ABSTRACT

EXOCELLULAR ENZYMES OF CORN ROOTS AND
A PARTIAL CHEMICAL CHARACTERIZATION
OF THE CELL WALL

John Edward Dever, Jr.

A partial characterization of the chemical composition of the corn root cell wall was achieved. Exocellular enzymatic activities of intact corn roots were then examined in an effort to determine the extent of their role in cell wall metabolism and growth.

To accomplish the chemical analysis of the cell wall, the three growth zones (meristematic, elongation and maturation) were excised and examined separately. The tissue was ground in a high speed homogenizer. Homongenization and subsequent cell wall isolation was done in a glycerol medium to avoid loss of water-soluble components. The suspensions were filtered through beds of glass beads to remove cytoplasmic components. The cell wall fragments were twice more suspended in fresh glycerol and filtered.

The cell wall was extracted with 0.5% ammonium oxalate-oxalic acid and a series of alkaline solutions (4 to 17.5% NaOH) to remove the pectic and hemicellulose fractions respectively. The residue was then treated with 72% H_2SO_4 to

dissolve the α-cellulose and separate it from the insoluble lignin-like material. Each fraction was then acid hydrolyzed and analyzed by paper chromatography.

The pectin fraction was found to constitute 27% of the meristem wall and decreased to 26 and 15% in the elongation and mature walls respectively. The α-cellulose portion represented only 5% of the meristem wall and increased steadily with the age of the tissue, accounting for 13% of the elongation and 25% of the mature walls. Hemicelluloses constituted 25% of the meristem wall and 46 and 40% of the elongation and mature walls respectively. Protein accounted for 9% of the meristem wall and 5% of the other zones.

Hydrolysis and chromatography indicated that the qualitative composition of each of the growth zones was similar: glucose, galactose, mannose, arabinose, xylose and galacturonic acid were present regardless of the age or stage of development of the tissue. Glucose was found to be the major sugar throughout the entire root and in each of the wall fractions. Xylose was the second most abundant sugar while all the others appeared as minor components. Only galactose was found to change significantly in concentration with root development. The prominent decrease with age is suggestive of metabolic turnover.

Examinations of exocellular root enzymes was conducted with five day old corn seedlings. The latter had been germinated on moist paper towelling placed in the dark at 25°C.

The roots of these intact seedlings were suspended in mineral nutrient solution to which a suitable substrate for determining enzyme activity had been added. Pectin methylesterase, acid phosphatase, β-glucosidase and fructofuranosidase activities were demonstrated in this manner. These enzyme activities could be substantially inhibited by treating the roots for short periods of time with 0.1 N HCl. This treatment exerted no apparent effects on overall growth as measured by root elongation. This suggests that these enzymes are not rate limiting in cell wall growth.

EXOCELLULAR ENZYMES OF CORN ROOTS AND A PARTIAL CHEMICAL CHARACTERIZATION OF THE CELL WALL

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INTRODUCTION

It has become quite evident in recent years that we can no longer think of the plant cell wall as simply an inert shell surrounding and protecting the living protoplasm. Evidence is accumulating to indicate that significant metabolic reactions occur within the cell wall itself.

But the presence of the cell wall is not without certain encumbrances. Indeed, one of the primary problems facing a cell is the increase in size after a rigid cell wall has been established around it. In order for growth to occur, there must be a mechanism for plasticizing this barrier that physically limits expansion. This mechanism may involve either a loosening of the bonds between adjacent polysaccharide chains, or actual cleavage of those chains. In either case, enzymes most likely are active at the site of the cleavage and future synthesis.

The plant's possession of exocellular enzymes may be of equal significance in facilitating its capture of nutrients. The availability of hydrolytic enzymes, particularly in those regions of the plant intimately associated with its soil environment, would greatly increase an organism's chance to survive in a medium deficient in small usable molecules. These enzymes, acting on complex organic substrates, could

provide minerals and organic molecules of a size that would more easily penetrate through the cell membrane.

There are some very basic questions concerning cell wall composition and structural relationships that must be answered before more rigorous investigations of the type which have been conducted using other cellular organelles, such as the choloroplasts, mitochondria, and nuclei can be undertaken.

This thesis is divided into two parts, each of which treats the examination of a fundamental process involved in growth and metabolism in the cell wall of corn roots.

In Part I the chemical composition of the cell wall is examined with emphasis being placed on the carbohydrate content.

Part II involves a survey of the exocellular enzymes of the intact corn root, and an attempt to correlate their presence and activities with overall root growth.

PART I

PARTIAL CHEMICAL CHARACTERIZATION OF THE CELL WALL

LITERATURE REVIEW

Cell Wall Structure

The wall of a mature higher plant cell can be delineated into two quite distinctive parts: the primary wall and the secondary wall. They differ considerably in structure and function (1), but chemically are fundamentally similar (37).

Because of the high degree of complexity exhibited by both walls, however, most attempts to attach precise definitions to them have met with some objection. It would appear that Wardrop's suggested definition (121) of the primary cell wall is the most satisfactory. He describes it as, "that structure enclosing, or which enclosed the protoplast during the phase of surface growth."

Consequently, the term secondary wall would apply to any material deposited after cell expansion had been completed.

The distinctive characteristic of the primary cell wall is its two phase system. It possesses a woven texture of cellulose microfibrils, the discontinuous phase, suspended in an amorphous matrix, the continuous phase (107). Synthesis and deposition of new microfibrils in the young cell wall probably occur at the outer surface of the membrane. They are arranged transverse to the longitudinal axis of the cell.

This orientation is believed to be a prime factor in influencing further surface growth. Due to this precise structure, there arises a differential resistance to expansion, restricting a general radial growth and favoring elongation in the direction of the least tension (1,96,97). As growth continues, new material is added to the cell wall chiefly by intussusception (37,38,104,122), i.e. by insertion of new microfibrils between the older ones.

Secondary cell wall growth is by apposition. Generally several layers may be observed. Each layer is composed of parallel microfibrils, more tightly packed than in the primary wall. The microfibrils of each layer possess a specific orientation in relation to the cell axes and also to each of the other secondary wall layers (110). The secondary wall functions as a major form of support for the plant and additionally serves as a formidable barrier to microorganisms (1).

The chemical composition of the primary and secondary cell walls, while qualitatively similar does differ somewhat quantitatively (117,118,119). Generally, the pectic and noncellulosic polysaccharide materials predominate in the primary cell wall, while cellulose appears as the major constituent of secondary wall (37,39,86,87).

By far, the most predominant cell wall constituents are the polysaccharides: cellulose, hemicellulose and pectic materials. Some degree of lignification is commonly found

in older tissue (81). Minerals and ribonucleic acid have also been reported (63) as minor components. The presence of protein (32,42,62,63,67,68,92,95), while often attributed to cytoplasmic contamination, is of major importance. However, the true significance of such a phenomenon has yet to be fully appreciated. Reports of ribose in holocellulose preparations from carrot tissue cell walls (66) and in Avena coleoptile (78) constitute some support for the presence of nucleic acid with protein tending to substantiate the prospect of *in situ* cell wall biosynthesis. This is weak support, however, as ribose could be associated with something other than the wall structure itself.

Cell Wall Polysaccharides

Cellulose

Cellulose is the most extensively studied and best characterized of the cell wall polysaccharides. It is chemically defined as long chain molecules of D-glucopyranose residues joined together by β-1,4 linkages. Molecular weight estimates vary from several thousand to at least 1.5 million depending chiefly on the means of isolation. Estimations of cellulose in plant tissue range from 10% in young leaf tissue, to 50% in wood and bark, and 90% in cotton fibers (94).

While hydrolysis of isolated celluloses regularly yields over 95% β-D-glucose, other monosaccharide residues have been reported (86,104). The relationship of these residues to the cellulose structure, whether actually incorporated or

secondarily associated with the major component, is not yet understood.

The use of X-ray diffraction, and the birefringence and dichroic properties of the cellulose molecule, have led to a good understanding of its crystalline structure and spatial relationships within the wall (38,57). The basic unit into which the long chain cellulose molecules are arranged is the microfibril having the dimensions 250 Å in diameter and 500-600 Å long. Microfibrils are constructed of approximately 20 micelles, each measuring 30-50 Å in diameter and 500-600 Å long. These in turn are composed of approximately 32 cellulose molecules (38).

Since determinations on the degree of polymerization indicate that individual cellulose chains may be as long as 30,000-40,000 Å, it is evident that any one chain is associated with a number of microfibrils. An aggregate of about 250 microfibrils forms a fibril. Some 1500 fibrils are associated into a macroscopic fiber as found in cotton.

Cellulose is completely insoluble in water and most organic solvents but may be extracted from the cell wall by treatment with a cuprammonium solution. More commonly, however, cellulose is identified as the residue following successive extraction with oxalate, bisulfite and alkali to remove the other wall components. This fraction, insoluble in alkali, is referred to as α-cellulose.

Hemicellulose

The hemicelluloses constitute, at best, a rather loosely defined group of non-cellulosic polysaccharides. Collectively, they include those classes of polysaccharides soluble in hot or cold alkali, but insoluble in water (104). They are composed of a variety of monosaccharides: D-glycose, D-galactose, D-mannose, L-arabinose, D-xylose, L-rhamnose, and uronic acids. Homopolymeric hemicelluloses, upon hydrolysis, yield only one type of residue. Examples are the xylans, glucans, arabans, and mannans. Just as common in nature are the heteropolymeric hemicelluloses composed of two or more sugars. Glucomannans, arabinoglactans and galactoglucomannans are representative of this class (8,9,88). An additional classification into acid and neutral hemicelluloses is based on the presence or absence, respectively, of a uronic acid.

The structure of the hemicelluloses may be linear or branched. The predominant linkage of linear hemicelluloses is of the β-1,4 class although β-1,3 type is known in oats and barley (10). A wider range of linkages is reportedly common among the branched hemicelluloses: β-1,6 in galactans, β-1,3 in arabans and xylans, and α-1,2 where galacturonic acid residues are linked to xylans (8,86).

The hemicelluloses are the major constituents of the amorphous matrix of the primary cell wall. Crystallinity has been reported among the wood xylans (120). Little is known about hemicellulose synthesis and deposition (85).

and since their removal from young tissue causes no major weakening of the cell wall structure, their functions also remain obscure. They have been reported as constituting 35-50% of growing cell walls, 70% of yeast walls, and 90% of ivory nut root.

Hemicelluloses are generally extracted by treatment with 5-20% alkaline reagents and can be precipitated from solution by neutralization followed by addition of alcohol (123). That the various classes of hemicelluloses have differences in solubilities is demonstrated by the fact that cupric hydroxide and borax in alkaline solutions have been employed to precipitate a xylan from straw and birch wood, and a mannan from ivory nut and yeast cells, while the fraction not precipitated yielded a galactan and an araban. The recent application of electrophoresis has demonstrated a new and very useful technique for more exacting analyses of the separated hemicelluloses (80).

Pectic Substances

The pectic substances are quite significant because of their postulated role in cell extensibility (11,12,40,52,102, 127). They comprise a group of polysaccharides whose major characteristic is the presence of a large amount of uronic acid residues joined linearly through α 1,4 linkages. A wide variety of monosaccharides are commonly associated with the pectins (45). Branching is reported in apple pectins (13,40) but in general relationship of the side chains to the

uronide backbone has not been well established. Molecular weights have been estimated to be between 25,000 and 360,000.

A great deal of the earlier confusion over nomenclature has been eliminated by recognition of three major classes of molecules (59,104).

Protopectin is the fraction which does not dissolve in neutral, hot or cold water. Its insolubility is believed to be due, in part, to the nature of its association with other cell wall components including lignin.

Pectin is that fraction soluble in hot water, and to some extent in cold water. A further distinction is some degree of esterification of its carboxyl groups with methanol. In low ester pectins about 4% of the molecule is methylated, while so-called high ester pectin contains 8-12% methylated carboxyls.

Pectic acid is relatively free of methylation and is soluble in water only as the pectate of sodium, potassium or ammonium. It can be precipitated from solution by divalent cations: calcium and magnesium are commonly used.

Until recently native pectin was thought not to be birefringent. When cellulose and hemicellulose are removed, a positive or a negative birefringence is observed (104) depending upon the refractive index of the liquid. It now appears that at least portions of the pectin chains possess crystalline form but the stronger patterns of cellulose obscure their faint optical effects.

The pectic materials are mostly found in the middle lamella and the primary wall. This is quite evident from staining with ruthenium red and other dyes (4), and the disruption of tissue organization when treated with the pectic enzymes.

Pectic materials may be extracted by a number of reagents other than water. Acids, glycerol, sucrose solution and, polyphosphates have all been used in addition to alkaline solutions (59). Alkaline conditions result in demethylation, however, and are therefore unsatisfactory. A milder extraction with a dilute ammonium oxalate-oxalic acid solution is presently the most favored. Ammonium oxalate can be used alone, but the presence of the acid apparently avoids the alkaline effect and tends to retard co-extraction of hemicelluloses.

Kertesz (59) provides a comprehensive review of the analytical methods for determining pectic contents. Most of these are based on estimation of galacturonic acid residues. Of the multitude of methods discussed, the measurement of carbon dioxide following decarboxylation (73,90) and some modification of the carbazole reaction (30) appear to be the most favorable and reliable.

Quantitative Cell Wall Analysis

A large number of independent analyses have been performed on plant cell walls. The primary cell wall has received particularly thorough attention owing to its relative

simplicity in comparison to cell walls possessing secondary growth and lignification. Table 1[1] presents a tabulation of some of the data accumulated on cell wall composition.

Considerable caution must be exercised, however, in any attempt to compare cell walls of the many different plant tissues and species examined. A great deal of variation exists in the methods by which the cell walls were isolated and fractionated, and in the subsequent hydrolyses and chemical analyses.

The techniques of cell wall isolation present the greatest degree of variability. Grinding is the most frequently used method of breaking up the fresh tissue, although pressing, as between glass plates (101), is also reported. Jenson and Ashton (55) isolated the cell wall as the residue after whole roots were suspended in various extraction media, without resorting to any mechanical form of cell rupture. In view of the fact that such preparations could contain a formidable amount of cytoplasmic contamination, their results may be questioned.

