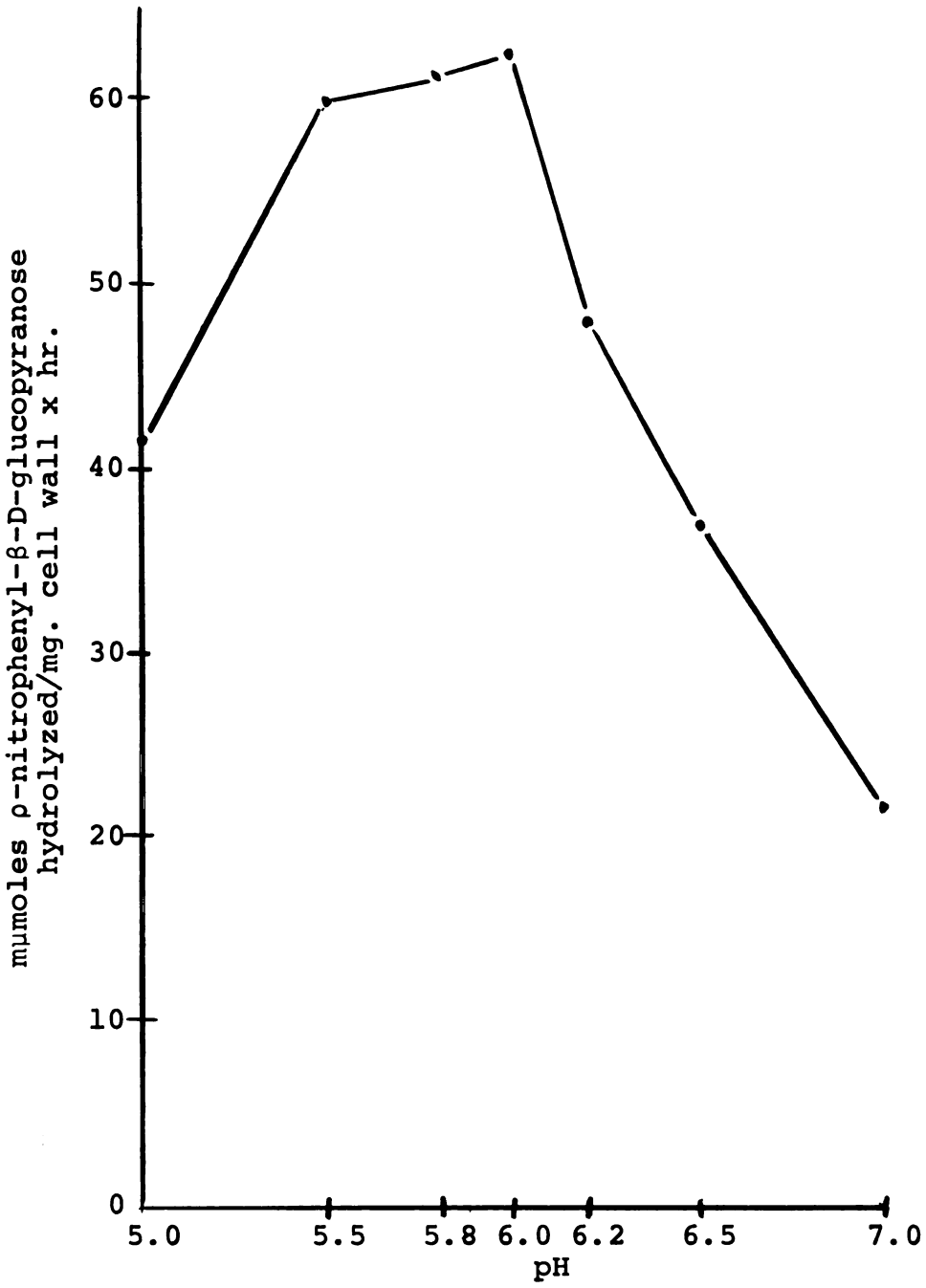


Figure 8. pH Optimum of cell wall β-glucosidase activity.

Substrate Conc.: 3 mM
Incubation Time: 30 min.
Wall Preparation III.
Buffer: 0.05 M MES

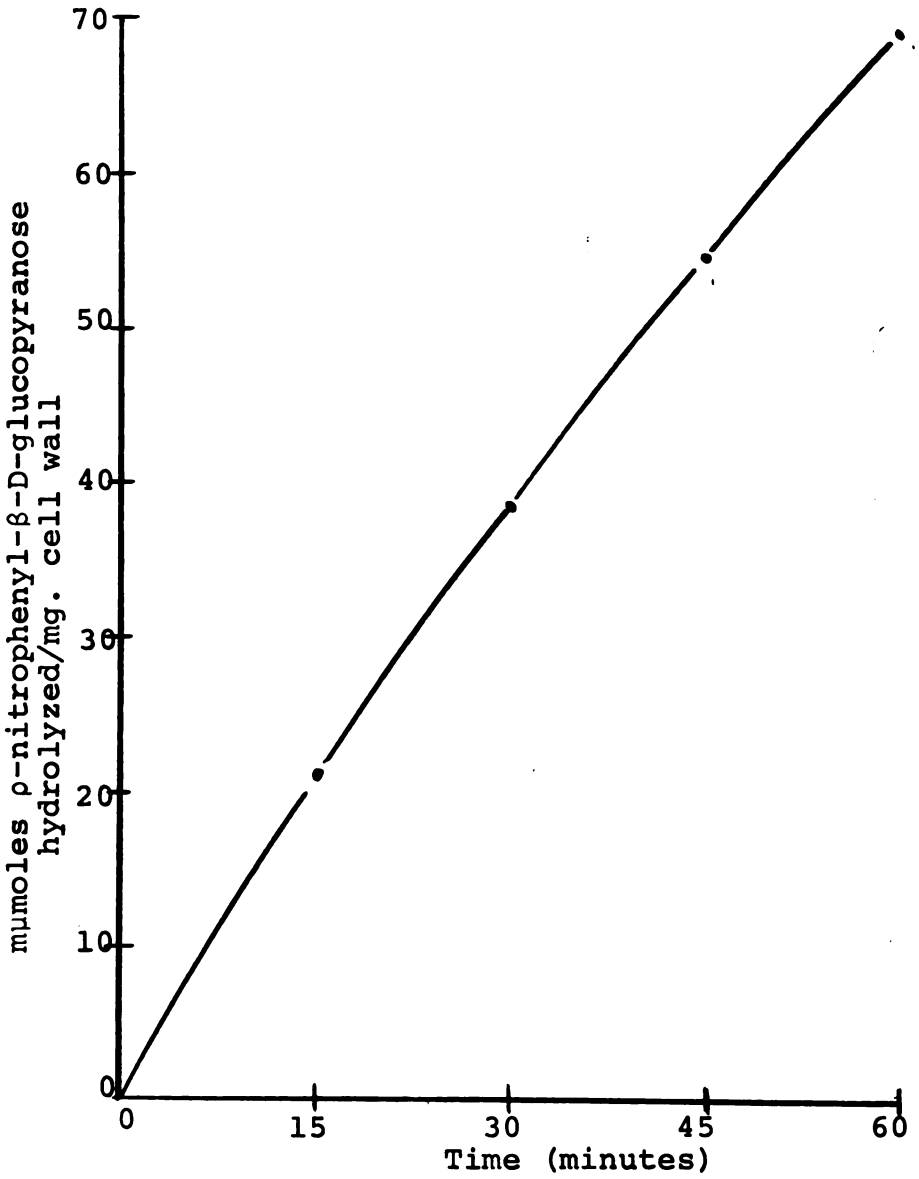


Figure 9. Time course of cell wall β-glucosidase activity.

Substrate Conc.: 3 mM
Wall Preparation X.

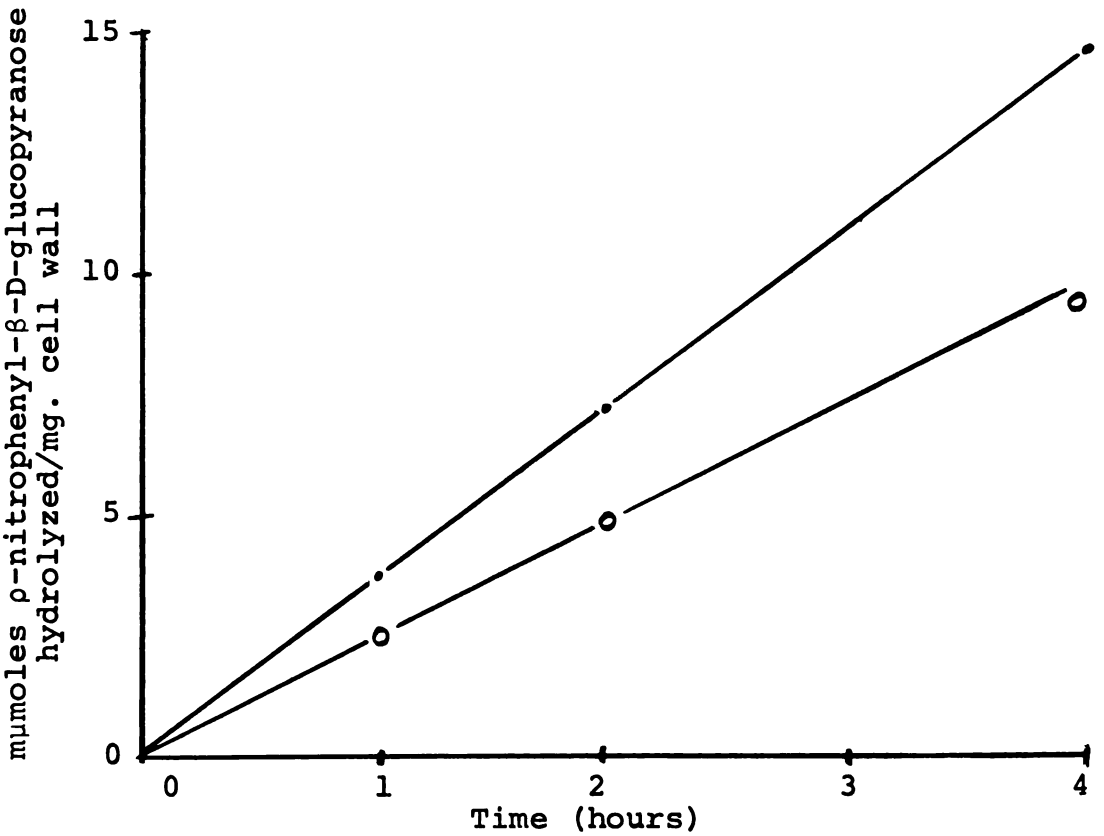


Figure 10. Time course of cell wall β-glucosidase activity.

Substrate Conc.: 0.05 mM
Preincubation Time: 3 hours
Wall Preparations: 1 - 4C ·
1 - 5C °

were prepared, were 7 days old and incubated for 16 hours at 24° in 2% sucrose in 0.01 M phosphate buffer (pH 6.5) and for these reasons the activity is much lower than in the cell walls used in most of this work. The interesting effect of this tissue incubation will be discussed later.

β-glucosidase activity is linear with respect to the amount of cell wall material used in the assay as is shown in Figures 11 and 12. Figure 11 also shows the variation between different cell wall preparations. The experiment in Figure 11 was also one of the earlier experiments of this work and the departure from linearity for the 10 mg tubes can be accounted for by the lack of a preincubation period to insure complete hydration and the utilization of substrate, 30% of which was consumed. The lower activity of the walls used in Figure 12 is again owing to the fact that they were from incubated tissue sections as were those for Figure 10.

The effect of substrate concentration on β-glucosidase activity is shown in the data of Figure 13. Saturation is not attained at the concentrations of substrate employed in these experiments, 0.3 to 12 μmoles/ml. For reasons of economizing with substrate and to minimize non-enzymatic hydrolysis, the lower concentrations were employed. Since, however, only about 5% of the substrate

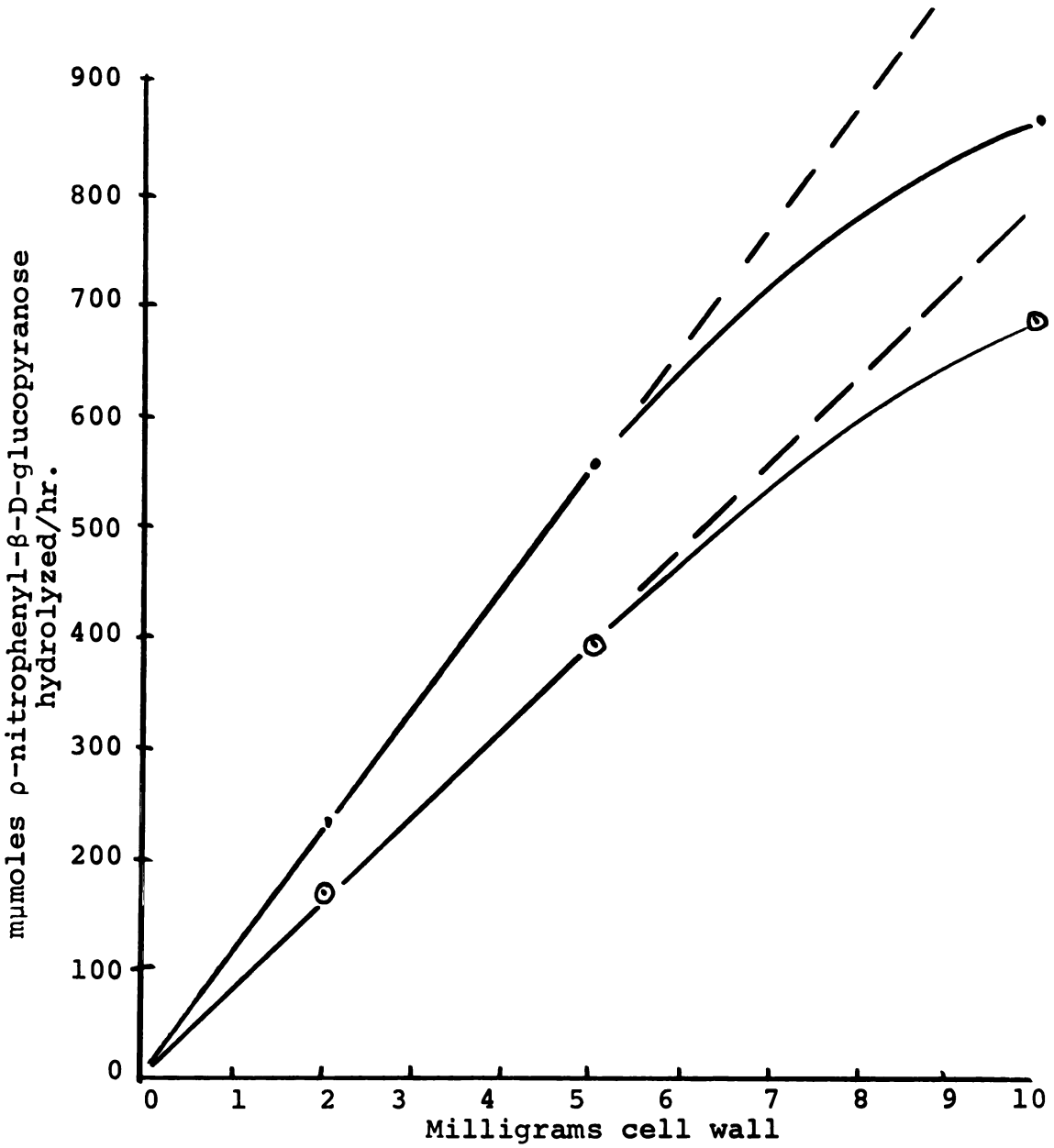
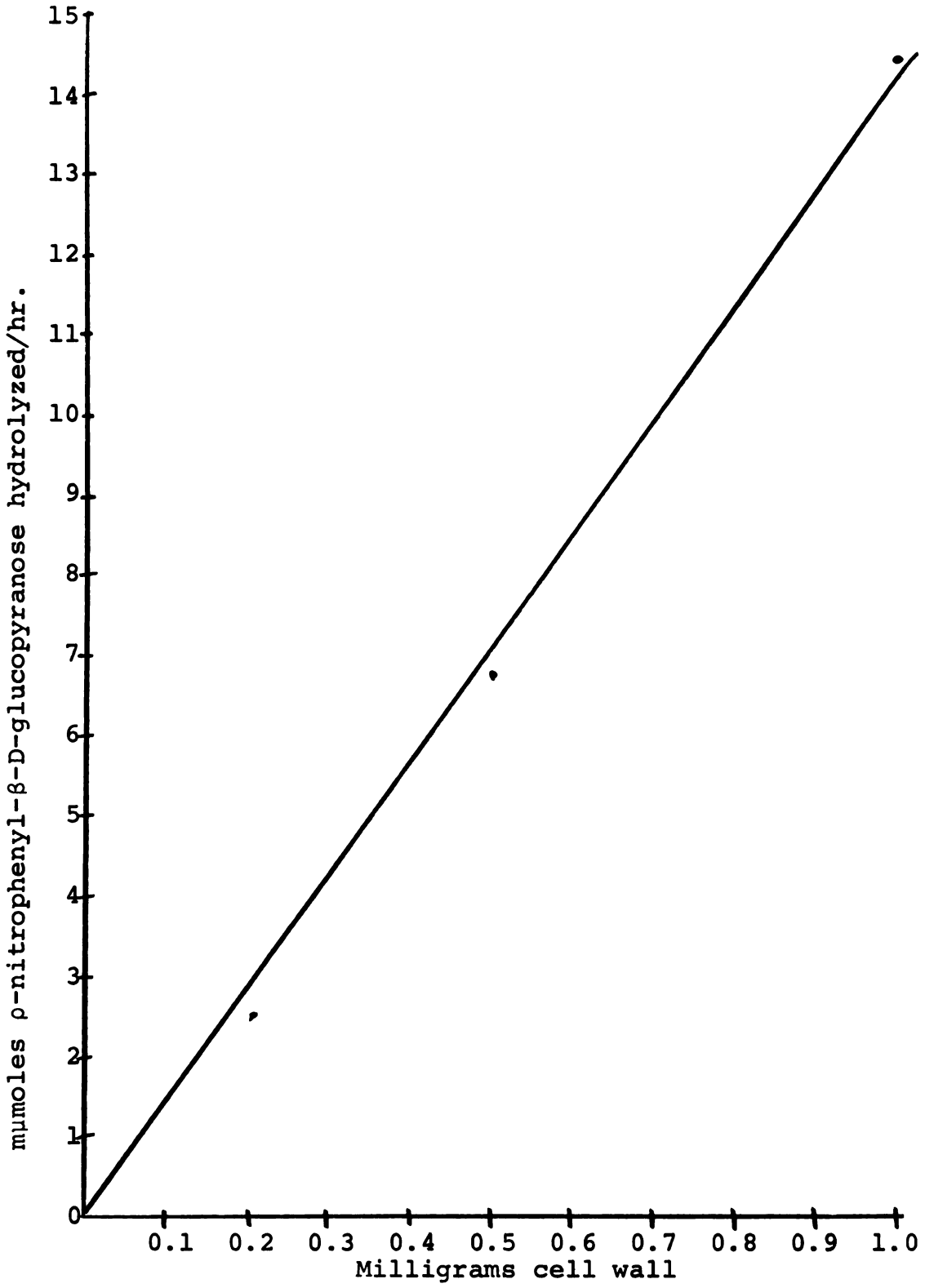


Figure 11. β-Glucosidase activity vs. amount of cell wall.

Substrate Conc.: 3 mM, Volume = 1.0 ml
Incubation Time: 15 minutes
Wall Preparations: IV = ·
V = ⊙

Figure 12. β-Glucosidase activity vs. amount of cell wall.

Substrate Conc.: 0.05 mM, Volume = 1.0 ml.
Incubation Time: 2 hours
Preincubation Time: 1 hour
Wall Preparation: 1 - 6B


mμmoles ρ-nitrophenyl-β-D-glucopyranose hydrolyzed/hr.
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Milligrams cell wall

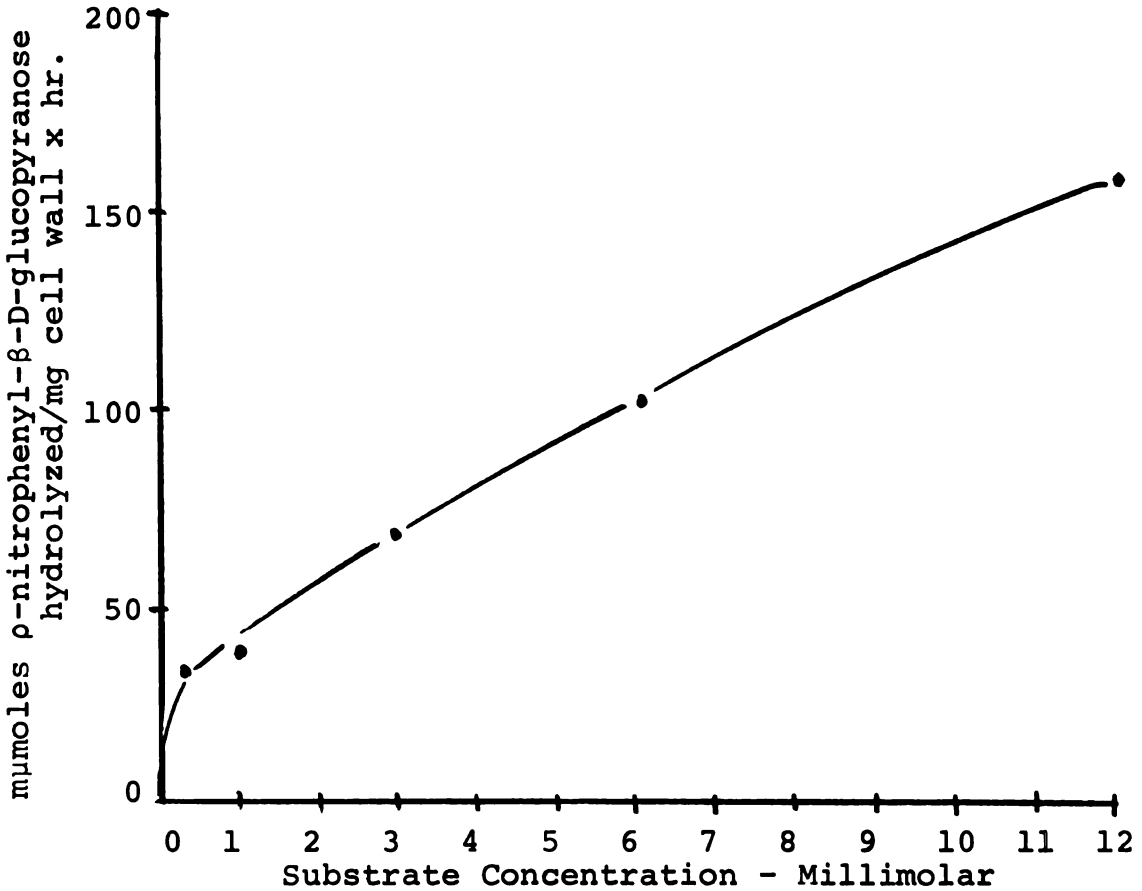


Figure 13. β-Glucosidase activity vs. substrate concentration.