The choice of a medium to suspend the tissue in during grinding presents the broadest range of variability. Ray (101), Bishop et al. (17), Thimann and Bonner (116), and Christiansen and Thimann (25) all use water; Albersheim and Bonner (3), Jansen et al. (52) and Morré (77) used 0.15N

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[1]This table has been constructed principally from data presented in Setterfield and Bayley (107).

Table 1. Chemical composition of plant cell walls.

Plant Tissue	Reference	Percent Dry Weight				
		Cellulose	Hemicellulose	Pectin	Protein	Lipid
Corn coleoptile 9 mm.	128	38	46	10		7
Corn coleoptile 32 mm.	128	33	34	10		23
Corn coleoptile 50 mm.	128	35	30	13		21
Corn coleoptile	79	28	22		30	7
Corn coleoptile	63	27		8	2.5-5	
Corn mesocotyl	104	30	50		3	2
Oat coleoptile	116	42	38	8	12	
Oat coleoptile	17	25	51	1	9	
Oat coleoptile	52				5	
Oat coleoptile	103				5	
Pea stem	25	26	40	20	13	1
Young wheat leaves	22	16	5	11		
Young wheat roots	22	26	10	11		
Oat roots	93	30-35	25			
Bean roots	89	21	15	7		
Sunflower epicotyl	46	37	4	53		
Sunflower hypocotyl	46	38	9	46		
Ash, elm, pine cambium	6	20-25	2-6	10-20	21-29	
Black locust cambium	7			9-13		
Melampyrum seeds	27	59-63[1]	37-40			
Melampyrum stem	27	24	40			

[1]75% of this alkali insoluble fraction was mannose.

acetate buffer, pH 4.4; Tavakoli and Wiley (114), ethanol; Curtis and Cantlon (27), benzene, ethanol and water; and Kivilaan et al. (63), and King and Bayley (61), glycerol. Bean and Ordin (14) compared cell walls isolated with four different media: water, ethanol, glycerol, and glycerol-ethylene glycol. Their evaluations indicate all except ethanol give reliable and correlative results.

Of particular note, although not wholly quantitative, are the cytochemical studies of Jensen (54), Flemion and Topping (36), and Albersheim et al. (4) in which various cell wall components were selectively removed and the remaining one stained to indicate its distribution throughout the several wall layers.

Another precaution that must be taken when evaluating Table 1 arises from the fact that the data, for the most part, represent percentages of total dry weight material. With respect to cellulose, which possesses rather distinctive solubility properties, the data are in close agreement and are reliable enough to suggest that it constitutes 25-40% of the cell wall.

Far less reliable are the calculations of pectic substances and hemicelluloses. The limits of solubility of these substances are just as arbitrary as their accepted definitions. As previously stated, both groups are composed of simple sugars and uronic acids. As a result, it is sometimes difficult to determine the extent of cross contamination during extraction (107).

The solvents used for extracting pectic substances are not completely specific for the polyuronides. Therefore, in addition to the sugars closely associated with the pectic molecules, one often extracts hemicellulosic components. Thus, on a total weight basis, pectic fractions will appear much larger than if specific determinations, based on uronic acid content alone, were performed. In light of the presently accepted definitions of pectins as polyuronides, together with the above considerations, the most acceptable values appear to be within the range of > 1% (Bishop et al., 17) to 8% (Kivilaan et al., 63 and Thimann and Bonner, 116).

EXPERIMENTAL

Methods and Materials

Preparation of Plant Material

Corn kernels of the variety Michigan 300 Hybrid were obtained from the Michigan Farm Bureau and used for all experimentation. Kernels were soaked in running tap water for 15 hours and then surface sterilized for 5 minutes in a 1% solution of Chlorazene (Frost Laboratories, Inc., Boston, Mass.). The kernels were then rinsed four times with distilled water. Six kernels were next placed between two moist paper towels which were then rolled and placed in polyethylene buckets. The kernels were always placed in the towelling, and the latter oriented in such a way that the long axis of the kernel lay in the same plane as normal growth. The buckets were covered with four layers of cheesecloth and placed in the dark at 25°C for 5 days. Enough water was added during the germination period to keep the towelling moist.

With the aid of a razor blade, three zones of the root were excised and stored in the freezer until enough tissue was collected for examination: The 0-2 mm. section included the root cap and was classed as the meristematic zone; the section 2-17 mm. (15 mm. length) was called the elongation

zone; and the section 17-32 mm. (15 mm. length) was called the maturation zone.

Preparation of the Cell Wall

The cell wall was isolated by the method described by Kivilaan et al. (63) and outlined in Figure 1. Root tissue was weighed in 25 gm. samples, each of which was placed in a Servall omnimixer cup along with 180 mls. of glycerol and 37 gms. of glass beads (200 μ dia., purchased from Minnesota Mining and Manufacturing Co., St. Paul, Minnesota). The mixture was homogenized for 15 minutes at 16,000 r.p.m. During homogenization, the omnimixer cup was suspended in ice water to keep the temperature at a minimum.

The homogenate was poured into a beaker and allowed to stand at 0°C. for one-half hour in order that the bulk of the glass beads would settle out. The suspension was then decanted onto a mat of 200 μ glass beads (1 cm. deep) placed on the surface of a sintered glass funnel surrounded by an ice pack. Suction was applied and the surface of the bead mat agitated periodically to prevent packing and plugging. The supernatant was discarded and the bead mat with the cell residue scraped into a beaker and resuspended in fresh cold glycerol. Again, the beads were allowed to settle out. The procedure of filtration, resuspension and sedimentation was repeated two more times. After the final cycle, the cell wall suspension was decanted into pre-weighed cellulose nitrate centrifuge tubes and cell wall pellets were collected

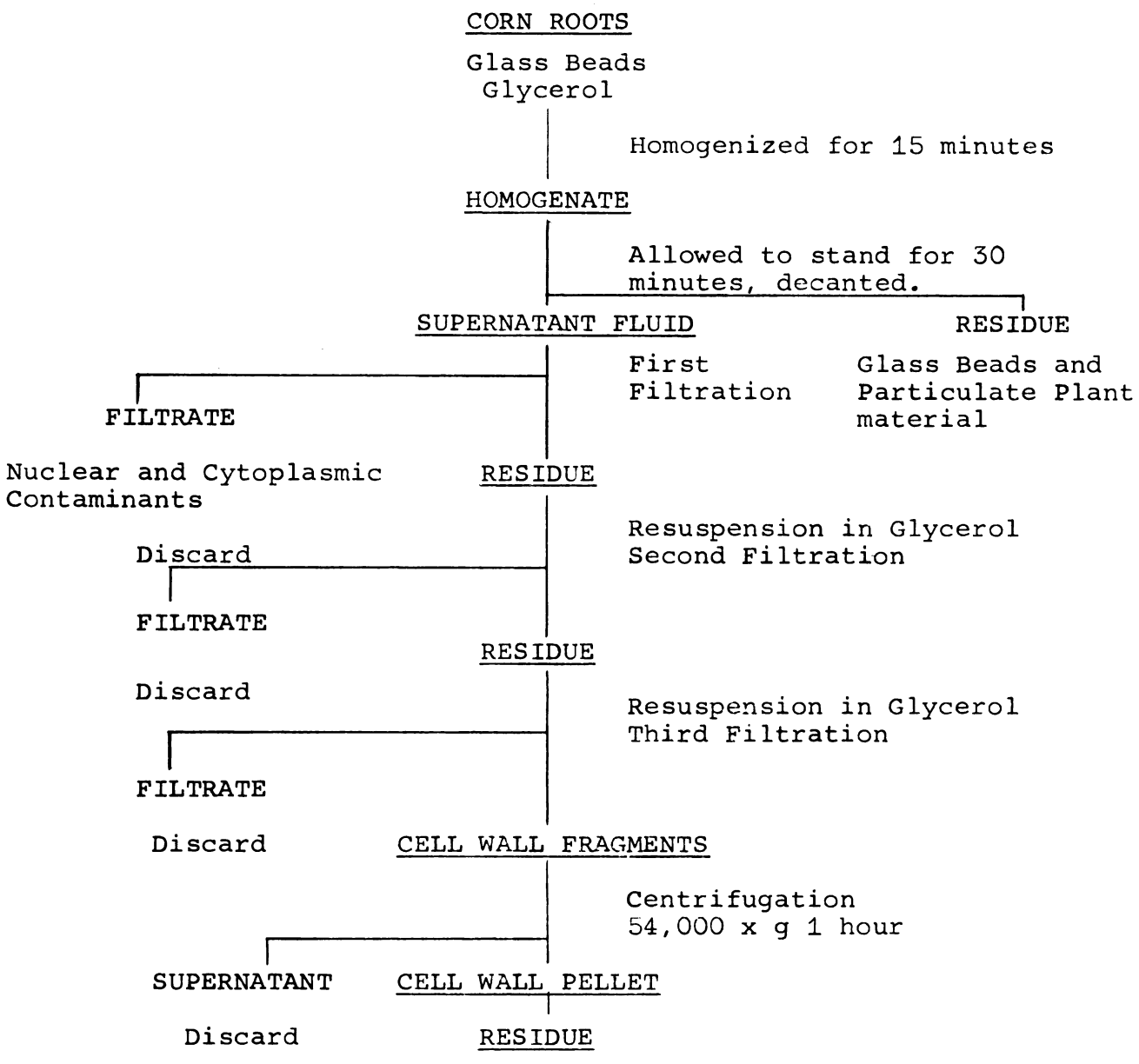

Figure 1. Scheme for the isolation of cell wall from corn roots.

by centrifugation at 54,000 x g. for 1 hour in a Spinco ultracentrifuge, model L; in an SW 25.1 swinging bucket rotor. The glycerol was discarded and the pellets weighed.

The pellets were twice resuspended in absolute ethanol and the residue collected by filtration under vacuum. This was followed by one washing with acetone and one washing with ether. For each washing, 10 volumes of solvent were used.

The cell wall material was placed in a vacuum dessicator with anhydrous $CaSO_4$ for three hours. It was then transferred to a dessicator over P_2O_5, evacuated, and stored overnight. Upon microscopic examination, the cells were found to be completely broken and free of any detectable cytoplasmic contamination. The resulting powdery cell wall material was weighed and stored in the freezer. This procedure was repeated for each of the three root zones.

Cell Wall Fractionation

Samples of cell wall from each of the three root zones (500 mg. of the elongation zone and maturation zone and 200 mg. of the tip fraction) were treated according to the scheme presented in Figure 2. After each extraction, the undissolved residue and the solvent were separated by filtration or centrifugation as indicated.

The proportions of solvents used were similar to those of Bishop et al. (17) except that the total volume was adjusted in relation to the amount of tissue used.

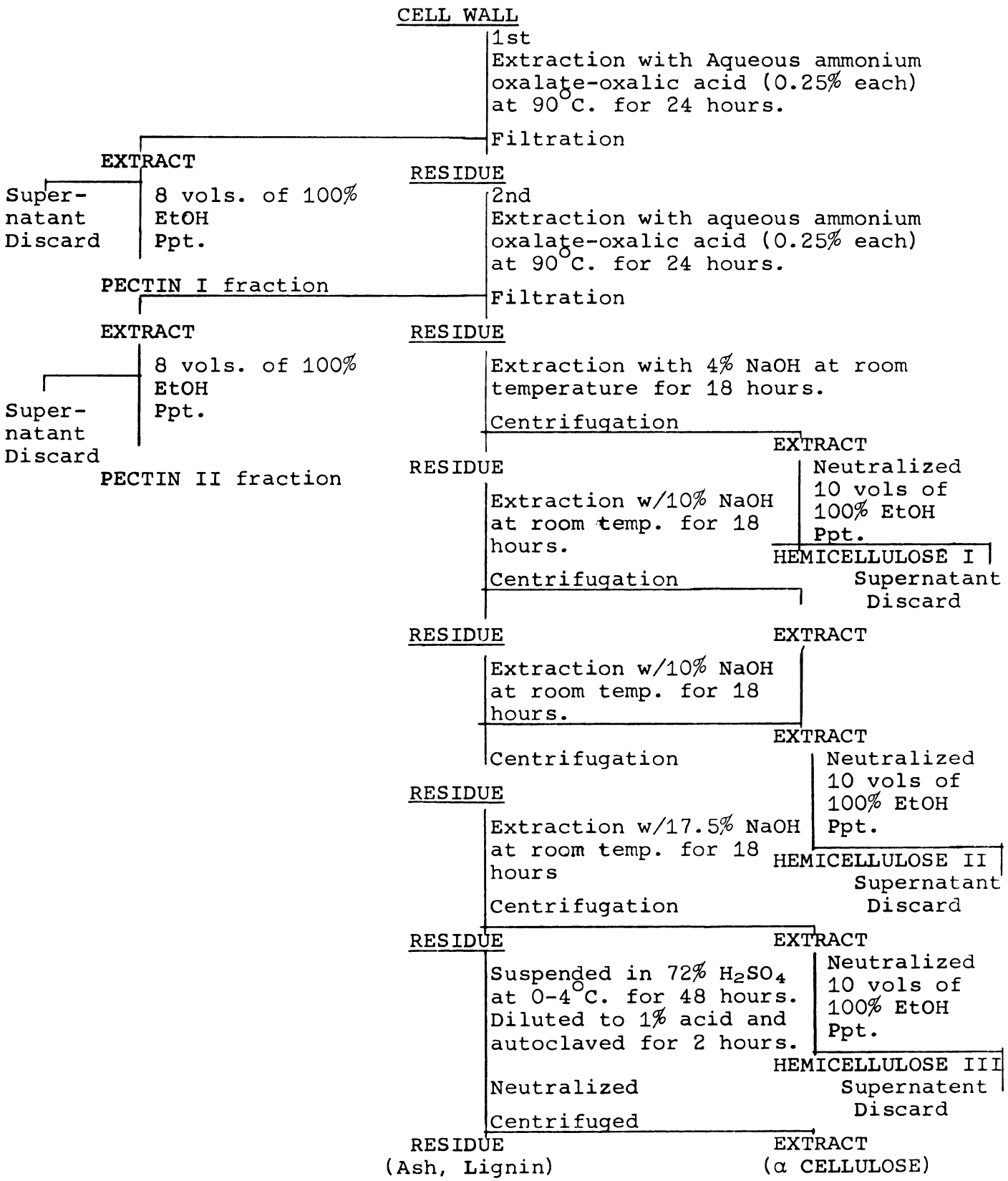

Figure 2. Fractionation of corn root cell walls.