Incubation Time: 30 minutes
Preincubation Time: 1 hour
Wall Preparation XI

was hydrolyzed during a typical experiment the error involved is small.

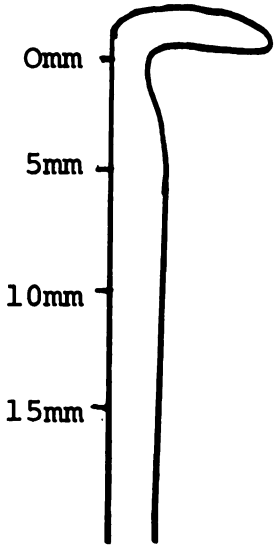
Table 2 shows the effect of preincubation on β-glucosidase activity. Two hours is sufficient time to hydrate the cell walls and that the activity is retained at least up to 54 hours at 0°. In another experiment, not shown, there was no loss of activity after 24 hours at room temperature. Thus, the enzyme is quite stable after the walls have been prepared.

Table 2. Effect of preincubation time on β-glucosidase activity. (Cell walls were suspended in buffer and kept at 0° prior to assay. Wall Prep. 2-2-C. Incubation time 2 hours.)

Preincubation Time (hrs.)	Activity: (mμmoles ρNP-β-D-Glu hydrolyzed/mg cell wall x hr.)
2	4.20
24	4.35
54	4.13

Figure 14 illustrates diagramatically the origin of the tissue sections used for cell wall preparations and their relative growth rates at the time of harvest. The DNA content and the relative number of cells in the sections are also shown. These are the data which should enable me to see whether wall enzyme activity increases and decreases in response to cell growth itself. Table 3 shows the protein content of cell walls isolated by the

PEA STEM SECTIONS



Section	Growth Rate (mm/hr)	Relative Rate
0-5mm	0.47	100
6-10mm	0.07	15
11-15mm	0.01	2

Section	DNA Content (mg/g fr. wt.)	Relative Cell Number
0-5mm	0.53 (100)	100
6-10mm	0.12 (23)	35
11-15mm	0.055 (10)	26

Figure 14. Regions of pea epicotyl studied.

Table 3. Protein content of cell wall preparations.

Tissue Section	Glycerol Prep. Walls Water Wash	Glycerol Prep. Walls Solvent Wash	Aqueous Prep. 4 x g Pellet
0-5mm	7.9%	7.2%	14.0%
6-10mm	6.7%	6.0%	9.0%
11-15mm	6.1%	6.0%	14.0%

three methods employed. It is evident that there are not large differences in protein content in cell walls prepared by grinding in glycerol with glass beads, although

there are differences in the case of the aqueous preparation. The values for the glycerol prepared walls agree with the values determined by King and Bayley (42) for cell walls prepared by the same method from the same tissue, but utilizing the micro-Kjeldahl method for determination of nitrogen.

The enzymatic activities of the cell walls isolated from the tissue sections are shown in Table 4. In all cases the specific activity on a weight basis is highest in the walls from the 0-5mm section, in most cases intermediate in the walls from the 6-10mm section, and lowest in the walls from the 11-15mm section. The specific activity was expressed on a wall weight basis because this seems to be more meaningful in this work than to express specific activity on a protein basis. However, if the activities are expressed on a protein basis, as shown for β-glucosidase activity in Table 5, the ratios of the specific activities for the various sections are not changed significantly. The β-galactosidase activity is predominantly a cytoplasmic enzyme and thus a contaminant cell wall activity, which is in keeping with the notion that the 4 x g pellet is contaminated with cytoplasmic protein as is suggested by its higher protein content. Table 6 gives the enzymatic activities of the 4 x g pellet on a DNA basis and on a cell number basis. In all cases the enzymatic activity expressed on a DNA

Table 4. Enzymatic activities of cell walls isolated from tissue from regions of differing growth rates.

	Glycerol Prep. Walls Water Wash (mμmoles pNP liberated/mg wall x hr.)	Aqueous Wall Prep. 4 x g Pellet (mμmoles pNP liberated/g fr. wt. x hr.)	***
Section	β-Glucosidase Activity		***
0-5mm	62.0 (100)	2942 (100)	289
6-10mm	15.0 (24)	1230 (42)	114
11-15mm	14.0 (23)	676 (23)	139
Section	α-Galactosidase Activity		***
0-5mm	60.5 (100)	2682 (100)	253
6-10mm	29.2 (48)	1264 (47)	117
11-15mm	22.9 (38)	1154 (43)	241
Section	Acid Phosphatase Activity		***
0-5mm	168.0 (100)	7645 (100)	749
6-10mm	42.2 (25)	4128 (54)	383
11-15mm	59.8 (35)	3526 (47)	734
Section	β-Galactosidase Activity		***
0-5mm	4.0 (100)	3272 (100)	321
6-10mm	1.8 (45)	1930 (59)	179
11-15mm	1.1 (28)	1284 (39)	268

***=Expressed on a per mg cell wall basis from the 4 x g pellet using the weight of the 4 x g pellet corrected for protein content down to the protein content of the glycerol-water walls.
( ) = relative activity.

Table 5. β-Glucosidase activity on a protein basis.

	Glycerol Prep. Walls Water Wash (mμmoles pNP liberated/μg protein x hr.)	Aqueous Prep. 4 x g Pellet (mμmoles pNP liberated/μg protein x hr.)
Section		
0-6mm	0.785	10.9
6-10mm	0.224	7.95
11-15mm	0.23	4.98

basis is lowest in the 0-5mm sections, intermediate in the 6-10mm sections and highest in the 11-15mm sections. The activity expressed on a cell number basis does not show the same pattern, and the differences are not as great as on a DNA basis.

The apparent Km values for the β-glucosidase activity for the cell walls from the 0-5mm and 11-15mm sections were determined and found not to be significantly different for walls from both sections as is illustrated in Figure 15.

The relative distribution of β-glucosidase, α-galactosidase, acid phosphatase, and β-galactosidase activities in the various fractions from the aqueous cell wall preparations are shown in Table 7. From this table it is evident that the most interesting cell wall enzymatic activity is the β-glucosidase, since more of the

Table 6. Cell wall enzyme activity vs. cell number.

	4 x g Pellet/DNA (mμmoles pNP liberated/mg. DNA x hr.)	4 x g Pellet/Rel. Cell No. (mμmoles pNP liberated/Rel. Cell No. x hr.)	
Section	β-Glucosidase		**
0-5mm	5,560	29.4	0.62
6-10mm	10,250	35.8	0.43
11-15mm	12,300	26.0	0.54
Section	α-Galactosidase		
0-5mm	5,070	26.8	0.60
6-10mm	10,530	36.1	0.83
11-15mm	21,000	44.4	0.88
Section	Acid Phosphatase		
0-5mm	14,420	76.4	1.68
6-10mm	34,400	118.0	1.20
11-15mm	64,250	142.0	2.30
Section	β-Galactosidase		
0-5mm	6,170	32.7	0.040
6-10mm	16,100	55.2	0.051
11-15mm	23,400	49.5	0.042

**=Glycerol prepared walls (water wash).

Figure 15. β-Glucosidase activity vs. substrate concentration.

Lineweaver-Burk plot; Km determination
Top and Bottom walls

Incubation Time: 2 hours
Preincubation time: 24 hours
Wall Preparation: 3-1 T lower line on L-B plot
upper line on V vs. S
3-1 B upper line on L-B plot
lower line on V vs. S

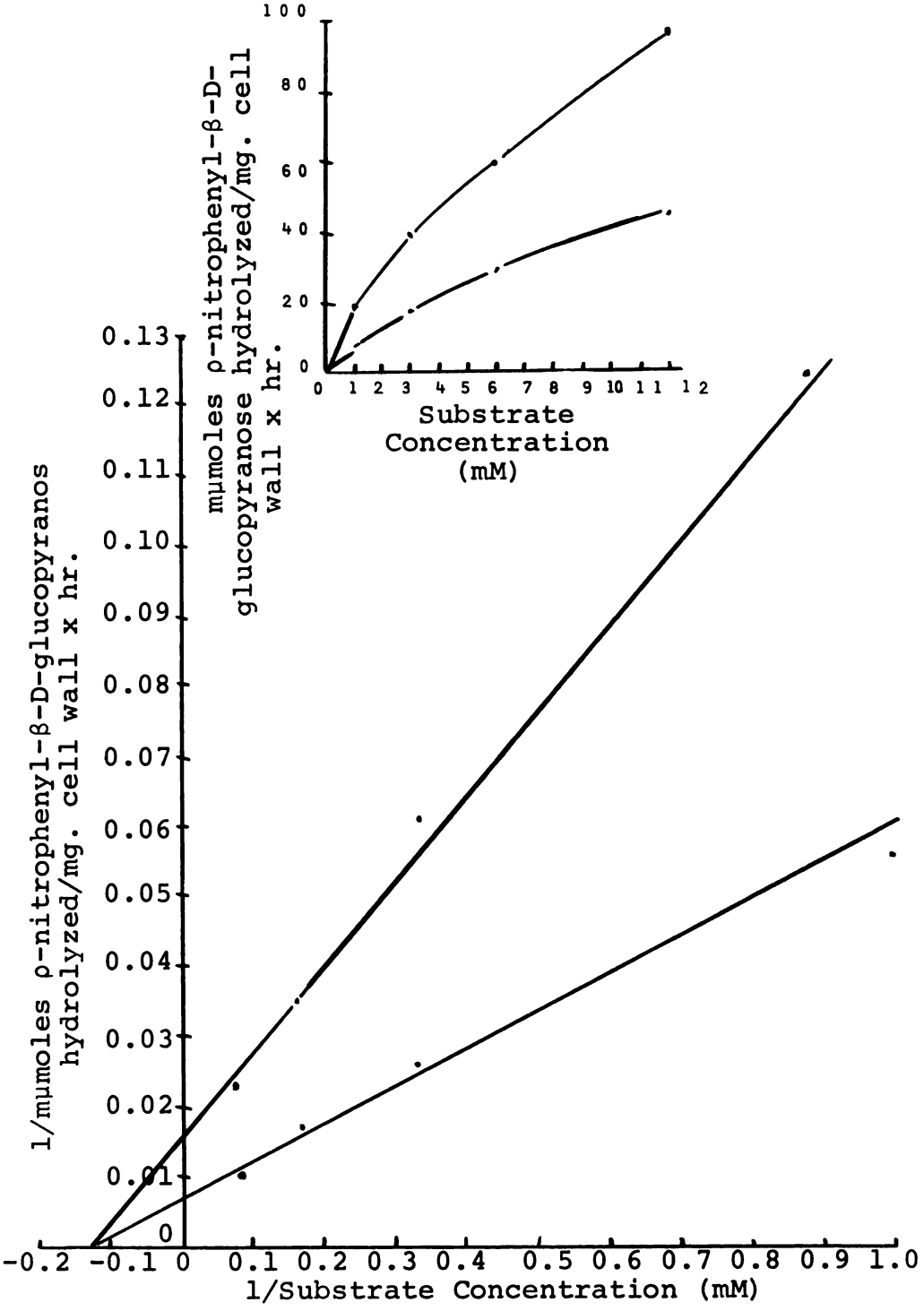

1/mμmoles ρ-nitrophenyl-β-D-glucopyranos hydrolyzed/mg. cell wall x hr.
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.2 -0.1 0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
1/Substrate Concentration (mM)
mμmoles ρ-nitrophenyl-β-D-glucopyranose hydrolyzed/mg. cell wall x hr.
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Substrate Concentration (mM)

Table 7. Relative distribution of enzymatic activities (as % total activity; homogenate = 100%).

Fraction	β-Glucosidase	α-Galactosidase	Acid Phosphatase	β-Galactosidase*
0-5mm Section				
Homogenate	100	100	100	100
4 x g Pellet	121	13.4	2.4	5.4
130 x g Pellet	40	9.2	2.9	6.2
100,000 x g Supernatant	14	41.4	84.6	46
6-10mm Section				
Homogenate	100	100	100	100
4 x g Pellet	126	14.1	2.4	7.1
130 x g Pellet	42	8.7	3.2	6.6
10,000 x g Supernatant	12	50.1	88.5	123
11-15mm Section				
Homogenate	100	100	100	100
4 x g Pellet	101	16.3	2.4	3.8
130 x g Pellet	40	10.3	3.2	3.8
10,000 x g Supernatant	18	45.9	98.8	90

*only represents one experiment.

(All activities were assayed at their pH optimum, Substrate Concentration = 1mM)
pH Optima: β-Glucosidase 5.8; α-Galactosidase 5.1; Phosphatase 5.5; β-Galactosidase 4.8.

activity is associated with the cell wall than any other fraction. The fact that more β-glucosidase activity is found in the 4 x g pellet than in the crude homogenate from which the pellet was isolated indicates that there is a substance in the homogenate which is inhibitory to the β-glucosidase associated with the 4 x g pellet. That this is, in fact, the case, is illustrated by the data of Table 8. No attempt has been made to isolate and identify the substance inhibitory to the β-glucosidase activity, although it was noted that 40% of the inhibitor was bound by MB-3 or Dowex-50 resins, while 60% passed through the columns, as is shown in Table 8.

One important question concerns the total amount of cell wall enzymatic activity per cell or in the tissue regions in question. Unfortunately, this question is left unanswered by this work since there is no way of determining the percentage recovery of cell wall. Dr. A. Kivilaan (personal communication), based on recovery experiments involving repeated reworking of cell extracts and pellets, feels that the glycerol method gives about a 25% yield. The yield of cell wall material with the methods utilized is given in Table 9; however, one can not say if the percent yield is the same for each of the tissue sections examined.

The time course of incorporation of radioactivity into the cell wall material is shown in Figure 16 as well

Table 8. Presence of substance inhibitory to cell wall β-glucosidase.

Incubation Mixture	mμmoles ρ-nitrophenol liberated/hr.	Inhibition (%)
0.5 ml Suspension 4 x g Pellet	142.0	---
0.3 ml 10,000 x g Supernatant	24.4	---
0.5 ml Suspension 4 x g Pellet + 0.3 ml 10,000 x g Supernatant	94.7	43%
0.5 ml Boiled 10,000 x g Supernatant	0.0	---
0.5 ml Suspension 4 x g Pellet + 0.3 ml Boiled 10,000 x g Supernatant	72.3	49%
0.5 ml Suspension 4 x g Pellet + 0.3 ml Boiled 10,000 x g Supernatant after Amberlite MB-3	97.0	32%
0.5 ml Suspension 4 x g Pellet + 0.3 ml Boiled 10,000 x g Supernatant after Dowex-50	99.1	30%

The 4 x g Pellet was from 0.5 g. of tissue and suspended in 6.0 ml. The boiled 10,000 x g Supernatant was recentrifuged after boiling for five minutes.

Concentration of inhibitory substance vs. inhibition

0.5 ml Suspension 4 x g Pellet	62.7	---
0.5 ml Suspension 4 x g Pellet + 0.03 ml Boiled 10,000 x g Supernatant	72.7	15% stimulation ??
0.5 ml Suspension 4 x g Pellet + 0.1 ml Boiled 10,000 x g Supernatant	53.0	15%
0.5 ml Suspension 4 x g Pellet + 0.3 ml Boiled 10,000 x g Supernatant	34.0	46%

In concentration experiment the 4 x g pellet of 0.5g tissue was suspended in 9.0ml.

Figure 16. Time course of radioactivity incorporated into cell wall material and β-glucosidase activity.

p-nitrophenol liberated
radioactivity
Substrate: p-nitrophenyl-β-D-($U^{14}C$)-glucopyranoside
Substrate Concentration: 1mM
Specific Activity: 0.12 μc/μmole

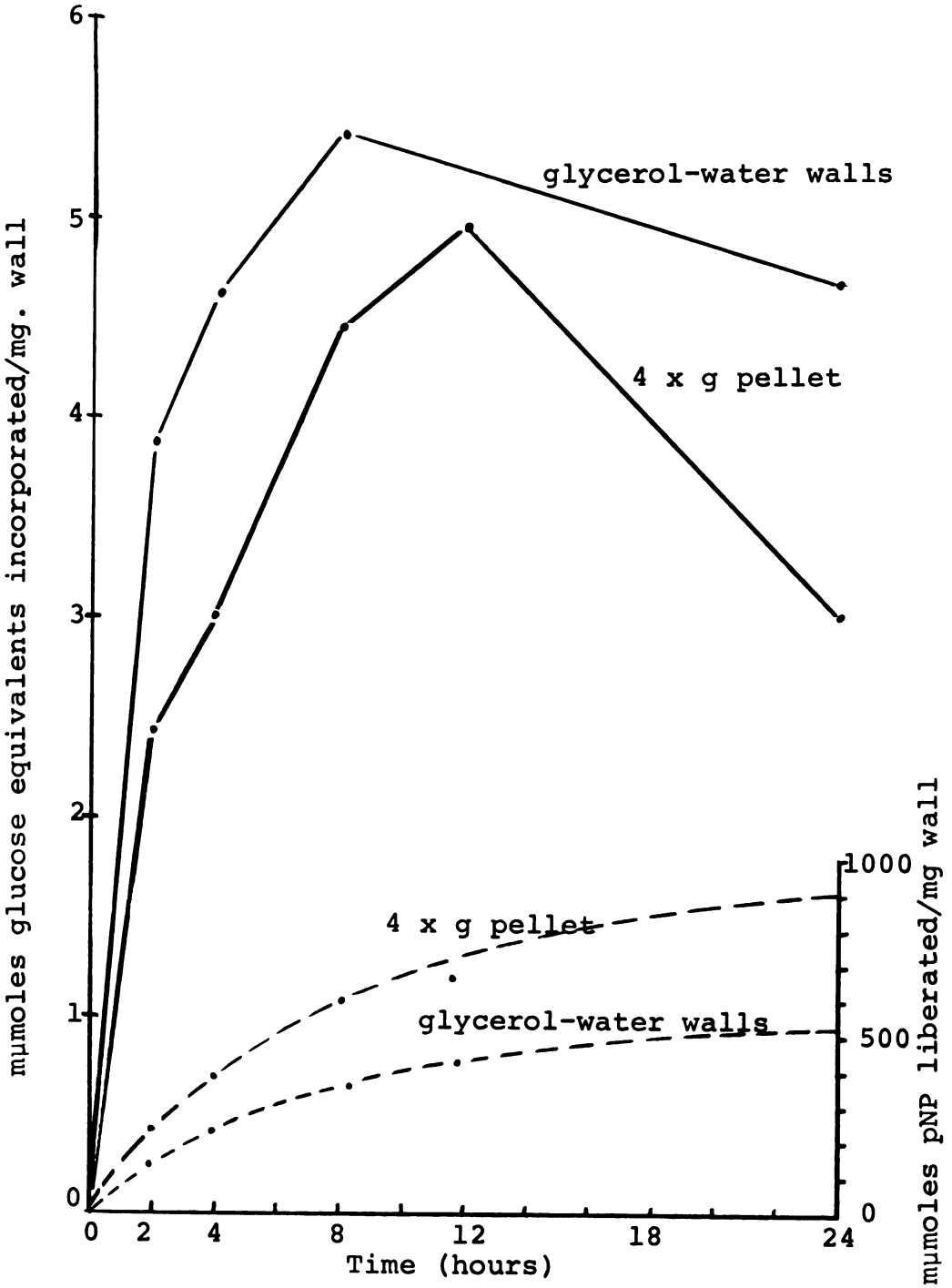

glycerol-water walls
4 x g pellet
mμmoles glucose equivalents incorporated/mg. wall
0
1
2
3
4
5
6
4 x g pellet
glycerol-water walls
1000
500
0
mμmoles pNP liberated/mg wall
0 2 4 8 12 18 24
Time (hours)

Table 9. Yield of cell wall material by isolation method.

Section	Method of cell wall isolation (mg cell wall material/g fr. wt. tissue)			
	Glycerol Prep. Walls			Aqueous Prep. 4 x g Pellet
	Water Wash	Solvent Wash		
		(Exp. 3-1)	(Exp. 3-2)	
0-5 mm	4.74	6.55	6.52	10.2
6-10 mm	3.39	5.55	4.25	10.8
11-15 mm	3.84	4.38	3.70	4.8

as the time course for the β-glucosidase activity. It appears that wall labeling activity, as determined by radioactivity on the walls, has saturated by 8 hours while the hydrolytic activity is still increasing at 24 hours. The apparent decrease in incorporated label into the wall at 24 hours might be attributed to exchange or hydrolysis of wall bound glucose, if it is, in fact, a real phenomenon. Table 10 illustrates the fact that nucleotide triphosphates do not affect the wall labeling activity. There is a stimulation by 1mM Mg^{++} as well as a possible stimulation by a lipid extract from the 4 x g pellet of the same tissue. Unfortunately, insufficent experiments were done to determine if the lipid extract effect is real. Lipid extractions of the labeled walls failed to remove any radioactivity.

Table 10. Factors affecting cell wall labeling activity.

Addition	mμmoles p-nitrophenol liberated/mg wall x hr.	CPM/mg. wall
Experiment 1		
Control	40.0	607
ATP (1mM) + $MgCl_2$ (1mM)	32.0	918
GTP (1mM) + $MgCl_2$ (1mM)	50.2	918
CTP (1mM) + $MgCl_2$ (1mM)	50.2	910
UTP (1mM) + $MgCl_2$ (1mM)	49.5	797
ATP (0.5mM) + GTP (0.5mM) + $MgCl_2$ (1mM)	39.0	887
$MgCl_2$ (1mM)	53.0	921
Experiment 2		
Control	46.7	889
$MgCl_2$ (10mM)	45.0	915
$CaCl_2$ (10mM)	40.0	793
0.05ml. Lipid extract in toluene	39.5	981
0.10ml. Lipid extract in toluene	43.0	1055

Cell walls used were glycerol-water wash cell wall preparation 4-2-T. Lipid extract was from one half of 4 x g pellet from 0.5g tissue. Four hour incubation time.