Samples were first extracted twice with 0.5% ammonium oxalate-oxalic acid (1:1) at 90°C. for 24 hours. To each extract, 8 volumes of absolute ethanol were slowly added with stirring. The mixtures were placed in a freezer at -10°C. overnight. The resulting precipitates were collected by centrifugation at 1,150 x g., labeled pectin fractions 1 and 2 respectively, dried in vacuo over anhydrous $CaSO_4$ for 3 hours, and stored in vacuo over P_2O_5.

The samples were next extracted successively, once with 4% NaOH, twice with 10% NaOH, and finally once with 17.5% NaOH. Each extraction was carried out at room temperature for 18 hours. The residue was collected by centrifugation at 3000 r.p.m. The supernatant solutions were neutralized with HCl, diluted with 10 volumes of absolute ethanol, and allowed to stand overnight at -10°C. The precipitates were collected by centrifugation, washed successively with ethanol, acetone, and ether and dried in vacuo over P_2O_5. The two 10% extracts were combined and the fractions designated hemicellulose 1, 2, and 3 for the 4, 10 and 17.5% alkaline extractions respectively.

The residue from the alkaline extractions was washed with ethanol, acetone and ether, then dried. It was then suspended in 72% H_2SO_4 and kept at 0-4°C. for 48 hours with occasional stirring. This mixture was then diluted with distilled water to make a final concentration of 1% H_2SO_4, autoclaved for two hours and, upon cooling, neutralized with NaOH.

The non-hydrolyzable residue was filtered off, weighed and classified simply as lignins and ash. The acid hydrolysates were labeled as α-cellulose. Aliquots of these fractions were desalted with a Warner-Chilcott Laboratories electric desalter, Model 1930C and used for paper chromatography.

Samples of the unfractionated cell wall were sent to Micro-Tech Laboratories, Skokie, Illinois for determination of carbon, nitrogen, hydrogen and ash. The nitrogen analyses were done by the Dumas method; the temperature of combustion was 900°C.

Chemical Analyses

Pectin Fractions

Samples from each of the root zones were hydrolyzed according to the method of Jermyn (56). The amounts of solvents used were scaled down proportional to the amount of material available. Pectin fractions were refluxed with concentrated HNO_3 and a few crystals of urea at 100°C. for 12 hours. The hydrolysates were cooled and diluted to a standard volume. Qualitative identification of the sugars was made by paper chromatography in five solvent systems and with three color reagents. The solvent systems were: (I) pyridine: ethyl acetate: H_2O (2:8:1), 30 hours; (II) pyridine: ethyl acetate: acetic acid: H_2O (5:5:1:3), 48 hours; (III) butanol: ethanol: H_2O (3:1:1), 48 hours; (IV) 80% phenol, 30 hours; (V) butanol: acetic acid: H_2O (4:1:5), 66 hours (47,70). All chromatograms were on Whatman No. 1 paper,

descending, after equilibration of two hours. Standards were always run on the chromatograph with unknowns for direct comparison. Aniline hydrogen phthalate, diphenyl amine, or aniline-diphenyl amine was used to detect the sugar spots (28). Quantitative determinations of sugars were performed according to the technique described by Wilson (125). Chromatograms of pectin extracts were prepared and developed using solvent I. Aniline hydrogen phthalate was used to produce color. Spots were cut from the paper and eluted with an HCl-ethanol solution for one hours. Optical densities were determined with a Beckman DU spectrophotometer equipped with Gilford electronic components. Hexoses have an absorption peak at 390 mμ and pentoses a peak at 360 mμ. Concentrations of the individual sugars were calculated from standard curves plotted from data obtained when series of known sugars were developed on the chromatogram along with the unknown pectin extracts. Uronic acid determinations were performed on squares of paper cut away from the chromatogram prior to aniline hydrogen phthalate treatment for sugar analyses. The fact that uronic acid spots remain at the origin in the pyridine solvent makes it possible to remove those substances without the aid of a color reagent. The cut out spots were eluted with distilled water for 4 hours. This eluate was used for the uronic acid determination following the method described by McCready and McComb (74,75) with the addition of sodium tetraborate as suggested by Bitter and Muir (18).

Hemicellulose Fractions

A sample of each hemicellulose fraction was hydrolyzed with 3% HNO_3 in a boiling water bath for 4 hours according to the method of Jermyn (56). Qualitative identification of the constituents was done in solvents I, III, and V and colors developed with the three reagents described previously. Quantitative procedures used in the analyses of the pectin fractions were similarly applied to the hemicellulose fractions.

α-Cellulose

Qualitative determination of the constituents of the desalted α-cellulose fractions was achieved through paper chromatography using ethyl acetate: acetic acid: H_2O (9:2:2), descending 28 hours in addition to solvents I and III previously described. Spots were developed with silver nitrate or aniline hydrogen phthalate. Purified disaccharide samples containing mannose were generously supplied by Dr. C. T. Bishop, National Research Council, Ottawa 2, Canada. All other sugars used as standards were purchased from Nutritional Biochemical Corp., Cleveland, Ohio and General Biochemicals, Chagrin Falls, Ohio.

Total reducing sugar was determined by the arsenomolybdate method of Nelson (26,82), using a Klett-Summerson photoelectric colorimeter, model 800-3 with a 540 mμ filter.

Glucose concentration was assayed by the glucose oxidase reaction (Glucostat, Worthington Biochemical Corporation, Freehold, New Jersey) and measured with a Beckman spectrophotometer at a wave length of 400 mμ.

RESULTS

Isolation of Cell Wall

For the preparation of purified cell material, a total of 34.4 gm. of meristem tissue, 195.0 gm. of elongation zone tissue, and 243.3 gm. of maturation zone tissue were used. The isolated wall material represents 1.00, 0.74, and 1.03% of these original fresh weights respectively.

Fractionation of the Cell Wall

Table 2 presents an analysis of the three root growth zones based on the total weights of the precipitated fractions and expressed as percent of the cell wall. Several trends are immediately obvious. As expected, α-cellulose is present in very small amounts in the meristem tissue, increases through the elongation zone and finally reaches an amount in the mature zone approximately five times greater than that in the root tip. Pectin, on the other hand, constitutes about 1/4 of the cell wall in the lower two zones and decreases significantly in the mature zone. Hemicelluloses are in lowest proportions in the tip and increase in the upper two zones. Protein, calculated as 6.25 times the percent N, is greatest in the tip and gradually decreases with the age of the tissue. A curious phenomenon is the higher amounts of non-hydrolyzable

Table 2. Fractional analysis of corn root cell wall.

	Composition (Per cent of dry weight)		
	Meristem	Elongation	Mature
Pectin	27.1	25.8	15.0
Hemicellulose	25.2	46.4	40.4
α-Cellulose	4.6	12.9	24.5
Protein	9.0	5.4	4.8
Lignin-like Residue	0.7	3.7	1.3
Ash	0.7	8.7	2.5
Carbon	42.5	40.7	44.2
Hydrogen	7.3	5.9	6.3

material and ash found in the elongation zone as compared to the meristem and older tissue.

Almost 50% of the meristem cell wall preparation is left unaccounted for, while greater recovery is observed for the upper two root regions. An absolute amount of recovery is not readily calculable because the values of protein and ash are based on whole cell wall and it must be assumed that at least portions of these may be already accounted for in the pectin, hemicellulose and α-cellulose fractions.

Chemical Analysis

Pectin Fraction

No detectable residue remained after hydrolysis with refluxing. Paper chromatography indicated similar qualitative

composition in each of the three root zones (Figure 3). Glucose, xylose, galactose, arabinose, and galacturonic acid were found in each. Table 3 presents the quantitative composition of the two ammonium oxalate-oxalic acid extracts for each root zone. The values for the sugars are based on the average of 4 eluted chromatogram spots and those of the galacturonic acid, on 8 eluted spots. The identification of the uronide as galacturonic acid was based on the comparison of the behavior of glucuronic and galacturonic acids in solvents II and III (Figure 4). While the other solvents, especially I, gave satisfactory separation of the sugars, they failed to distinguish among the uronic acids.

Glucose was the most abundant sugar found throughout the length of the root. Xylose appeared as the second most abundant sugar except in the tip region where its concentration was equal to that of galactose. It was noted that the tip region possessed approximately 3 times as much galactose as the rest of the root. Arabinose and galacturonic acid were minor constituents in all sections.

Hemicellulose Fraction

Appreciable residues were left after the hemicelluloses were hydrolyzed with HNO_3. Subsequent hydrolysis with 72% H_2SO_4 in sealed tubes at 100°C. for 6 hours yielded an additional soluble fraction and a residue. Neither of these two

Figure 3. Diagram of a chromatogram of the pectic hydrolysates from corn root cell wall.

Solvent: pyridine: ethyl acetate: water (2:8:1), 30 hours, descending.
T1, T2, E1, E2, M1 and M2 refer to the first and second oxalate extractions of the meristematic, elongation and maturation zones respectively.

GAL A = galacturonic acid
GAL = galactose
GLU = glucose
MAN = mannose
FRU = fructose
ARA = arabinose
XYL = xylose
RHA = rhamnose

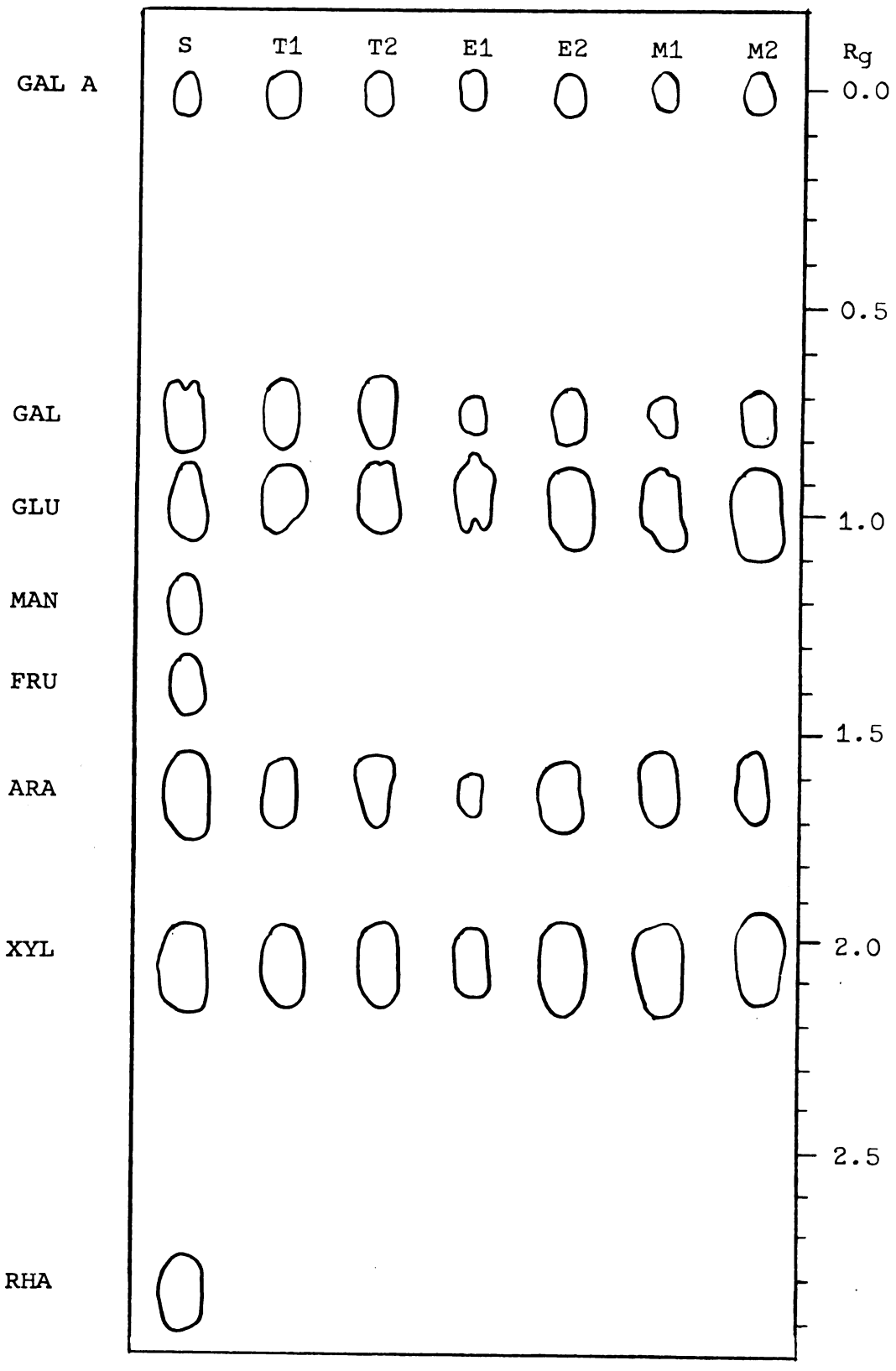
S
T1
T2
E1
E2
M1
M2
Rg
GAL A
0.0
0.5
GAL
GLU
1.0
MAN
FRU
1.5
ARA
XYL
2.0
2.5
RHA

Figure 3

Figure 4. Identification of galacturonic acid from pectic hydrolysates by paper chromatography.

(A) Pyridine: ethyl acetate: acetic acid: water (5:5:1:3), 48 hours, descending

(B) Butanol: ethanol: water (3:1:1), 48 hours descending

1. Galacturonic acid
2. Pectin unknown
3. Galacturonic acid + glucuronic acid

Table 3. Sugar analysis of pectin fractions from corn root cell wall.