A brief attempt was made to determine what the product of the wall labeling activity was and how it was bound to the cell wall material. A large batch of labeled walls was made (365,000 CPM) in 49 mg of wall) and subjected to a classical cell wall fractionation procedure. Ninety per cent of the label (327,000 CPM) appeared in

the oxalate extract and 96% of that label (315,000 CPM) was present in the 80% ethanol supernatant after precipitation of the pectin fraction. These results indicate that the radioactivity was originally cell wall bound but was hydrolyzed off as a low molecular weight (not alcohol precipitable) compound by the weak acid hydrolysis. After removal of the oxalate the labeled material was chromatographed on a Sephadex G-15 column for which the profile is shown in Figure 17. Aliquots from the major radioactive peak (fraction 63), in the disaccharide region, and the minor radioactive peak (fraction 69), in the monosaccharide region, as well as the reducing sugar peak (fraction 70) were subjected to thin layer chromatography (Figure 18). The thin layer chromatogram was sprayed with alkaline permanganate to detect oxidizable compounds, including sugars. The area above the origin, where the material from the radioactivity peak was spotted, was scraped into scintillation vials and counted. The radioactivity corresponded in the scrapings with the spots shown on an autoradiogram of the plate. The radioactive spots from fractions 69 and 70 runs behind the glucose spot as shown after the permanganate spray. The radioactive spot from fraction 63 is slightly above the cellobiose spot which suggested that it might be laminaribiose. To check this possibility, an aliquot of fraction 63 was silylated and subjected to gas-liquid-chromatography

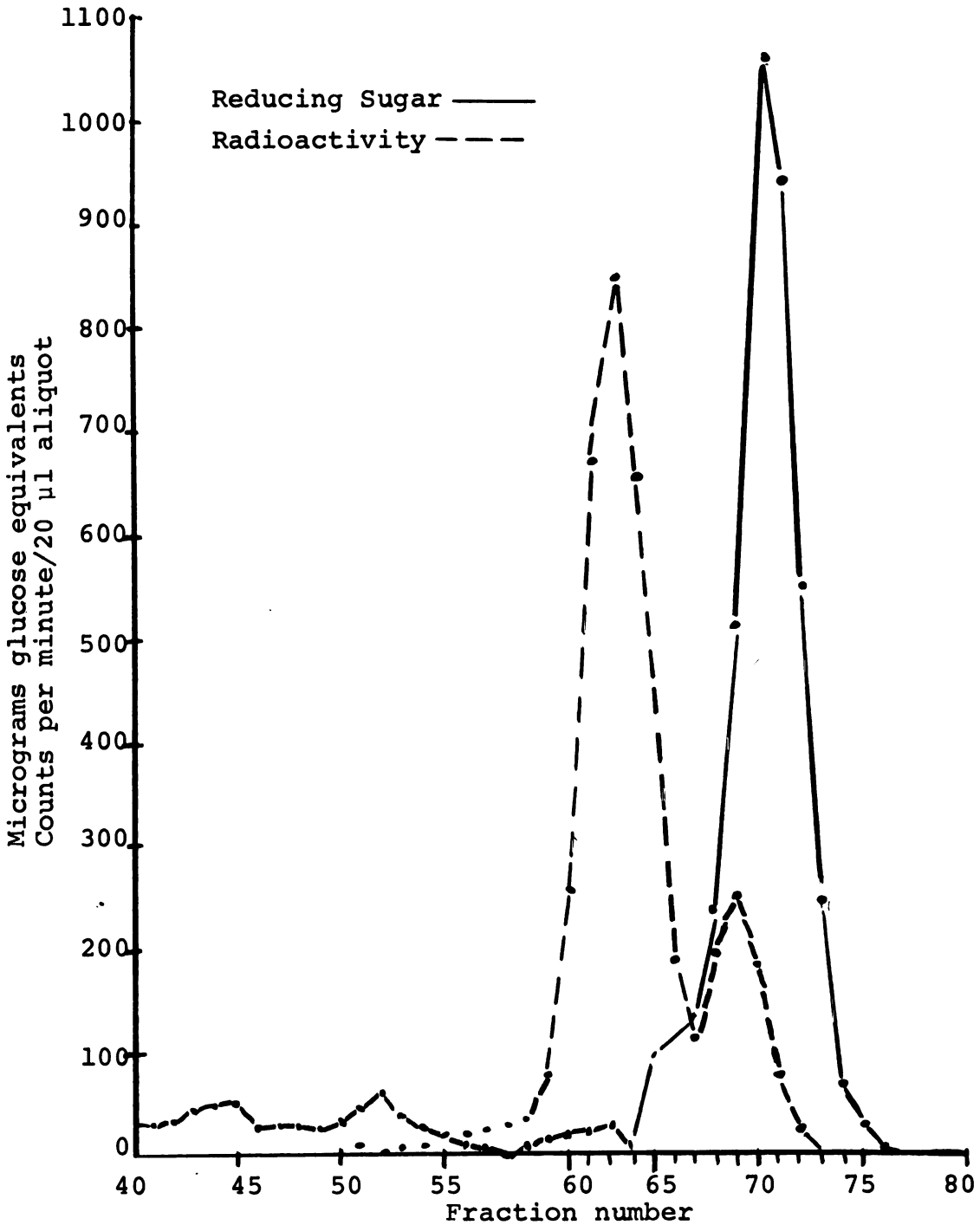


Figure 17. Profile of Sephadex G-15 column chromatography of radioactive product of cell wall labeling activity.

Figure 18. Thin layer chromatography of fractions from G-15 column.

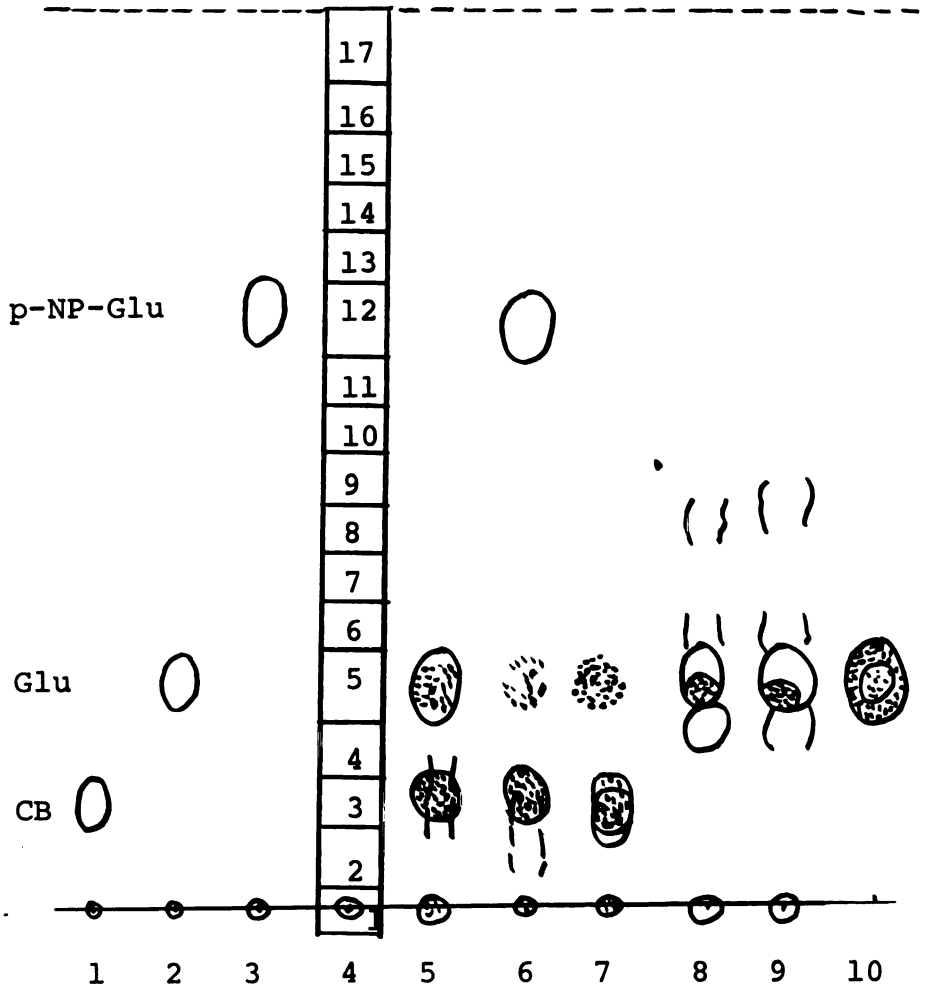
Spot	Material spotted
1	20 µg cellobiose
2	20 µg β-D-glucose
3	40 µg p-nitrophenyl-β-D-glucose
4	50 µl Fraction No. 63
5	50 µl Fraction No. 63 + 20 µg β-D-glucose (mixed before spotting)
6	50 µl Fraction No. 63 + 40 µg p-nitrophenyl-β-D-glucose
7	50 µl Fraction No. 63 + 20 µg cellobiose
8	50 µl Fraction No. 69
9	50 µl Fraction No. 70
10	20 µl ^{14}C-glucose

Spots which appeared after permanganate spray are outlined-- ○

Radioactive spots are represented by dotted spots-- ⁘

Radioactivity in regions scraped:

Region	Counts Per Minute
4-1	77
4-2	142
4-3	661
4-4	267
4-5	188
4-6	90
4-7	22
4-8	13
4-9	10
4-10	4
4-11	6
4-12	18
4-13	0
4-14	6
4-15	0
4-16	0
4-17	0