	(Sugars expressed as per cent of total fraction)					
	Meristem Zone		Elongation Zone		Mature Zone	
	Extract		Extract		Extract	
	1	2	2	2	1	2
Glucose	22.0	22.8	46.4	60.8	56.0	51.2
Xylose	11.2	9.2	35.6	21.6	12.5	10.8
Galactose	12.8	10.8	4.1	2.6	4.8	3.6
Arabinose	5.6	4.2	3.3	2.0	4.2	3.6
Galacturonic acid	4.6	3.8	2.4	3.2	2.8	2.6

factions were further analyzed. Table 4 indicates what proportion of the total hemicellulose fractions these residues represent. Qualitative paper chromatography indicated no major differences between the three alkaline extractions, nor between the three root regions (Figure 5).

Table 4. Acid insoluble residues of hemicellulose fractions.

Root Zone	Hemicellulose Fraction	Sample mg.	3% HNO_3 Insoluble mg.	%	72% H_2SO_4 Insoluble mg.	%
Meristem	1	10	.233	2	.189	2
	2	10	2.796	28	1.619	16
	3	5	.527	11	.520	10
Elongation	1	10	.599	6	.201	2
	2	10	3.302	33	1.580	16
	3	5	2.360	47	1.312	26
Mature	1	10	.219	2	.170	2
	2	10	.857	9	.761	8
	3	5	.710	14	.681	14

Figure 5. Diagram of a chromatogram of the 10% NaOH extractible hemicellulose hydrolysate of corn root cell wall.

The system was chromatographed in pyridine: ethyl acetate: water (2:8:1), 30 hours, descending.

1. Standards
2. Meristematic zone
3. Elongation zone
4. Maturation zone

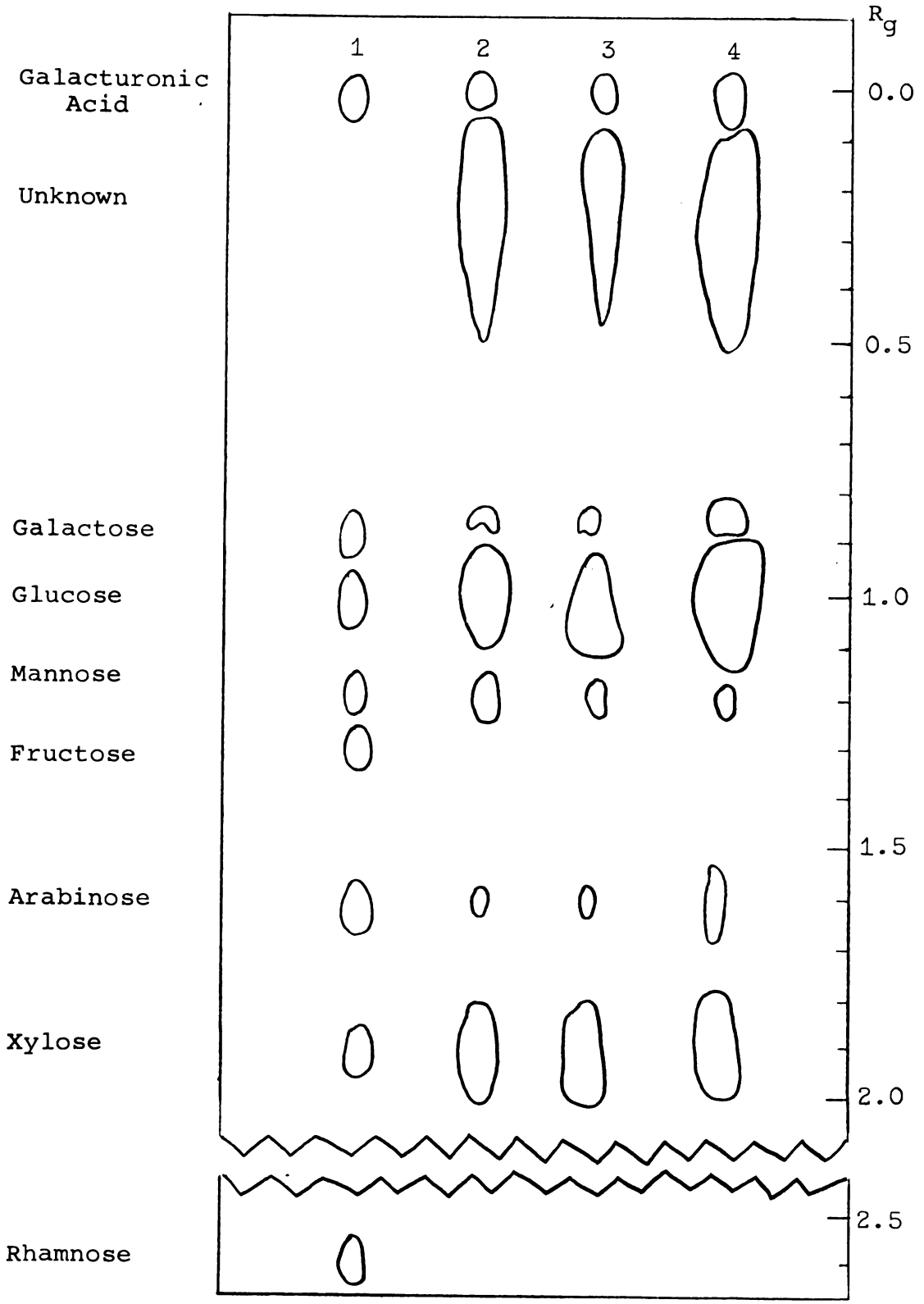
1
2
3
4
R_g
Galacturonic Acid
Unknown
Galactose
Glucose
Mannose
Fructose
Arabinose
Xylose
Rhamnose
0.0
0.5
1.0
1.5
2.0
2.5

Figure 5

The quantitative distribution of sugars is shown in Table 5. It is observed, as in the pectin fractions, glucose and xylose are the predominant sugars with galactose, arabinose and galacturonic acid occurring in lesser proportions.

Table 5. Sugar analysis of the hemicellulose fractions from corn root cell walls.

Hemicellulose Fraction	(Sugars expressed as per cent of total fractions) Meristem 1	Meristem 2	Meristem 3	Elongation 1	Elongation 2	Elongation 3	Mature 1	Mature 2	Mature 3
Sugar									
Glucose	8.6	15.8	3.1	15.7	6.8	4.3	27.4	17.6	22.4
Galactose	2.2	3.5	0.8	1.4	1.8	2.4	3.9	2.8	3.1
Mannose	2.2	1.8	1.4	1.2	0.6	2.3	0.4	0.9	2.4
Xylose	7.6	7.4	7.1	9.4	5.2	4.1	22.9	16.1	12.7
Arabinose	1.9	1.2	1.7	1.0	1.6	2.3	4.3	1.6	2.6
Galacturonic Acid	0.9	0.4	0.1	1.0	0.6	0.1	4.1	2.1	2.6

In distinct contrast to the pectin fractions, is the appearance of mannose in small amounts throughout the root. In all fractions assayed, significant and sometimes major quantities of the hemicellulose could not be accounted for as either simple sugars or acid resistant residues (Table 6). The presence of unknown spots (probably aldobiuronic acids although no identification was attempted) and material of higher molecular weight at the origin (probably oligosaccharides as a result of incomplete hydrolysis of the hemicellulose) undoubtedly prevent a more complete and quantitative analysis of the hemicellulose fractions.

Table 6. Per cent of hemicellulose fractions accounted for as simple sugars and acid resistant residue.

	Hemicellulose Fraction		
	1	2	3
Meristem zone	26	58	26
Elongation zone	36	50	63
Maturation zone	65	50	60

α-Cellulose Fractions

Paper chromatography of the α-cellulose fractions with solvent I indicated only glucose in the meristem and elongation regions, and glucose plus a small amount of mannose in the mature section (Figure 6). Streaking near the origin indicated the possibility of higher molecular weight material. Subsequent chromatography with solvent III resolved the streaking into three discrete spots with r_g values of 0.12-0.13, 0.42-0.43, and 0.60-0.65 respectively. Further analysis of the mature zone (Figure 7), in solvent III with cellobiose, gentiobiose, mannobiose, (M_p $1 \xrightarrow{\beta} 4$ M_p), glucosyl-mannose (G_p $1 \xrightarrow{\beta} 4$ M_p) and mannosyl-glucose (M_p $1 \xrightarrow{\beta} 4$ G_p) [2] indicated that one spot cochromatographs with mannobiose (r_g = 0.61) and another with gentiobiose (r_g = 0.47) while the third unknown could not be identified with any of the disaccharides. No further identification of these spots was attempted.

[2]The mannose containing disaccharides were gifts of Dr. C. T. Bishop.

Figure 6. Diagram of a chromatogram of the α-cellulose fraction hydrolysates of corn root cell wall.

The hydrolysates were chromatographed in pyridine: ethyl acetate: water (2:8:1), 28 hours, descending.

1. Standards
2. Meristematic zone
3. Elongation zone
4. Maturation zone

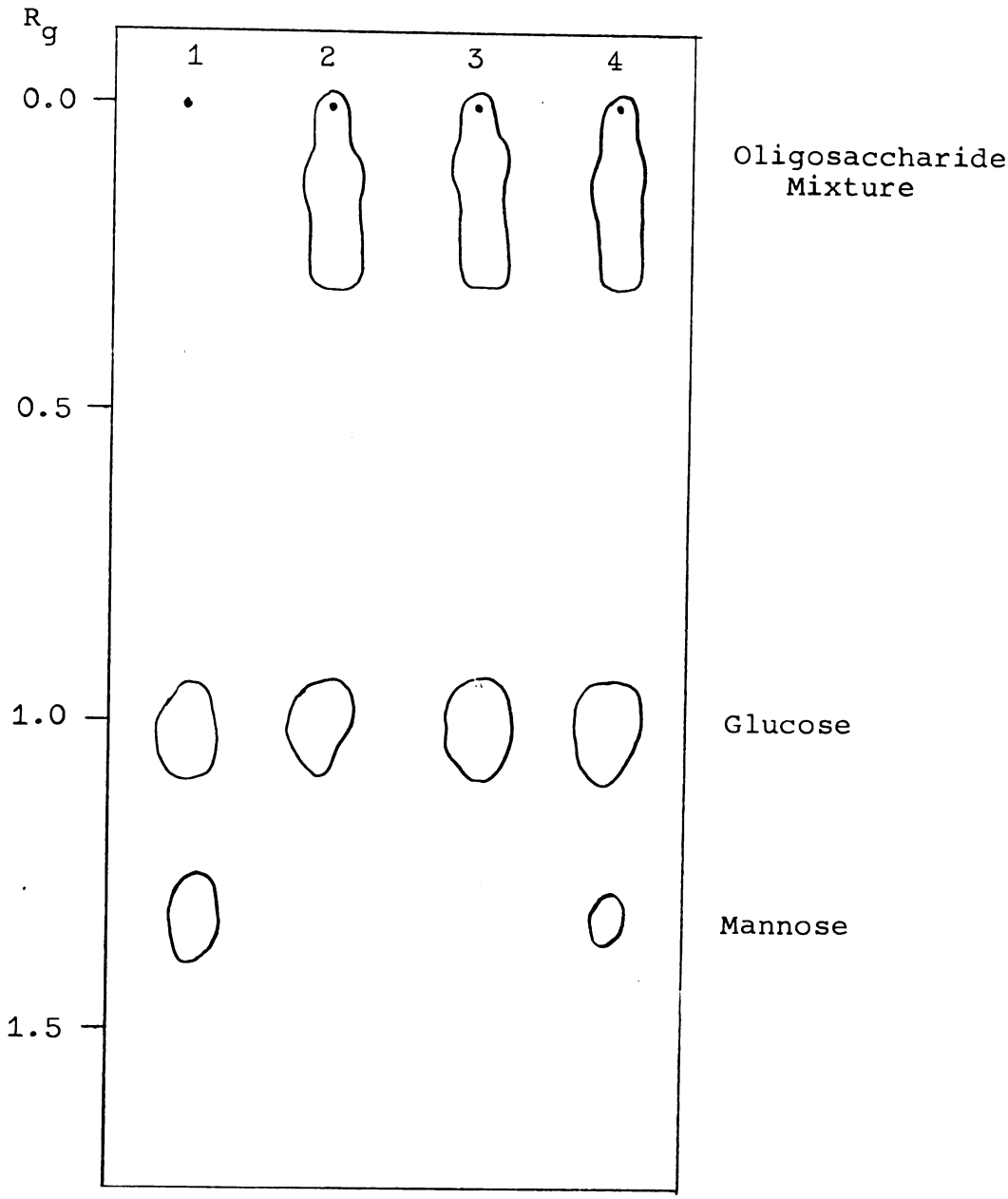
R_g
1
2
3
4
0.0
0.5
1.0
1.5
Oligosaccharide Mixture
Glucose
Mannose

Figure 6

Figure 7. Identification of disaccharide components of cell wall α-cellulose from maturation zone of corn root by paper chromatography.

The system was chromatographed in butanol: ethanol: water (3:1:1), 48 hours, descending.