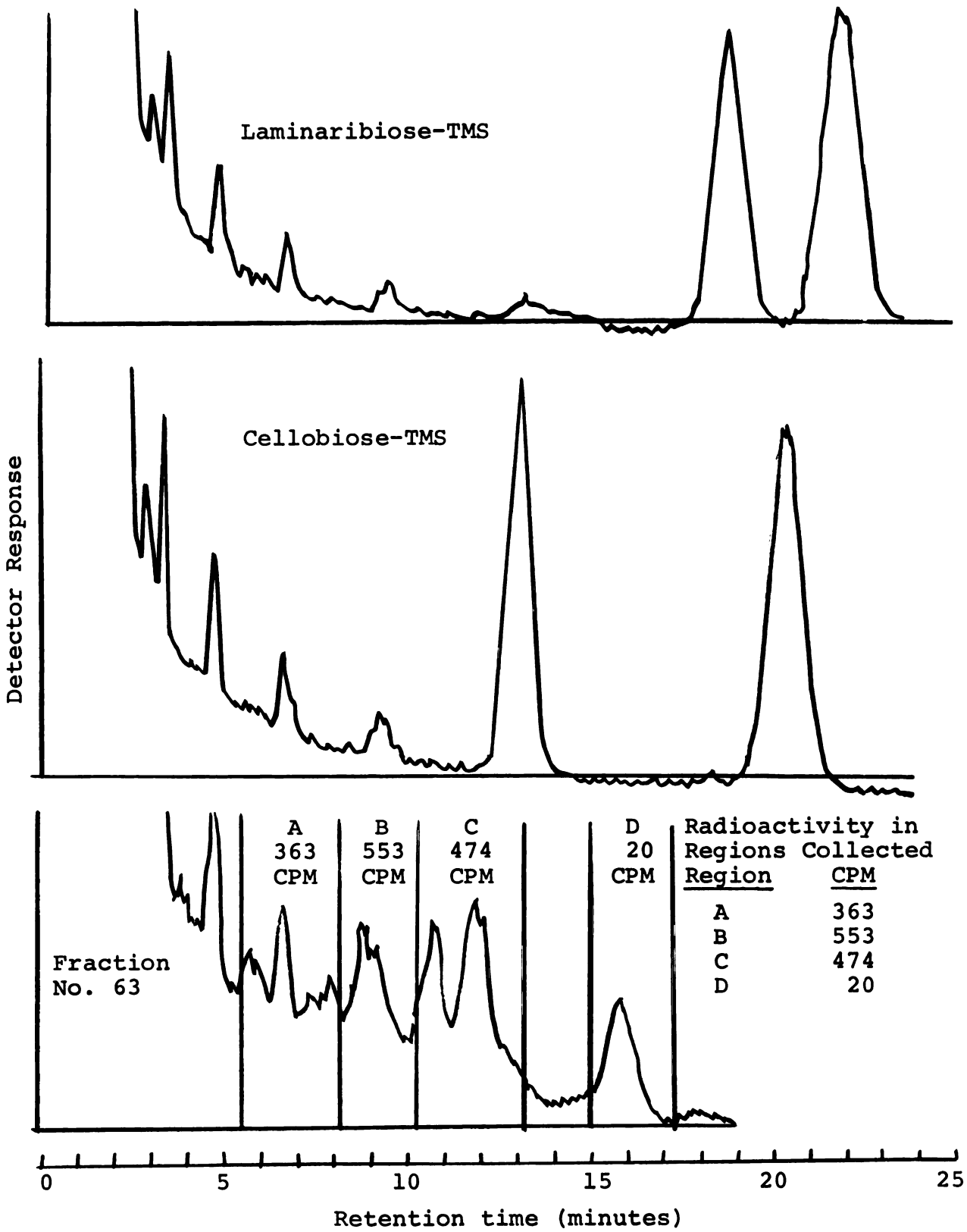

17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
p-NP-Glu
Glu
CB
1
2
3
4
5
6
7
8
9
10

along with cellobiose and laminaribiose standards. The sample gave no peaks in the region of either the α or β peaks of either cellobiose or laminaribiose, but it did yield several peaks which were collected and found to be radioactive. Based upon the behavior of the labeled compounds on Sephadex, TLC, and GLC (Figure 19), it would seem that they are about the size of disaccharides and have the chromatographic solubilities of disaccharides--but that they are not identical to laminaribiose, cellobiose, or gentiobiose.

Note: Further work has revealed that the radioactivity collected from GLC probably does not correspond to any of the observed peaks. The radioactivity comes off the column in the disaccharide region, but there is not enough of the compound(s) to detect on GLC.

Figure 19. GLC charts of TMS derivatives of products of wall labeling activity, cellobiose, and laminaribiose.

Samples silylated with pyridine:N-trimethylsilylimidazole (1:1) for 1 hour at 50°
Column: 2% OV-1 on Gas Chrom Z support (3mm x 6 ft.)
Oven temperature: 205°


Laminaribiose-TMS
Cellobiose-TMS
Detector Response
A
363
CPM
B
553
CPM
C
474
CPM
D
20
CPM
Fraction
No. 63
Radioactivity in
Regions Collected
Region CPM
A 363
B 553
C 474
D 20
0
5
10
15
20
25
Retention time (minutes)

DISCUSSION

Our present knowledge of cell wall structure is insufficient to determine which components of the wall are responsible for the rigid structure of the cell wall and, as a consequence, what types of linkages must be broken to permit extension growth. If we knew which bonds had to be broken we would then know what types of enzymatic activities might be expected to be quantitatively related to growth. Consequently, both structural and enzymatic approaches are valid since an enzymatic correlation with growth might direct the attention of investigators to the correct structural linkage. There is the possibility that a structural approach might fail if there is no net change in polysaccharide structure; that is, a linkage between macromolecules could be broken, the polysaccharides moved with respect to each other, and the cross linkages reformed. If a dynamic state of this break, move, and rejoin process does occur in cell walls, then it could be very hard to find structural changes associated with cell enlargement.

The fact that there are net changes in the levels of certain polysaccharides in cell walls that do occur during growth of plant cells seems to be fairly well

established (3, 56, 61, 68). It has been shown in Avena coleoptile sections (39, 73), Pisum sections (55) and in bean seedlings (68) that the acid soluble non-cellulosic glucose polymers of the cell wall increase and decrease in amount during extension growth. Several investigators have presented evidence that glucanases may play a role in the case of auxin induced extension growth of excised Avena coleoptile sections (58, 59, 60, 86, 93); however, there is some disagreement on this point (12, 79). Cell walls from rapidly growing tissues have been reported to have more glucanase or cellulase activity than cell walls isolated from slowly growing tissue (18, 33, 67), and a preliminary report of the present work (64).

The method employed for isolation of highly purified cell wall material in this work has been used in this laboratory for several years and by such criteria as homogeneity in the light and electron microscope appears to yield pure cell walls which compare favorably with walls isolated by related methods (6).

β-glucosidase activity has been reported to be localized in the cell wall (10) or associated with the cell surface external to the plasmalemma (41). In this work most of the β-glucosidase activity was found to be associated with a crude cell wall preparation. It is possible that the reason for very little soluble activity may be due to the presence of the inhibitory substance.

If the inhibitor is no more effective against soluble activity than it is against the cell wall associated activity, then it appears that most of the activity is associated with the cell wall. No attempt was made to characterize the inhibitor. D-glucono-(1-5)-lactone has been shown to be a potent inhibitor of β-glucosidase with 0.016 mM lactone giving 50% inhibition in the presence of 5 mM substrate (14). Another possibility is that the inhibitor may be the naturally occuring substrate for the β-glucosidase and, in fact, be favored over the p-nitrophenyl glucose, which is certainly an unnatural substrate.

It has been shown that ^{14}C-labeled cell wall polysaccharides, prepared by dilute acid extraction of prelabeled plant material, can be hydrolyzed first to oligosaccharides and then to free glucose by a cell wall preparation from Avena coleoptiles (40). Thus, there are enzymes in the wall which hydrolyze components extracted from the walls. Similarly, cell wall preparations from corn coleoptiles are capable of autolysis in vitro (51) and during this autolysis there is an enzymatic solubilization of a lichenan type glucan (β 1→3 and β 1→4 linked D-glucose) and some free glucose from the cell wall material (43). Clearly then, cell wall glucans can be hydrolytically cleaved from the wall by wall bound enzymes and there is thus the possibility that such cleavage would alter the mechanical rigidity of the wall.

From my own work, it can be said that the specific activity of β-glucosidase associated with the cell walls isolated from tissues with different growth rates is correlated with the growth rate. Thus, cell walls isolated from rapidly growing tissue have a higher β-glucosidase specific activity than do cell walls isolated from slowly growing tissue. On the basis of the relative cell number in the sections used it appears that the activity is directly related to the cell number. Each cell has a certain amount of enzymatic activity no matter what the cell size happens to be and thus the lower specific activity in the walls isolated from the larger non-elongating cells is due to the dilution of the enzyme activity by more wall material. This suggests that the cell wall associated β-glucosidase does not exert a tightly coupled control over cell wall extension. It may be that several bonds must be broken simultaneously to allow the cell wall to begin extending and that this can not occur until a critical enzyme concentration is reached in the wall. Once extension has started, the β-glucosidase could then be in excess during elongation. When the enzyme concentration falls below another critical level possibly extension can no longer occur. However, during the time in which the enzyme concentration is in excess the rate of elongation would be subject to some other control, such as availability of a cell wall precursor or a cofactor,

or, in some cases, the hydrostatic pressure of the cell contents. The β-glucosidase may be a necessary, but not sufficient, factor in the control of cell wall elongation.

One can thus propose two kinds of models, assuming that cell wall enzymes have anything at all to do with growth.

Model A--Enzyme Limiting and Controlling

In this model the rate determinant of extension growth would be the concentration of glycosidase per unit of wall. Very young, non-vacuolated cells would not extend due to lack of turgor. As turgor developed, the wall would be softened by glycosidase activity. As new cell wall material is deposited, glycosidases would be diluted until the enzyme could not hydrolyze sufficient bonds in a unit of volume to permit further wall deformation.

Model B--Enzyme Non-Limiting, Co-Factor or Precursor Control

In this model one would assume that there is always enough glycosidase to permit wall deformation and sufficient turgor to drive the deformation. The control mechanism then would be a co-factor stimulation or inhibition of glycosidase activity, or the availability of wall precursors.

Our present data do not enable a decision to be made between these possibilities and indicates only that glycosidase activity per cell is the same for growing and nongrowing cells while the specific activity per unit of wall mass changes.

Although the DNA content of the tissue was not a valid parameter of cell number in this work, it may be a valid parameter of cell number in older tissue as in the work of Maclachlan et al. (54). The discrepancy between the cell numbers and DNA content of the tissue may be due to all cells not having the same amount of DNA (88, 89, 90) or possibly the efficiency of extraction is not the same for all three sections examined.

The Lineweaver-Burk plot of the β-glucosidase activity of the cell walls from the fastest growing and the non-elongating sections shows that in both cases the enzyme has the same apparent Km (8.3×10^{-3}). This Km is within the range of those for other β-glucosidases (32). This fact along with the activity appearing to be a function of cell number leads one to believe that the reason for the lower specific activity in the cell walls from the non-elongating region is due to the presence of less enzyme per unit of wall weight. There is no reason to suspect an inhibitor being responsible for the difference in activity of the isolated cell walls. An inhibitor, if active, would be a non-competitive one on the basis of

the Lineweaver-Burk plot (22). One type of non-competitive inhibition is that in which the enzyme-inhibitor-substrate complex does not breakdown and results in a rate dependent on the breakdown of the enzyme-substrate complex which in effect reduces the amount of active enzyme (22).

In the following sections I intend to indicate a number of unsolved problems that I believe have relevance to the general problem of wall extension and wall metabolism.

Unfortunately, the one bit of data which would be desirable but which could not be obtained was the yield of isolated cell wall per cell. It is not possible to calculate how much of the total cell wall is recovered since we do not know the total amount of cell wall material in the tissue prior to disruption and wall isolation. The working assumption was that the β-glucosidase activity is uniformly distributed throughout (or associated with) all of the cell wall material so that in the event of different yields of cell wall from the different sections, the relative specific activities of the isolated walls would not be altered. The yield of the 4 x g pellet in the aqueous preparations from the three regions of tissue used does not appear to be the same by comparison of the specific activities. Alternatively, the contamination of the 4 x g pellet is not the same from each of the regions, as can be seen from the protein content (Table 3),

and this contaminating protein is enzymatically active. This contamination is possibly from membrane material adjacent to the cell wall, suggesting that most of the β-glucosidase may actually be attached to the membrane but functioning in the cell wall.