1. Cellobiose
2. Gentiobiose
3. α-Cellulose from maturation zone
4. Mannosyl-glucose
5. Mannobiose
6. Glucosyl-mannose

U Unknown

R_C Cellobiose used as reference

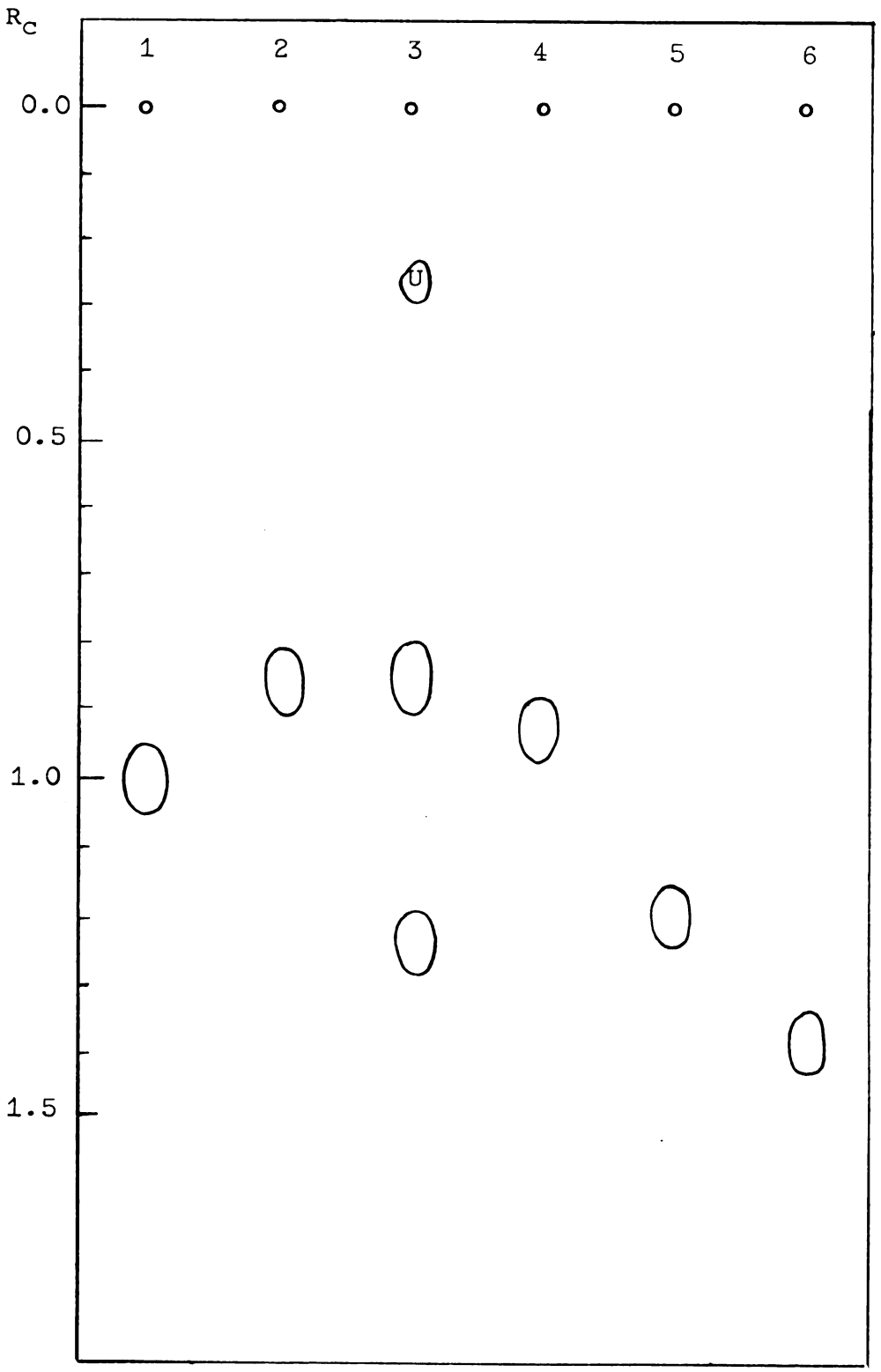

Figure 7

The results of total reducing sugar and glucose oxidase determinations are presented in Table 7. The 14 to 28% of the fraction not accounted for by the total reducing sugar determination can, at least in part, be attributed to the oligosaccharides created by incomplete hydrolysis of the α-cellulose. Significantly the glucose oxidase assay supports the chromatographic evidence that the α-cellulose is mainly glucose except in the mature region where a mannose spot was detectable.

Table 7. Analysis of the α-cellulose fractions from corn root cell wall.

Root Zone	α-Cellulose[1] mg.	Total[2] Reducing Sugar mg.	Glucose[3] mg.	Reducing Sugar per cent of α-cellulose	Glucose per cent of reducing sugar
Meristem	9.3	7.3	6.6	79	90
Elongation	64.8	55.4	54.7	86	99
Mature	122.7	88.2	73.9	72	84

[1]Calculated as the difference between the hemicellulose extracted residue and the residue after digestion with 72% H_2SO_4.

[2]Calculated from ammonium molybdate method of Nelson.

[3]Calculated from glucose oxidase assay.

When the total amount of each constituent sugar released upon hydrolysis is expressed on the basis of the whole cell wall, instead of on the separate fractions (pectin, hemicellulose, α-cellulose) a distribution as shown in Table 8 is

Table 8. Summary of the sugar composition as per cent of the corn root cell wall.

Component	Meristem	Elongation	Maturation
Glucose	12.8	29.2	31.9
Xylose	4.6	11.2	9.5
Galactose	3.3	1.6	2.0
Arabinose	1.7	1.4	1.8
Mannose	0.8	0.7	3.1
Galacturonic Acid	1.3	1.0	1.7
Total hydrolyzable carbohydrates	24.5	45.1	50.0

observed. Glucose is still the most abundant sugar throughout the root. Except for galactose in the meristem, the only other sugar in significant amount is xylose. Galactose, arabinose and mannose represent very small portions of the wall. It is significant to note that if pectin is defined on the basis of galacturonic acid content, it constitutes only 1-2% of the corn root cell wall, and if based on total dry weight of material extracted with oxalate solutions, the pectin fraction (Table 2) represents 15-27% of the cell wall. These data as in the other tables, do not take into consideration the disaccharide residues of the α-cellulose fractions, nor the aldobiuronic acid residues of the hemicellulose fractions.

DISCUSSION

The principle goal of this investigation was to achieve a more thorough understanding of root growth with special emphasis being placed upon primary cell wall development and metabolism. In contrast to the relatively large amount of research performed using coleoptile and shoot meristem tissue, much less attention has been directed to the root. Undoubtedly, a major factor for this is the difficulty of obtaining a detailed and meaningful analysis due to the amounts of secondary tissue encountered. While the present report makes no attempt to distinguish between primary and secondary wall composition and activity, it recognizes the absolute necessity of examining the entire root if a comprehensive knowledge of the growth sequence is to be achieved. As significant as coleoptile and meristem analyses are, it is important to recognize that the former represents only a transitory stage of the life cycle, and the latter, but one developmental stage of the root or shoot.

In order to gain perspective of a system as complex as the cell wall, it is necessary first to know what components are present and how they are made available for use. Each of these problems has been investigated to some extent, but little effort has thus far been expended to correlate the

separate results. For this reason, it was determined that a chemical analysis of the cell wall constituents in the various developmental regions of the corn root should be performed, and an attempt made to survey the enzymes associated with the wall and responsible for the control of those constituents.

The initial investigation, then, was a fractionation of the isolated root cell wall into the classical pectin, hemicellulose and cellulose fractions with subsequent hydrolysis and analysis of each fraction.

The present results indicate that the cell wall constitutes 1% of the fresh weight of the meristem tissue in corn roots. The percentage decreases to 0.74% in the elongation zone and increases again to 1.03% in the mature tissue, reflecting the metabolic changes accompanying root development. Kivilaan et al. (personal communication) report that cell walls of 4-day old corn coleoptiles comprise 1.22% of the fresh weight, and this percentage decreases to 0.77% in 8-day old plants.

Correlated with these developmental differences are significant changes in the major carbohydrate fractions of the isolated cell wall, and in the chemical composition of each of these fractions. The pectin fraction accounts for 27% of the meristem wall and decreases to 15% of the mature wall, while the α-cellulose constitutes only a small portion (4.6%) of the undifferentiated root tip, but increases five fold in

the mature tissue. Hemicelluloses account for one quarter of the meristem wall and approximately one-half of older walls.

Since there are only a few reports of root cell wall analyses, a comprehensive evaluation of the current results, and an absolute comparison with other plants is difficult and arbitrary. Burström (22) reported 11% pectin, 10% hemicellulose and 26% cellulose in wheat root cell walls. Odhnoff (89) calculated 7% pectin, 15% hemicellulose and 21% cellulose in bean root cell wall, and Phillips (93) found that the oat root cell wall contained 25% hemicellulose and 30-35% cellulose. The complications in drawing comparisons with the present data arise primarily from differences in germination periods and methods of extracting the various fractions. Burström worked with 10 day old wheat, Odhnoff with 7-14 day old bean and Phillips with 7-105 day old oat roots. These investigators extracted the wall components from the whole root without first isolating the cell wall. Phillips first dried and powdered the root tissue, while Burström and Odhnoff began with fresh or frozen tissue. Such procedures undoubtedly increase the chances of cytoplasmic contamination. Odhnoff's scheme of extraction with ethanol, 2% H_2SO_4 and 72% H_2SO_4, and Phillips' ethanol, cold water, hot water and 1% HCl differ considerably from the method here employed. Burström's system of ethanol water, KOH and 72% H_2SO_4 more nearly compares with the method of analysis reported here.

An additional source of variation is the differences in the length of the individual roots harvested. Since the above workers report only the total root weight used, with no reference to what proportion of each root was used, it appears likely that they excised more than the 23 mm. used in the present analysis. Therefore, beginning with older and presumably longer roots, it seems obvious that their analyses would involve more mature tissue and, as a result, would yield a high cellulose/pectin ratio.

If the pectin, hemicellulose, and cellulose contents reported in the present study are expressed on the basis of the entire 23 mm. root, instead of the individual growth zones, values of 21, 40, and 16% are achieved respectively. Despite the complications previously mentioned, the pectin and cellulose contents compare favorably with published data. Only the relatively high hemicellulose values appear to be in variance with previous analyses. Burström stated his values seemed low in this respect but offered no explanation. Phillips reported a considerable amount of xylan in his cellulose fraction. In view of this, and the fact that these earlier results were based mainly on total reducing sugars, without the benefit of qualitative analyses, it appears quite reasonable to assume that low hemicellulose values arise from (1) less rigorous techniques leading to incomplete extraction and cross-contamination with other fractions and (2) incomplete hydrolysis of the fractions leading to deceivingly low total reducing sugar values.

It should be noted that root cell wall characterization in each of the previous reports was based primarily on the solubility properties of the various fractions. But the specificity of some of the extraction solvents is questionable, as previously noted. The "pectin fraction" provides an excellent example of discrepancies thus arising. By extracting with ammonium oxalate-oxalic acid solution, pectin represents 27, 26 and 15% of the dry weight of the meristematic, elongation and maturation cell walls respectively. However, total galacturonic acid content for those same growth regions accounts for only 1.3, 1.0, and 1.7% of the wall. A similar occurrence was reported in corn coleoptiles (63), where Kivilaan et al. found that the ammonium oxalate fraction constituted 28% by dry weight of the wall, but the galacturonic acid totalled only 8%. It will be seen that the true pectin, based on galacturonic acid content, represents only a very small fraction of the cell wall. This is primarily why Ray (102) feels that the pectin fraction may not be responsible for the expansion properties of the cell. Nonetheless, Burström (22) and Griffioen (46), working independently, have reported higher cellulose-pectin ratios in roots than in wheat leaves and sunflower stems. Beginning with the meristem, the three growth regions of the corn root yield cellulose/pectin ratios of 4, 13, and 14 compared to a value of 3 for the coleoptiles based on Kivilaan's data. When one considers that cellular expansion of leaf and stem cells occurs along

several axes, while growth in root cells is usually undimensional, and that the former generally expand to a greater volume, it seems axiomatic that the root would require less of the substances involved in this expansion mechanism. The proportional distribution of pectin between root and shoot provides a reasonable argument in favor of their participation in cellular expansion.

A second significant fact may be observed concerning the galacturonic acid of the corn root cell wall. Not all of it is extracted in the pectin fraction; a considerable portion is removed during alkaline extraction of the hemicelluloses (Table 5). The differential solubilities thus observed may be interpreted as an indication of separate pectin species, possibly related to the degree of association with accompanying sugars or simply the result of differences in molecular size. Of the total galacturonic acid in the meristematic wall, 88% is found in the pectin fraction. This proportion decreases significantly with age; only 67 and 24% of the sugar acid is located in the pectin fraction of the elongation and maturation zones respectively. This is taken as evidence of metabolic turnover during growth and development and could conceivably reflect a change in the role of this component with age.

Changes in two other carbohydrate components are concomitants of root development. Glucose is observed to be the most abundant sugar present throughout all of the growth zones, but a transition in its distribution between the

cellulosic and non-cellulosic fractions is evident. While only 29% of the total glucose in the meristem wall is deposited as cellulose, this proportion increases to 37% and finally to 47% in the elongation and maturation zones. The latter value is probably an underestimation due to the significant amount of glucose present in disaccharides resulting from incomplete hydrolysis of the cellulose fraction.
In view of the fact that the total glucose contents in the upper two growth regions are approximately equal (29 and 32%) the net increase in the cellulosic fraction tends to support MacLachlin and Duda's report (70) that, in the apical section of pea epicotyls grown without substrate for cell wall synthesis glucose disappears from their dilute-acid-soluble wall fraction while cellulose increases. Similar results were reported by Ray for oat coleoptile cell wall.

According to the evidence presented by the previously mentioned workers, and by Bishop's group (17), 1-22% of these non-cellulosic glucans exhibit the starch-like sensitivity to the iodine test. It seems probable that at least part of the remaining non-cellulosic glucose is in the form of short chain dextrins, possibly breakdown products of the extraction processes. Regardless of the native form the evidence suggests that the glucans represent an important reserve for further growth, especially in the absence of appropriate substrates.

The present analysis also indicates a decrease in the galactose content of the pectin fraction concomitant with secondary growth (Table 3). Galactose constitutes 12% of the meristematic pectin and decreases dramatically to 3 and 4% in the elongation and maturation zones. Similar loses have been reported in pea epicotyl (72), oat coleoptile (101), and various angiosperm cambiums (118). Although it is suggested in these reports that the plasticity of the wall and the mechanism of enlargement are associated with the breakdown of certain polysaccharides including galactans and starch-like glucans, the underlying relationships remain obscure.

The current data contribute little to illuminate the function of the hemicelluloses in cell walls. Hemicelluloses of corn roots possess a composition similar to those found in other plants except for the absence of rhamnose and a low arabinose content. Since rhamnose is usually found in trace amounts only, as in oat coleoptile and angiosperm cambium, its apparent absence in corn roots may be a function of the sensitivity of the color reagents and techniques used. Rhamnose has been reported in significant amounts in the holocellulose of carrot tissue cultures (66). The low arabinose content is interesting since it is generally found in a 1:1 ratio with xylose. However, this probably illustrates simply that there are differences in the hemicellulose species in different parts of the plant. This supposition can only be evaluated after a more extensive separation and analysis of

the individual hemicelluloses. With respect to this problem, caution should be exercised with regard to the hemicelluloses as extracted by the procedures used. Although the sugar content of the pectin and hemicellulose fractions are similar, it remains to be seen whether this indicates inadequacies in the extraction technique or that, in fact, there are real differences in the arrangement of the sugars leading to entirely different species possessing different solubilities.

The chemical analysis of the corn root cell wall presents, with few minor exceptions, a profile quite similar to those of bean, oat, and wheat roots, and in the cambial, coleoptile and leaf tissue of numerous plants. There is evidence of metabolic turnover of glucose, galactose, and galacturonic acid during secondary growth. The extremely small amount of galacturonic acid present in the roots has prompted some doubt regarding the classic role normally attributed to the pectins in cellular expansion. However, if the galacturonic acid residues were arranged throughout the wall in short chains interspersed with and in some unknown manner attached to the non-cellulosic polysaccharides, it still seems possible that the reactive uronide carboxyl groups could control the plastic qualities contributing to cell expansion. The higher pectin concentration in leaf and stem cells, where, in contrast to root cells, a greater degree of expansion occurs, tends to support the idea that these molecules participate in the expansion process. Ray suggests that the hemicelluloses

may be involved in expansion because of the large volume of the wall that they occupy. This speculation presents a most intriguing possibility when considered in light of Burström's report that 70-85% of the cell wall protein is associated with the hemicellulose fraction. Thus, Lamport's theory suggesting that a wall protein, which he calls "extensin," is the primary constituent controlling cell extensibility, is worthy of further attention. It is however, difficult to ignore the vast amount of data implicating the pectins in this process. We must also remember that, by applying any extraction scheme, we are imposing arbitrary boundaries and restrictions upon the various wall components. Obviously the artificial fractionation tells us little concerning their natural association. The possibility exists, therefore, that the expansion mechanism is indeed more complex than presently suspected and that it involves pectin, hemicellulose, and protein.

SUMMARY

1. Cell wall was isolated from corn roots and found to constitute 1.00, 0.74, and 1.03% of the weight of meristematic, elongation, and maturation tissues respectively.

2. Sequential extraction of the wall provided three major polysaccharide fractions: pectins which decreased with secondary growth; α-cellulose which increased with age of the tissue; and hemicelluloses whose concentration increased during the transition from meristematic to elongation growth and then remained constant.

3. Glucose, galactose, xylose, arabinose, and galacturonic acid were found in the pectin and hemicellulose fractions. Traces of mannose were also observed in the hemicellulose fractions. Glucose and mannose were the only monosaccharides found in the α-cellulose fractions. Glucose was the most abundant sugar throughout all of the fractions.

4. Although the total galacturonic acid content of the root remains reasonably constant throughout the developmental phases of growth, a smaller portion of it is found in the pectin fraction as the tissue ages. A concomitant increase in the hemicellulose galacturonic acid is noted. This is interpreted as evidence of metabolic turnover and possibly reflects a change in the function of the wall component.

5. Glucose undergoes a transition from predominantly non-cellulosic glucans in meristem tissue to cellulose in mature tissue. Galactose content of the meristem is approximately 3 to 4 times higher than in older tissue. Both of these are widely observed phenomena and are generally believed to be associated with polysaccharide dissolution during cellular expansion.

6. The significance of pectin in the expansion mechanism is discussed and alternate systems suggested in view of the extremely small amount of galacturonic acid present in the walls of most plant tissues.

PART II

EXOCELLULAR ENZYMES OF CORN ROOTS

LITERATURE REVIEW

Although the presence of exocellular enzymes is well documented in the bacteria, yeast, and lower plants, much less attention has been focused on the possibility of a similar situation in higher plants. In part, at least this can probably be attributed to the inherent difficulties of examining complex tissue systems.

The term exocellular enzymes is used to denote enzymes exterior to the plasmalemma permeability barriers as opposed to those of the protoplasmic portion of the cell. The presence of protein in the cell wall was previously noted in Part I. The structural and metabolic aspects of these compounds, as well as their possible relationship with cytoplasmic components have been reviewed by Newcomb (84).

Examinations of exocellular enzyme activity have been conducted by three different methods: with complete tissue, either intact or excised; with isolated cell wall; and histochemically in the specific case of acid phosphatase.

It is of little surprise that the enzyme systems most thoroughly studied are those showing a possible response to auxin treatment and therefore, at least indirectly, linked to cell extension. Newcomb (83) reported that ascorbic acid oxidase activity is greatly enhanced by the addition of

auxins to tobacco pith tissue. Honda (50), using wheat roots, and Mertz (76), working with maize root tips, have demonstrated that over 90% of the total ascorbic acid oxidase activity, based on oxygen consumption measured manometrically, is associated with the isolated cell wall fraction. Thus, it is the generally accepted theory that ascorbic acid is not the cytoplasmic counterpart to the cytochrome oxidases and that any relationship to respiration is secondary to its role in wall expansion and metabolism. Mertz also demonstrated the differential activities of ascorbic acid oxidase as a result of cellular development: the greatest activity being observed in the region of elongation.

The presence of pectin methylesterase activity in cell walls has similarly attracted attention, but with some contradictory results. Glasziou (43) and Jansen et al. (53) have shown that pectin methylesterase can be extracted from Avena coleoptile cell wall with dilute salt solutions but not with water and therefore the latter suggests that the enzyme is linked to the cell wall through an ionic bond. Since this extraction can be influenced by salt concentration and pH, Glasziou theorizes that the enzyme is located in the free space, a concept that is generally accepted for many of the cell wall enzymes (106). Glasziou also reports that the partitioning of the enzyme between solution and tissue can be altered by the presence of auxin; a phenomenon in disagreement with Jansen's results. Another point in dispute is the

effect of auxin on pectin methylesterase activity. Bryan and Newcomb (20) report a significant increase in enzyme activity with addition of auxin while Jansen could find no effect. In each of these investigations, the tissue was first treated *in vivo* and then the enzyme activities assayed *in vitro*. While the controversy over the effect of auxin on pectin methylesterase activity has yet to be satisfactorily resolved, the presence of that enzyme in the cell wall is well documented.

Probably one of the earliest observed exocellular enzyme activity was that of invertase (β-fructofuranosidase). In 1920, Knudson (65) observed an increase in reducing sugar in the medium when corn plants were grown on sucrose. Finding no invertase in the medium, he attributed the reaction to root excretions. Dormer and Street (31) reported that tomato plants grown with sucrose added to the medium developed much thicker and healthier root systems. This could not be demonstrated with another disaccharide, maltose. They proposed a scheme for a phosphorylase mechanism in which the glucose moiety was preferentially phosphorylated and absorbed while the fructose was left in the medium. Burström (21,23) also attributed sucrose inversion to root surface action. More specifically, through plasmolytic experiments, he showed that the amount of inversion could be increased and suggested that the catalytic mechanism was in the free space. Since he could also demonstrate maltose hydrolysis with his wheat roots,

he raised the possibility that such reactions were merely due to the presence of hydrogen ions absorbed to the root surface. However, activity curves in relation to pH, and evidence that not all substrates (including maltose) are hydrolyzed in other systems tend to invalidate this possibility. Hellebust and Forward (49) demonstrated the change in invertase with development. Using corn radicles they were able to show that there was no difference in the quality of the invertase in tissues of various age, but the greatest amount of activity was observed in the region of elongation. Further evidence that invertase activity is a surface phenomenon has been supplied by Hassid (47) and Straus (112). Hassid floated lily leaves on a sucrose rich medium and demonstrated that the surface area, and not the total volume of the leaf, influenced the amount of invertase activity. Straus isolated cell wall fractions from corn endosperm, cypress cones, tobacco and rose stems, and several other plant tissues and demonstrated abundant invertase activity on sucrose and raffinose in all but one strain of tobacco. Chang and Bandurski (24) report that not only is invertase activity associated with the intact roots of corn seedlings, but at least a portion of the enzyme is released into the surrounding medium. They report a similar phenomenon for a nuclease in addition to surface bound cellobiase, adenosine triphosphatase and pyrophosphatase activities which are active only when the root is in intimate contact with the substrate.

They, therefore, refer to these as surface bound as opposed to soluble enzymes. The release of enzymes from several other tissues has been described by Straus and Campbell (113). Peroxidase, acid phosphatase, amylase, and indoleacetic acid oxidase have all been shown to be released by washing tissue cultures with water. An additional fraction of each enzyme was released by treatment with $CaCl_2$. This especially emphasizes the complexity of the cell wall enzyme association and the role that salts might play in mediating the binding of enzymes to specific surfaces.

Kivilaan et al. (64), have demonstrated adenosine triphosphatase, UDPG pyrophosphorylase, inorganic pyrophosphatase and α-glycerophosphatase in cell walls of corn celeoptiles isolated by the glycerol technique. Phosphatases appear to be among the most ubiquitous enzymes found in cell walls. Interestingly enough, early experimentation with phosphatases was agriculturally inspired. The works of Rogers et al. (195), Ratner and Samoilova (98,99,100), and Hayashi and Takijima (48) all consider absorption and utilization of organic phosphorous by crop plants. Okuda et al. (91), found that sterile cultures of 5-6 week old rice plants excreted phosphatase. Estermann and McLaren (35) have examined the often questioned relationship between true root enzymes and those of micro-organisms in what they call the rhizosphere. Using sterile, intact barley roots, they have demonstrated phosphatase and invertase activities attributable mainly to

the plant and a urease attributable to micro-organisms in the root environment. Dubovenko (33) also discusses the relationship of root phosphatase and the effect of micro-organisms on its activity. Histochemical examinations of barley roots by Estermann and McLaren, and onion roots by DeJong (29) have confirmed the localization of an acid phosphatase in the walls of epidermal and cortical cells.

In addition to the well defined activities of the enzymes already mentioned, fragmentary reports of other reactions should be noted. Amylase activity and its relation to growth regulators has been reported by Gall (41) in kidney bean tissue cultures and by Karstens and deMeester-Manger Cats (58) in tobacco tissue cultures grown on an agar-starch substrate. Siegel (109) demonstrated a peroxide dependent increase in the lignin components of plant cell walls. He proposes the presence and activity of cell wall peroxidases. Stenlid has reported the presence of β-glucosidases and β-galactosidases in varying degrees as surface enzymes in excised roots of wheat, barley, corn and several other plants (111).

EXPERIMENTAL

Methods and Materials

General

Corn seedlings were grown in a liquid medium with a series of supplementary substrates in order to examine two basic metabolic questions. First, a simple survey of root enzymes was performed to determine which systems were operative. Then, the roots were subjected to mild acid treatment. It has been shown that controlled acid treatment can be employed to inactivate enzymes external to the permeability barrier without causing disruption of cellular integrity. Thus, it was hoped that such treatment might indicate certain enzymes to be rate limiting by correlating enzyme inactivation directly to overall root growth.

Nutrient Solution

The nutrient medium was composed of 11 inorganic salts as described by Taylor (115) and used by Chang (24). The organic portion of Taylor's medium was not included. The final concentration of the inorganic constituents were 0.8 mM KNO_3, 0.87 mM KCl, 0.15 mM KH_2PO_4, 0.15 mM $MgSO_4$, 0.1 mM $Ca(no_3)_2$, 4 μM $FeCl_3$, 6 μM tartaric acid, 9 μM H_3BO_3, 1.8

μM $MnCl_2$, 4.5 μM $ZnSO_4$, and 0.15 μM $(NH_4)_6Mo_7O_{24}$. The pH of the medium was adjusted to 7.0 with 1 N KOH and sterilized by autoclaving at 100°C. for 1 hour.

Enzyme Substrates

Whenever possible, substrates were added to the nutrient solution prior to sterilization. Labile substrates were added just before assays were performed. Maltose, lactose, sucrose, raffinose, xylan, pectin, pectic acid, and all monosaccharide standards were obtained from Nutritional Biochemicals Corp.; laminarin from K & K Laboratories, Plainview, New Jersey; ρ-nitrophenyl-β-D-glucopyranoside, O-nitrophenyl-β-D-galactopyranoside, and ρ-nitrophenyl phosphate, disodium salt from Calbiochemicals, Los Angeles, California; and ρ-nitrophenylsulfate from Sigma Chemical Co., St. Louis, Missouri.

Seedling Preparation and Incubation

Corn kernels of variety Michigan 300 hybrid, "medium flat," were treated and germinated as described in Part I. Five day old seedlings were carefully unwrapped from the towelling. Throughout the handling of the seedlings, precaution was taken to avoid injury insofar as possible. Some broken root hairs must be expected, but only healthy seedlings with uninjured primary roots were selected. These seedlings were floated in distilled water until a sufficient

quantity had been unwrapped. Nine seedlings, with roots ranging from 100 to 110 mm. in length, were wrapped with a cotton plug about the kernel and the roots immersed into a 15 x 100 mm. Pyrex test tube containing 13 ml. of nutrient solution and substrate. This tube was nested within a 25 x 150 mm. tube (Figure 8). This outer tube served to support the seedlings in such a way that the roots were freely suspended in the solution without resting on either the bottom or sides of the inner tube. All glassware had previously been sterilized. Charcoal filtered air was bubbled through the solution for aeration. Seedlings were allowed to grow at 25° under 700-800 foot-candles of fluorescent light as measured by a Weston light meter, model 756. At regular time intervals, three or four replicates were harvested. The roots were excised, dried in an oven at 100°, and weighed. Aliquots of the nutrient solutions were used in the various enzyme assays. Blanks of roots in plain nutrient solution, and substrate without roots were maintained for each assay in order to correct for changes not attributable to enzyme action.