It should be mentioned that the β-glucosidase activity associated with the highly purified cell wall preparation is quite stable while the cell walls are in suspension, as illustrated by the results in Table 2. Not only is the β-glucosidase activity stable for several days at 0°, in one experiment it was noted that there was no loss of activity when a cell wall suspension was allowed to stand at room temperature for 24 hours. There is variability between cell wall preparations for reasons unknown at this time. It should be noted that the enzymatic activity associated with the glycerol isolated-solvent washed cell walls is significantly lower than that for glycerol isolated-water washed cell wall preparations. This lower activity is presumably due to partial inactivation of the enzyme by the solvent wash and could explain the variability between different preparations. The variability between different preparations of the glycerol isolated-water washed cell wall preparations was not critically studied.

It is also possible that the variability between cell wall preparations has something to do with tissue

variability from one batch of tissue to another although this would seem unlikely since the tissue was grown under uniform conditions. In some experiments the tissue was split in two parts and separate cell wall preparations were made by the glycerol-solvent wash method. In these cases the β-glucosidase activities of the two preparations were always within one or two per cent of each other. Another point which would suggest tissue variability is that all preparations of the 4 x g pellet made from tissue harvested at the same time had enzyme activities within 5 to 10 per cent of each other. On the other hand, there was more variability between β-glucosidase activity of the 4 x g pellet from tissue harvested several months apart. This may be due to the subjectivity in selection of seedlings to harvest, that is to say, I may have chosen slightly different seedlings after an absence from cutting sections. The point is that there is variability between cell wall preparations (see Figures 10 and 11) and I don't know what the reason is for the variability. The reason for lower β-glucosidase activity in cell walls that were isolated from tissue sections that had been incubated for 16 hours on 2% sucrose and phosphate buffer is also unknown. These sections were also taken from seedlings 7 days old, if that makes any difference. No experiments were done to look at the effect of tissue age or the effect of incubation on the excised sections. Thus, an

extension of these experiments would be to determine the stability of glycosidases in excised stored tissue and also to examine sections from much older and much younger tissue.

The substrate specificity of the cell wall β-glucosidase was not studied and can not be determined unless the enzyme is isolated and purified. There is no evidence to suggest that there is more than one β-glucosidase associated with isolated cell wall material. However, it is certain that p-nitrophenyl-β-D-glucose is not the naturally occurring substrate for the enzyme. This substrate was chosen due to the highly sensitive assay possible. It can not be determined from this work if the observed cell wall labeling activity is significant in cell wall metabolism. The assay conditions used may not favor transferase activity. Although transferase and hydrolase activity may reside in the same enzyme molecule, it is not possible to say if the two activities are possessed by the same enzyme from this work. Such determination also depends on utilization of the isolated and purified enzyme. The Tris inhibition of the cell wall β-glucosidase suggests that the glucosidase also has transferase activity. Nelson et al. (66) have shown that protonated hydroxyalkyl-substituted amines inhibit the glucosidase-transferase activity of amylo-1,6-glucosidase-oligo-1, 4→1,4-transferase from rabbit muscle.

The cell wall labeling activity observed, unfortunately, has not shown incorporation of β-D-glucose into a polysaccharide. This may be due to the fact that the ammonium oxalate-oxalic acid extraction was too harsh and caused extensive hydrolysis as demonstrated by the large amount of free glucose obtained. Keeping this in mind, it is somewhat surprising that all of the label was not present as free glucose. Most of the label appears to be present in a disaccharide-like compound, possibly a sugar and a sugar alcohol or uronic acid. If this is the case, then one asks why the label was not removed from the wall be the washings before the oxalate extraction. It is most interesting that the label is not present in cellobiose, laminaribiose, or gentiobiose. At any rate the experiments with the ^{14}C labeled substrate ask more questions than they answer. The time course of the cell wall labeling activity suggests an incomplete system or saturation of acceptor sites or possibly even turnover if the decrease in incorporation between 12 and 24 hours is real.

It is hard to say that the α-galactosidase or acid phosphatase activities may be involved directly in cell wall metabolism during growth since these activities associated with the cell wall are only a small portion of the total activities. It is not surprising that the activities of enzymes not associated with cell wall extension are also increased in rapidly growing tissue

since all metabolic activity would be higher in the growing cells. Possibly these enzymes are not associated with the cell wall *in vivo* and they are due to contamination of the isolated cell walls. I feel that the β-galactosidase activity is a contaminant on the cell wall material since there is very little activity associated with the more highly purified cell walls and most of the activity is in the soluble fraction.

It may appear that there is a contradiction between my findings and those of Nevins (67), who found that β-glucosidase activity in bean hypocotyls increased from 3 days after imbibition to a maximum at 5 days and then declined. The activity peak immediately preceeded the logarithmic phase of growth. It should be noted that he was looking at total β-glucosidase activity in whole bean hypocotyls with respect to the age of the seedlings. My results give insight into what is going on in different regions of the pea seedling at a definite time and this a very short time. The time at which my tissue was harvested was during the log phase of growth and no attempt was made to study the enzyme activity of isolated cell walls from the same section of tissue with respect to age. Data comparable to those of Nevins could be obtained by marking the sections that I did use but not harvesting them until two or three days later.

SUMMARY

In conclusion, the present work shows that there is a correlation between the specific activity of β-glucosidase associated with isolated cell walls and the growth rate of the tissue from which the cell walls were isolated. The β-glucosidase activity of the sections examined was found to be a function of the number of cells in the section. Most of the β-glucosidase activity is associated with the cell wall and is inhibited by something in the soluble fraction. Finally, there appears to be a wall labeling activity associated with the isolated cell walls when p-nitrophenyl-β-D($U^{14}C$)-glucopyranoside is used as the substrate. However, the product(s) of the reaction has not been identified and it is not known if the β-glucosidase and wall labeling activity are present in the same enzyme molecule.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Albersheim, Peter. 1965. Substructure and function of the cell wall. In Plant Biochemistry. J. Bonner and J. E. Varner, Eds. Academic Press Inc., New York and London. 151-186.

2. _____. 1965. Biogenesis of the cell wall. In Plant Biochemistry. J. Bonner and J. E. Varner, Eds. Academic Press Inc., New York and London. 298-321.

3. Baker, David B. and Peter M. Ray. 1965. Direct and indirect effects of auxin on cell wall synthesis in oat coleoptile tissue. Plant Physiol. 40: 345-352.

4. Barczai-Martos, M. and F. Korosy. 1950. Preparation of acetobrome-sugars. Nature. 165: 369.

5. Barker, S. A., E. J. Bourne, G. C. Hewitt, and M. Stacey. 1955. Studies of Aspergillus niger. Part IV. The Synthesis of β-linked glucosaccharides. J. Chem. Soc. 3734-3740.

6. Bean, R. C. and L. Ordin. 1961. A study of procedures for isolation and fractionation of plant cell walls. Anal. Biochem. 2: 544-557.

7. Bonner, J. 1935. Zum Mechanismus der Zellstreckung auf Grund der Micellarlehre. Jahrb. Wiss. Bot. 82: 377-412.

8. Bryan, Walter H. and E. H. Newcomb. 1954. Stimulation of pectin methylesterase activity of cultured tobacco pith by indoleacetic acid. Physiol. Plantarum. 7: 290-297.

9. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315-323.

10. Chkanikov, D. I., G. A. Tarabrin, A. M. Shabanova, and P. F. Konstantinov. 1969. Localization of β-glucosidase in the cells of higher plants. Fiziologiya Rastenii. (Transl. from Russian) 16: 322-325.

11. Clark, John M., ed. 1964. Experimental Biochemistry. W. H. Freeman and Co., San Francisco. 95 and 217.

12. Cleland, Robert. 1968. Auxin and wall extensibility: reversibility of auxin-induced wall-loosening process. Science. 160: 192-194.

13. _____. 1971. Cell wall extension. Ann. Rev. Plant Physiol. 22: 197-222.

14. Conchie, J., A. L. Gelman and G. A. Levvy. 1967. Inhibition of glycosidases by aldonolactones of corresponding configuration: The C-4- and C-6-specificity of β-glucosidase and β-galactosidase. Biochem. J. 103: 609-615.

15. Cooil, Bruce J. and James Bonner. 1967. The nature of growth inhibition by calcium in the Avena coleoptile. Planta. 48: 696-723.

16. Courtois, J. E. and M. Leclerc. 1956. 'Etude de l'action transferante de la β-glucosidase de l'emulsine d'amandes. Bull. Soc. Chim. Biol. 38: 365-375.

17. Crook, E. M. and B. A. Stone. 1957. The enzymic hyrolysis of β-glucosides. Biochem. J. 65: 1-12.

18. Datko, Anne Harmon and G. A. Maclachlan. 1970. Patterns of development of glycosidase activities in the pea epicotyl. Can. J. Bot. 48: 1165-1169.

19. DeJong, Donald W. 1967. An investigation of the role of plant peroxidase in cell wall development by the histochemical method. J. Histochem. Cytochem. 15: 335-346.

20. Dever, John E., Jr., Robert S. Bandurski, and A. Kivilaan. 1968. Partial chemical characterization of corn root cell walls. Plant Physiol. 43: 50-56.

21. Dippel, L. 1878. Die neue Theorie uber die feinere Struktur der Zellhulle, betrachtet an der Hand der Tatsachen. Frankfurt.

22. Dixon, Malcolm and Edwin C. Webb. 1964. Enzymes. Second edition. Academic Press Inc., New York.

23. Duncan, W. A. M., D. J. Manners, and J. L. Thompson. 1959. Enzyme systems in marine algae. 3. Trans-β-glucosylation by extracts of Cladophora rupestris and Ulva lactuca. Biochem. J. 73: 295-298.

24. Frey-Wyssling, A. 1950. Physiology of cell wall growth. Ann. Rev. Plant Physiol. 1: 169-182.

25. Ghuysen, Jean-Marie. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bact. Rev. 32: 425-464.

26. Glaser, Erhard and Wilhelm Wulwek. 1924. Uber neue synthetisch dargestellte Nitrophenolglucoside mebst Beitragen zur Desinfektionskraft und Giftigkeit der Nitrophenole. Biochem. Zeit. 145: 514-534.

27. Glasziou, K. T. 1969. The localisation and properties of pectin methylesterase of Avena coleoptiles. Physiol. Plantarum. 12: 670-680.

28. Good, Norman E., G. Douglas Winget, Wilhelmina Winter, Thomas N. Connolly, Seikichi Izawa, and Raizada M. M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry. 5: 467-477.

29. Green, P. B., R. O. Erickson, and J. Buggy. 1971. Metabolic and physical control of cell elongation rate: in vivo studies in Nitella. Plant Physiol. 47: 423-430.

30. Hall, M. A. and L. Ordin. 1967. Subcellular location of phosphoglucomutase and UDP glucose pyrophosphorylase in Avena coleoptiles. Physiol. Plantarum. 20: 624-633.

31. Hallaway, Mary, P. D. Phethean, and J. Taggart. 1970. A critical study of the intracellular distribution of ascorbate oxidase and a comparison of the kinetics of the soluble and cell-wall enzyme. Phytochem. 9: 935-844.

32. Hestrin, Shlomo, David S. Feingold, and Michael Schramm. 1955. Hexoside hydrolases. In Methods in Enzymology, Vol. I., Sidney P. Colowick and Nathan O. Kaplan, Eds. Academic Press, Inc., New York. 231-257.