Enzyme Assays

Pectin Methylesterase

Pectin (2.5 g) was dissolved with boiling in 300 ml. of 0.1 N NaCl and mixed with nutrient solution so that the final concentration was 0.5%. The pH of the solution was

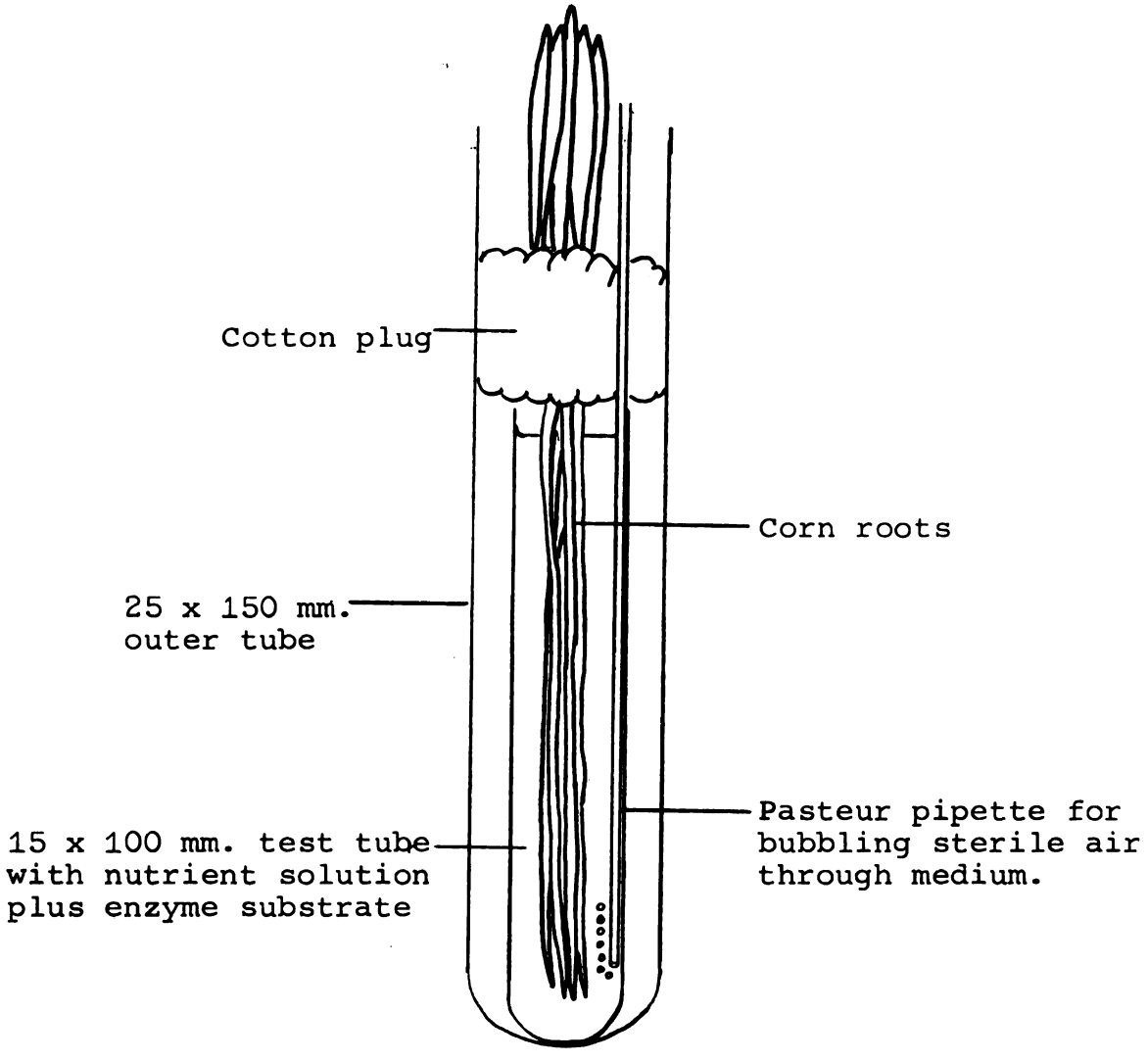

Figure 8. Method of seedling incubation for enzyme determinations.

adjusted to 7.0. After incubation with the corn roots, 10 ml. aliquots of solution were removed and titrated to the original pH with 0.02 N NaOH (60): the milliequivalents of alkali being equal to the milliequivalents of methoxyl liberated by the pectin methylesterase.

Pectinase

Roots were incubated in mineral solutions containing 0.5% of either pectin or pectic acid adjusted to pH 7.0 with 1N KOH. The media were subsequently tested for total reducing power by the method of Benedict (15). Aliquots of the incubation mixture were chromatographed in 77% ethanol; 15% formic acid (85:15), ascending for 8 hours, and butanol: acetic acid: H_2O (5:1:2), descending for 20 hours. Silver nitrate reagent was used to develop the spots.

β-Fructofuranosidase (invertase)

Roots were incubated in 0.95 mM sucrose or raffinose in nutrient solution at pH 7.0. When sucrose was used as substrate, enzyme activity was expressed in terms of glucose production as measured by the glucocose oxidase assay (Glucostat). When raffinose was the substrate, Glucostat, Galactostat and total reducing sugar tests were performed. The latter was assayed by the arsenomolybdate method of Nelson (82). Aliquots of the incubation mixture with the latter substrate were chromatographed in pyridine: ethyl

acetate: H_2O (2:3:1) descending for 24 hours and color developed with anisidine-HCl.

Hemicellulase

A 0.5% solution of laminarin or 0.3% xylan in nutrient solution, pH 7.0 was used as the substrate. Glucostat assay was used for determinations with laminarin and Nelson's reducing sugar method with xylan.

α-Glucosidase

A 0.5 mM concentration of maltose was added to the nutrient solution at pH 7.0. After incubation with roots, samples were assayed for the appearance of glucose by the glucose oxidase assay (Glucostat).

Acid Phosphatase

Roots were incubated in a 3.0 mM solution of ρ-nitrophenyl phosphate in nutrient solution at pH's of 5.0, 7.0 and 8.9. For determining the amount of substrate hydrolyzed, 0.5 ml aliquots of incubation medium was made basic with 2.0 ml of 0.2 N NaOH and the optical density recorded with a Beckman spectrophotometer, model DU, at 410 mμ (16). Values were compared to a standard curve prepared from a serial dilution of ρ-nitrophenol.

β-Galactosidase

Roots were incubated in either 0.5 mM lactose or 3.0 mM o-nitrophenyl-β-D-galactopyranoside in nutrient solution at pH 7.0. When lactose was the substrate, the Glucostat reagent was used to assay for glucose release. When the substituted phenol was the substrate, the assay was performed as discussed for acid phosphatase. A wavelength of 410 mμ was used (51).

β-Glucosidase

Determinations were carried out according to the directions for β-galactosidase except that ρ-nitrophenyl-β-D-glucopyranoside was the substrate.

Sulfatase

Roots were incubated in 3.0 mM nitrophenyl sulfate in nutrient solution at pH 7.0. The assay was that used for all other chromogenic substrates at 410 mμ (51).

Acid Treatment of Roots

Roots of corn seedlings were treated with HCl in an effort to determine if cell wall enzyme activity could be directly correlated to overall root growth. Acid inactivation of surface enzymes was first reported by Willtstätter and Lowry (124) in their investigations of yeast invertase. More recently, a similar technique has been used on fungal

spores by Mandels (71). Roots were immersed in 0.1 N HCl for periods of ½ to 5 minutes, after which they were rinsed in distilled water for an equal time interval. The roots were then used to repeat the pectin methyl esterase, invertase, and phosphatase assays.

Measurement of Root Growth

Normal and acid treated roots were marked with india ink at 10 and 30 mm. from the tip. They were allowed to grow for 10 hours in nutrient solution, and the increase in root length was recorded. Since no increase was observed in the upper region, the elongation in the first 10 mm. of root was the lone parameter used to define root growth.

RESULTS

Enzyme Activities

The root systems of intact corn seedlings were found to possess pectin methylesterase, β-fructofuranosidase, non-specific phosphatase, and β-glucosidase activities. No activities of pectinase, hemicellulase, sulfatase, α-glucosidase, α-galactosidase, or β-galactosidase could be demonstrated under the conditions used.

Figure 9 indicates the response of roots to a pectin substrate. After correction for a slight acidification of the medium attributed to normal root metabolism and any spontaneous dissociation of the methoxyl component from pectin, a linear change in pH due to the appearance of free carboxyls can be demonstrated by the amount of alkali necessary to titrate the medium to its original neutrality. It can be shown that this response is not due to a metabolite excreted from the root by taking an aliquot of plain inorganic nutrient medium in which roots had been growing for various periods of time and incubating it with the pectin substrate. When this is done, no pH change indicating demethylation is observed.

An effort was made to identify the de-esterification product as methanol by forming the hydrazone derivative.

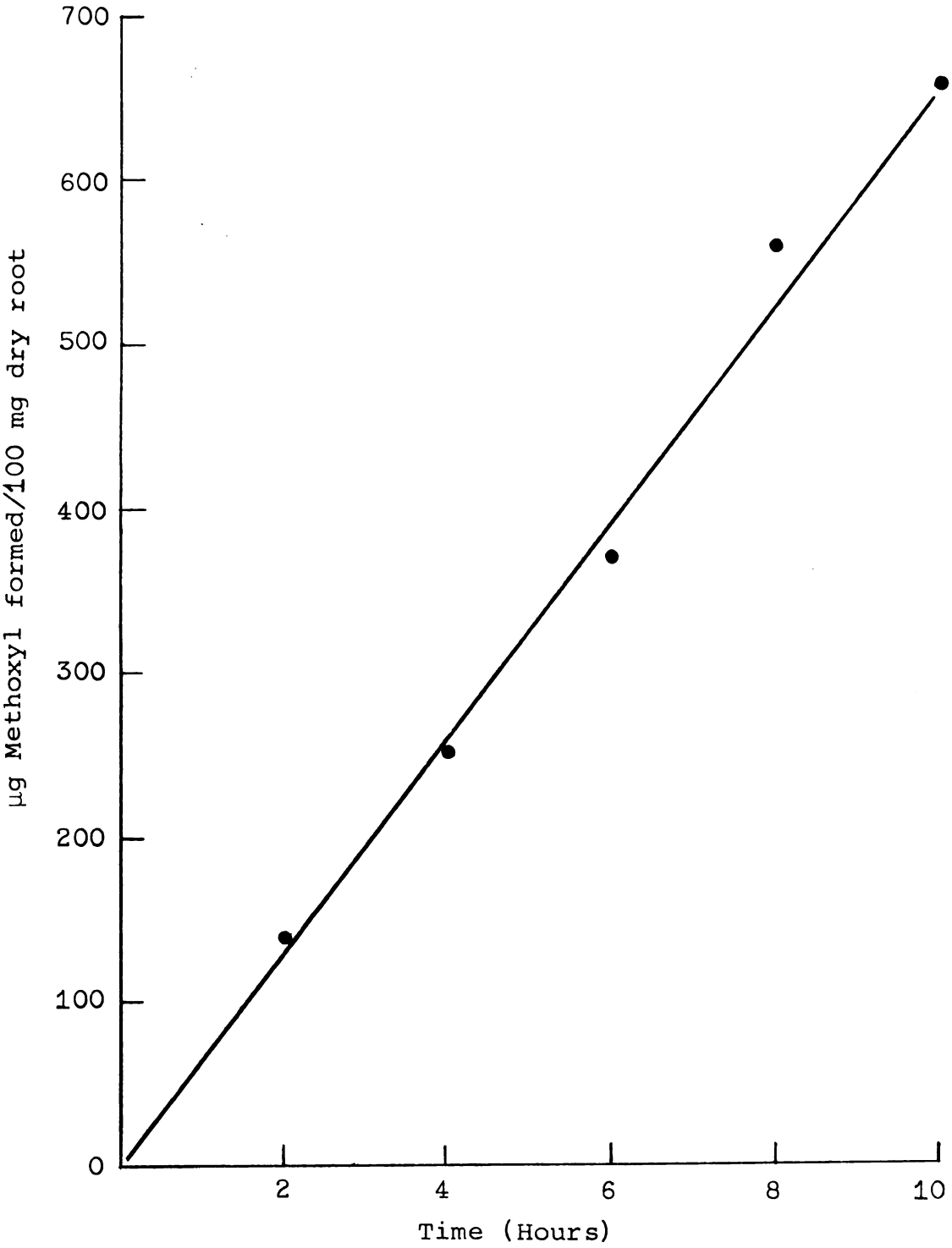

Figure 9. Exocellular pectin methylesterase activity in corn roots.

Aliquots of the incubation mixture and a pectin control were distilled until 80% of the medium had been collected. Since methanol forms an azeotrope with water, no attempt was made to fractionate the distillate further. To approximately 60 ml. of distillate, 5 ml. of 3% $KMnO_4$ was added and the mixture was allowed to stand at room temperature with occasional agitation for 15 minutes. A 5% oxalic acid solution (5.9 ml.) was added and the mixtures were allowed to stand overnight at $0^{o}C$. This procedure was repeated with distilled water and inorganic nutrient solution. Yellow red crystals were formed in the distillates of the incubation mixture (5.6 mg.) and pectin control (3.6 mg.). No crystals were observed in the distilled water or mineral solution. The melting point of the crystals was 164-167^{o}C. The melting point of the 2,4-dinitrophenyl hydrazone derivative of formaldehyde is reported as 166^{o}C. (108). Some of the crystals were dissolved in ethyl acetate and chromatographed using an ethanol: petroleum ether solvent (80:20), ascending for 8 hours (Figure 10). The color of yellow spots were intensified by spraying with 10% NaOH. The samples compare favorably with hydrazone derivatives of commercial formaldehyde and methanol which had been oxidized by permanganate.