33. Heyn, Anton N. J. 1969. Glucanase activity in coleoptiles of Avena. Arch. Biochem. Biophys. 132: 442-449.

34. _____. 1970. Dextranase activity and auxin-induced cell elongation in coleoptiles of Avena. Biochem. Biophys. Res. Comm. 38: 831-837.

35. Horikoshi, Koki and Yonosude Ikeda. 1965. Studies on the spore coats of Aspergillus oryzae. II. Conidia coatbound β-glucosidase. Biochim. Biophys. Acta. 101: 352-357.

36. Hutson, D. H. 1964. Trans-β-glucosylation by alfalfaseed enzymes. Biochem J. 92: 142-146.

37. Jansen, Eugene F., Rosie Jang, and James Bonner. 1960. Binding of enzymes to Avena coleoptile cell walls. Plant Physiol. 35: 567-574.

38. Jervis, L. and Mary Hallaway. 1970. Isolation of ribosomes from cell-wall preparations of barley (Hordeum vulgare). Biochem. J. 117: 505-507.

39. Katz, M. and L. Ordin. 1967. Metabolic turnover in cell wall constituents of Avena sativa L. coleoptile sections. Biochim. Biophys. Acta. 141: 118-125.

40. _____. 1967. A cell wall polysaccharide-hydrolyzing enzyme system in Avena sativa L. coleoptiles. Biochim. Biophys. Acta. 141: 126-134.

41. Keegstra, Kenneth and Peter Albersheim. 1970. The involvement of glycosidases in the cell wall metabolism of suspension-cultured Acer pseudoplatanus cells. Plant Physiol. 45: 675-678.

42. King, N. J. and S. T. Bayley. 1965. A preliminary analysis of the proteins of the primary cell walls of some plant cells. J. Expt. Bot. 16: 294-303.

43. Kivilaan, A., Robert S. Bandurski, and A. Schulze. 1971. A partial characterization of autolytically solubilized cell-wall glucan. Plant Physiol. In press.

44. Kivilaan, Aleksander, Teofila C. Beaman, and Robert S. Bandurski. 1969. A partial chemical characterization of maize coleoptile cell walls prepared with the aid of a continually renewable filter. Nature. 184: B.A. 81-82.

45. Kivilaan, A., T. Cabrera-Beaman, and R. S. Bandurski. 1961. Enzymatic activities associated with cell wall preparations from corn coleoptiles. Plant Physiol. 36: 605-610.

46. Knox, K. W. 1965. Enzymic synthesis of O-β-D-glucopyranosyl-(1-6)-D-galactose. Biochem. J. 94: 534-535.

47. Knox, R. B. and J. Heslop-Harrison. 1969. Cytochemical localization of enzymes in the wall of the pollen grain. Nature. 223: 92-94.

48. Lai, Yee Foong and J. E. Thompson. 1970. 5'-nucleotidase and glucose-6-phosphatase in a purified cell wall fraction from Phaseolus vulgaris. Phytochem. 9: 1017-1021.

49. Lamport, Derek T. A. 1965. The protein component of primary cell walls. Adv. Bot. Res. 2: 151-213.

50. _____. 1970. Cell wall metabolism. Ann. Rev. Plant Physiol. 21: 235-270.

51. Lee, Su-hwa, A. Kivilaan, and Robert S. Bandurski. 1967. In vitro autolysis of plant cell walls. Plant Physiol. 42: 968-972.

52. Lennarz, W. J. and Barbara Talamo. 1966. The chemical and enzymatic synthesis of mannolipids in Micrococcus lysodeikticus. J. Biol. Chem. 241: 2707-2719.

53. Lockhart, J. A. 1965. Cell Extension. In Plant Biochemistry. J. Bonner and J. E. Varner, eds. Academic Press, Inc., New York and London. 826-849.

54. Maclachlan, G. A., Anne Harmon Datko, John Rollit, and Elaine Stokes. 1970. Sugar levels in the pea epicotyl: regulation by invertase and sucrose synthetase. Phytochem. 9: 1023-1030.

55. Maclachlan, G. A., and C. T. Duda. 1965. Changes in concentration of polymeric components in excised pea-epicotyl tissue during growth. Biochim. Biophys. Acta. 97: 288-299.

56. Maclachlan, G. A., and M. Young. 1962. Breakdown and synthesis of cell walls during growth. Nature. 195: 1319-1321.

57. Manners, D. J., J. R. Stark, and D. C. Taylor. 1971. Studies on carbohydrate-metabolising enzymes. Part XXIII. Trans-β-glucosylation by extracts of Tetrahymena pyriformis and Ochromonas malhamensis. Carbohyd. Res. 16: 123-132.

58. Masuda, Yoshio. 1968. Role of cell-wall-degrading enzymes in cell-wall loosening in oat coleoptiles. Planta. 83: 171-184.

59. Masuda, Yoshio, Susumu Oi, and Yukio Satomura. 1970. Further studies on the role of cell-wall-degrading enzymes in cell-wall loosening in oat coleoptiles. Plant and Cell Physiol. 11: 631-638.

60. Masuda, Yoshio and Shunji Wada. 1967. Effect of β-1,3-glucanase on the elongation growth of oat coleoptiles. Bot. Mag. Tokyo. 80: 100-102.

61. Matchett, William H. and James F. Nance. 1962. Cell wall breakdown and growth in pea seedling stems. Am. J. Bot. 49: 311-319.

62. Mertz, Dan. 1961. Distribution and cellular localization of ascorbic acid oxidase in the maize root tip. Am. J. Bot. 48: 405-413.

63. Muhlethaler, Kurt. 1967. Ultrastructure and formation of plant cell walls. Ann. Rev. Plant Physiol. 18: 1-24.

64. Murray, Allen K. and Robert S. Bandurski. 1970. Cell wall enzymes and growth rate. Plant Physiol. (Suppl.) 46: S-13.

65. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

66. Nelson, T. E., E. Kolb, and J. Larner. 1969. Purification and properties of rabbit muscle amylo-1, 6-glucosidase-oligo-1,4→1,4-transferase. Biochemistry 8: 1419-1428.

67. Nevins, Donald J. 1970. Relation of glycosidases to bean hypocotyl growth. Plant Physiol. 46: 458-462.

68. Nevins, Donald J., Patricia D. English, and Peter Albersheim. 1968. Changes in cell wall polysaccharides to bean hypocotyl growth. Plant Physiol. 46: 922.

69. Nisizawa, K. and Y. Hashimoto. 1970. Glycoside hydrolases and glycosyl transferases. In The Carbohydrates: Chemistry and Biochemistry. W. Pigman and D. Horton, eds. Academic Press, Inc., New York and London. 241-300.

70. Northcote, D. H. 1969. The synthesis and metabolic control of polysaccharides and lignin during the differentiation of plant cells. In Essays in Biochemistry. Vol. 5. P. N. Campbell and G. D. Greville, eds. Academic Press, Inc., New York and London. 90-137.

71. Phethean, P. D., L. Jervis, and Mary Hallaway. 1968. The presence of ribonucleic acid in the cell walls of higher plants. Biochem. J. 108: 25-31.

72. Pollock, M. R. 1962. Exoenzymes. In The Bacteria. Vol. IV. I. C. Gunsalus and Roger Y. Stanier, eds. Academic Press, New York. 121-178.

73. Ray, P. M. 1963. Sugar composition of oat-coleoptile cell walls. Biochem. J. 89: 144.

74. _____. 1969. The action of auxin on cell enlargement in plants. In Communication in Development. A. Lang, ed. Academic Press, Inc., New York and London. Devel. Biol. Suppl. 3: 172-205.

75. Roelofsen, P. A. 1959. The plant cell wall. In Encyclopedia of Plant Anatomy. V. 3. Part 4. Gebruder Borntrager, Berlin. 335.

76. _____. 1965. Ultrastructure of the wall in growing cells and its relation to the direction of the growth. Adv. Bot. Res. 2: 69-145.

77. Rogers, H. J. and H. R. Perkins. 1968. Cell Walls and Membranes. E. and F. N. Spon Ltd., London. 90-113.

78. Roggen, H. P. J. R. and R. G. Stanley. 1969. Cell-wall hydrolysing enzymes in wall formation as measured by pollen-tube extension. Planta. 84: 95-303.

79. Ruesink, Albert W. 1969. Polysaccharidases and the control of cell wall elongation. Planta. 89: 95-107.

80. Samen, J. F., W. E. Moore, and M. A. Millett. 1963. Methods in Carbohydrate Chemistry. Vol. III. Roy L. Whistler, Ed. Academic Press, New York. 66.

81. Setterfield, G. and S. T. Bayley. 1957. Studies on the mechanism of deposition and extension of primary cell walls. Can. J. Bot. 35: 435-444.

82. _____. 1961. Structure and physiology of cell walls. Ann. Rev. Plant Physiol. 12: 35-62.

83. Somogyi, Michael. 1952. Notes on sugar determination. J. Biol. Chem. 195: 19-23.

84. Sutherland, I. W. and Mary Norval. 1970. The synthesis of exopolysaccharide by Klebsiella aerogenes membrane preparations and the involvement of lipid intermediates. Biochem. J. 120: 567-576.

85. Suzuki, Hiroshi. 1967. Acceptor specificity of β-glucosidases of higher plants. Sci. Rept. Tokyo Kyoiku Daigaku. 8B: 80-94.

86. Tanimoto, Eiichi and Yoshio Masuda. 1968. Effect of auxin on cell wall degrading enzymes. Physiol. Plantarum. 21: 820-826.

87. Tsou, Kwan-Chung and Arnold M. Seligman. 1952. Preparation of naphthyl β-glucopyranosides as chromogenic substrates for β-D-glucopyranosidase. J. Am. Chem. Soc. 74: 3066-3069.

88. Van Parijs, R. 1967. Quantitative changes of ribonucleic acid (RNA), desoxyribonucleic acid (DNA), protein and dry matter in different organs of pea seedlings, during germination and cell elongation. Arch. Int. Physiol. Biochim. 75: 125-138.

89. Van Parijs, R. and L. Vanderdriessche. 1966. Changes of the DNA content of nuclei during the process of elongation in plants. I. The formation of polytene chromosomes. Arch. Int. Physiol. Biochim. 74: 579-586.

90. _____. 1966. Changes of the DNA content of nuclei during the process of elongation in plants. II. Variations, other than doublings, of the amount of feulgenstain in maturing plant cells. Arch. Int. Physiol. Biochim. 74: 587-591.

91. Veen, B. W. 1970. Orientation of microfibrils in parenchyma cells of pea stem before and after longitudinal growth. Koninkl. Nederl. Akademie Van Wetenschappen - Amsterdam. Proceedings Ser. C. 73: 113-117.

92. _____. 1970. Control of plant cell shape by cell wall structure. Koninkl. Nederl. Akademie Van Wetenschappen - Amsterdam. Proceedings Ser. C. 73: 118-121.

93. Wada, Shunji, Eiich Tanimoto, and Yoshio Masuda. 1968. Cell elongation and metabolic turnover of the cell wall as affected by auxin and cell wall degrading enzymes. Plant and Cell Physiol. 9: 369-376.

94. Walker, Waldo S. and Thomas Hoeltgen. 1969. Carbohydrate breakdown in cell walls of collenchyma of celery. Proc. Iowa Acad. Sci. 76: 32-37.