When corn roots are incubated with raffinose (galactopyranosyl-glucopyranosyl-fructofuranose), a linear increase in total reducing power is observed with time (Figure 11). Glucostat and Galactostat assays, however, showed that no

Figure 10. Chromatography of 2,4-dinitrophenylhydrazone derivatives of the de-esterification products of pectin.

System: Ethanol: petroleum ether (80:20), ascending, 8 hours.

O = origin
F = front
1 = 2,4-dinitrophenylhydrazine
2 = formaldehyde-hydrazone (prepared from stock formaldehyde)
3 = formaldehyde-hydrazone (prepared by oxidizing methanol to formaldehyde)
4 = pectin
5 = pectin incubated with corn roots

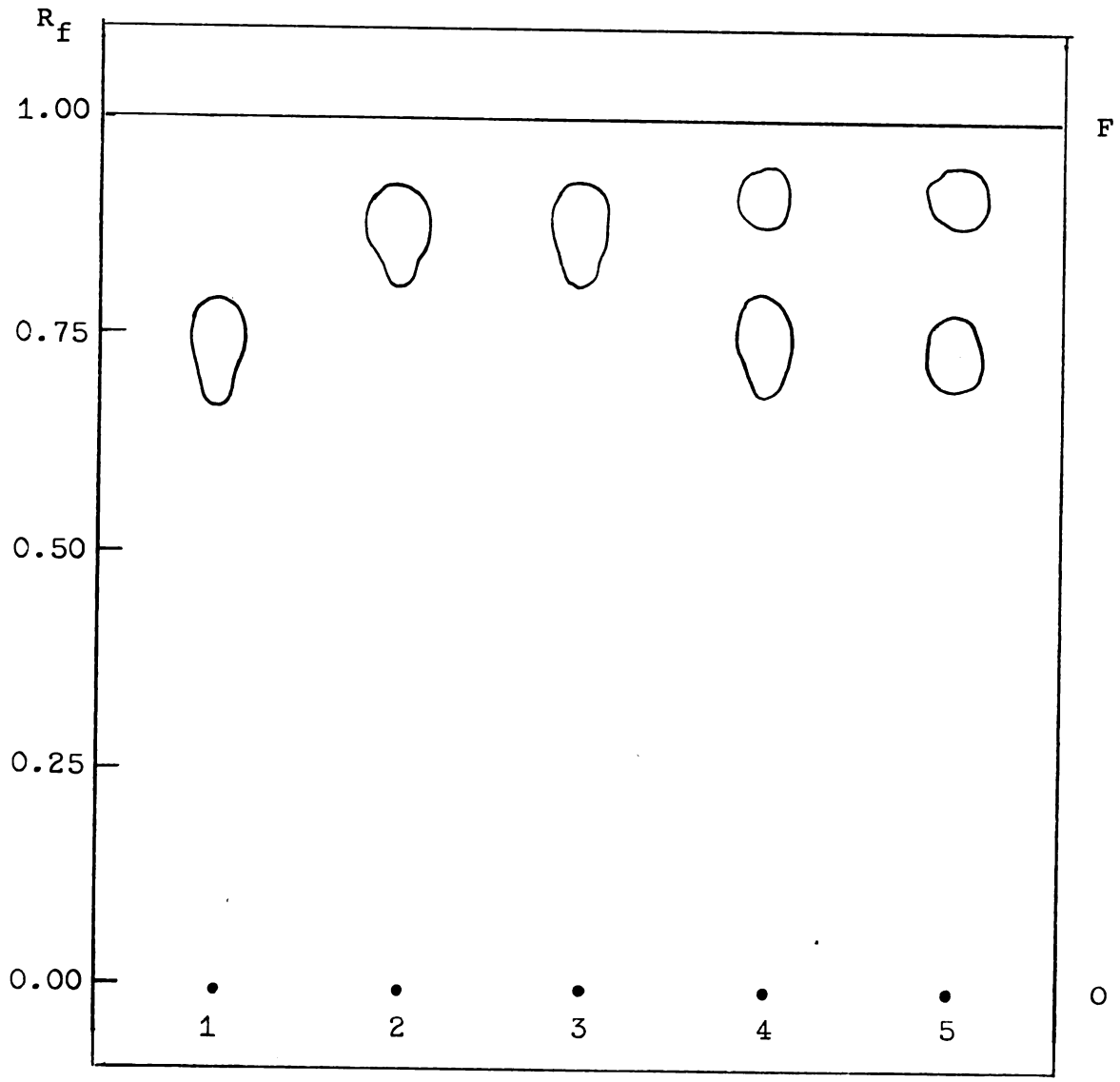

Figure 10

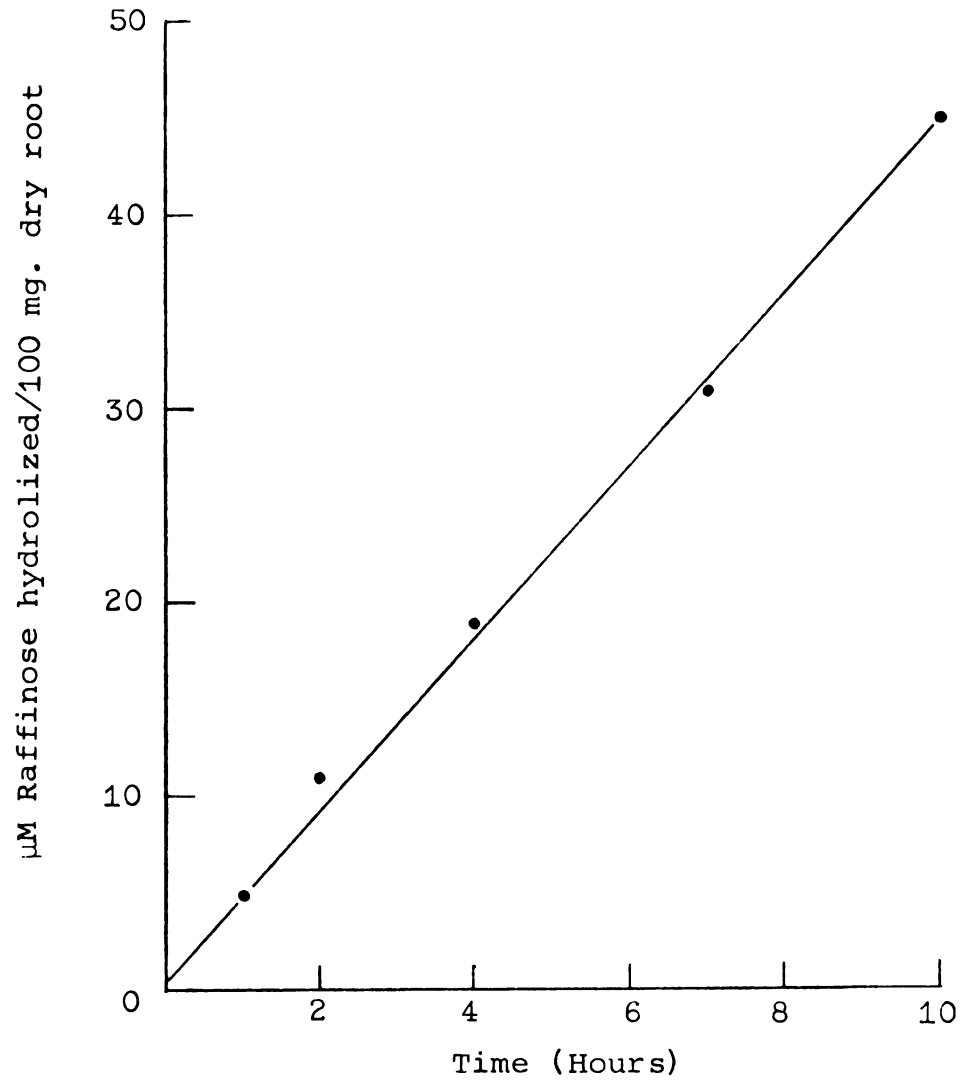

Figure 11. Exocellular β-fructofuranosidase activity on raffinose: in corn roots.

free glucose or galactose had been liberated. When the incubation mixtures were chromatographed, as schematically demonstrated in Figure 12, the reason for this phenomenon became clear. Roots have the ability to hydrolyze the sucrose portion of the trisaccharide but lack the enzyme necessary to attack the melibiose moiety, a 6-O-α-D-galactopyranosyl-β-D-glucopyranose. The only products of raffinose hydrolysis, therefore are melibiose and fructose. Figure 13 indicates that the cell wall enzyme has an affinity for sucrose 3 times greater than that for raffinose.

The availability of chromogenic substrates truly facilitates the accuracy, ease, and reproducibility with which a survey of enzyme activities may be conducted. Actually, the substituted nitrophenol substrates are colorless or nearly colorless at acid or neutral pH's. Upon hydrolysis of the substituted moiety, the nitrophenol can be made to undergo a tautomeric change in an alkaline medium to produce a yellow color which can be measured quantitatively at 410 mμ.

Preliminary experiments using ρ-nitrophenylphosphate were run at 3 pH values to determine the optimum conditions. The results appear in Table 9.

It will be noted that the maximum phosphatase activity for corn roots grown in a mineral solution with ρ-nitrophenyl phosphate was 7.0. In comparison a pH of 5.0 gave only 74-95% of that activity, and, interestingly, a pH of 8.9 gave 59-71%. The range in percent of activity is undoubtedly due to small

Figure 12. Chromatographic evidence of exocellular β-fructofuranosidase in corn roots.

Solvent: pyridine: ethyl acetate: water (2:8:1), 24 hours, descending.

A1, 2, 7 and 10 = nutrient solution, in which roots were incubated for 1, 2, 7 and 10 hours.

B1, 2, 7 and 10 = nutrient solution + raffinose in which roots were incubated for 1, 2, 4, 7 and 10 hours.

S = standards
R, RAF = raffinose
MEL = meliboise
SUC = sucrose
GAL = galactose
GLU = glucose
FRU = fructose

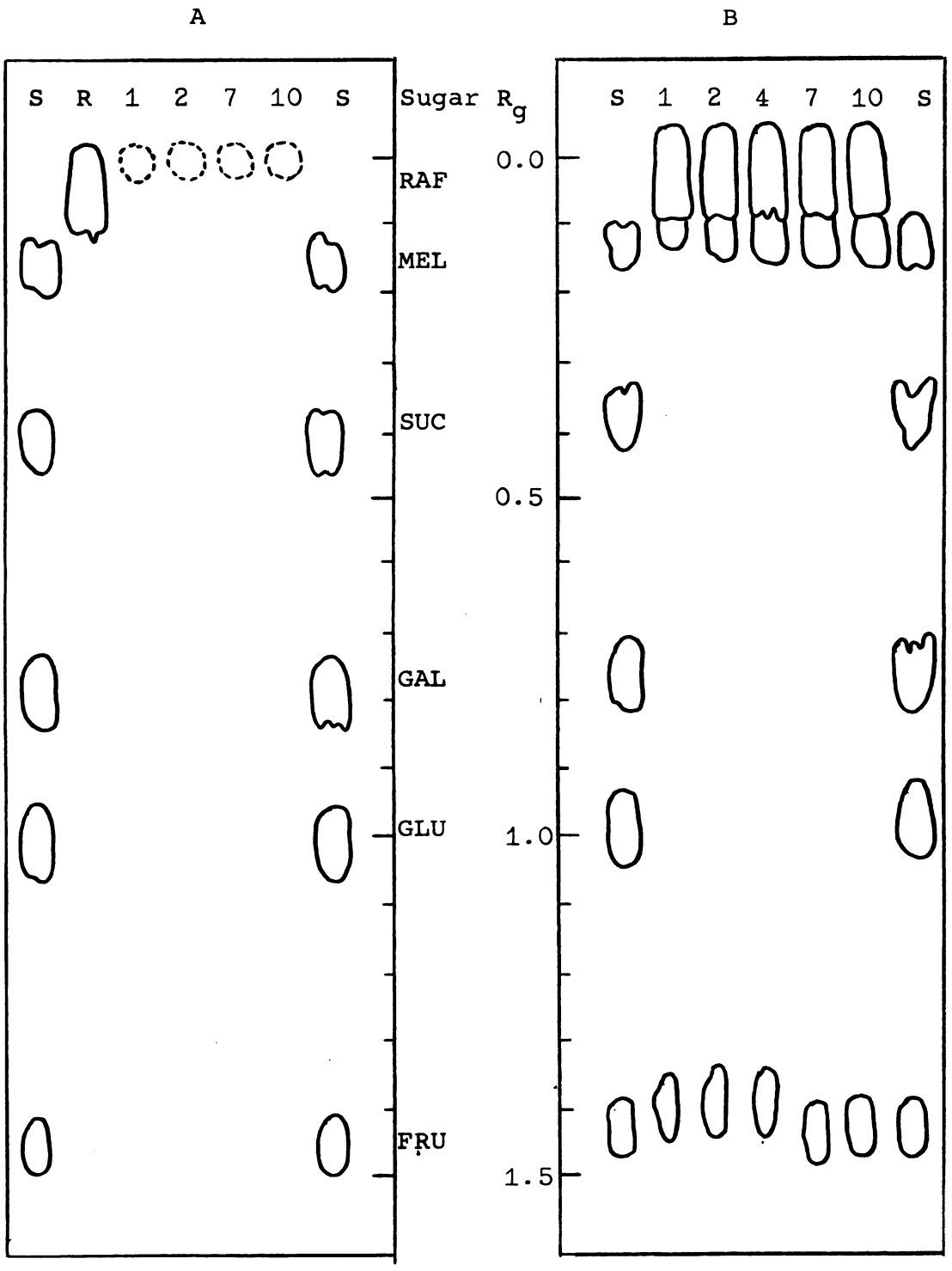
A
B
S R 1 2 7 10 S
Sugar
R_g
RAF
MEL
SUC
GAL
GLU
FRU
0.0
0.5
1.0
1.5
S 1 2 4 7 10 S

Figure 12

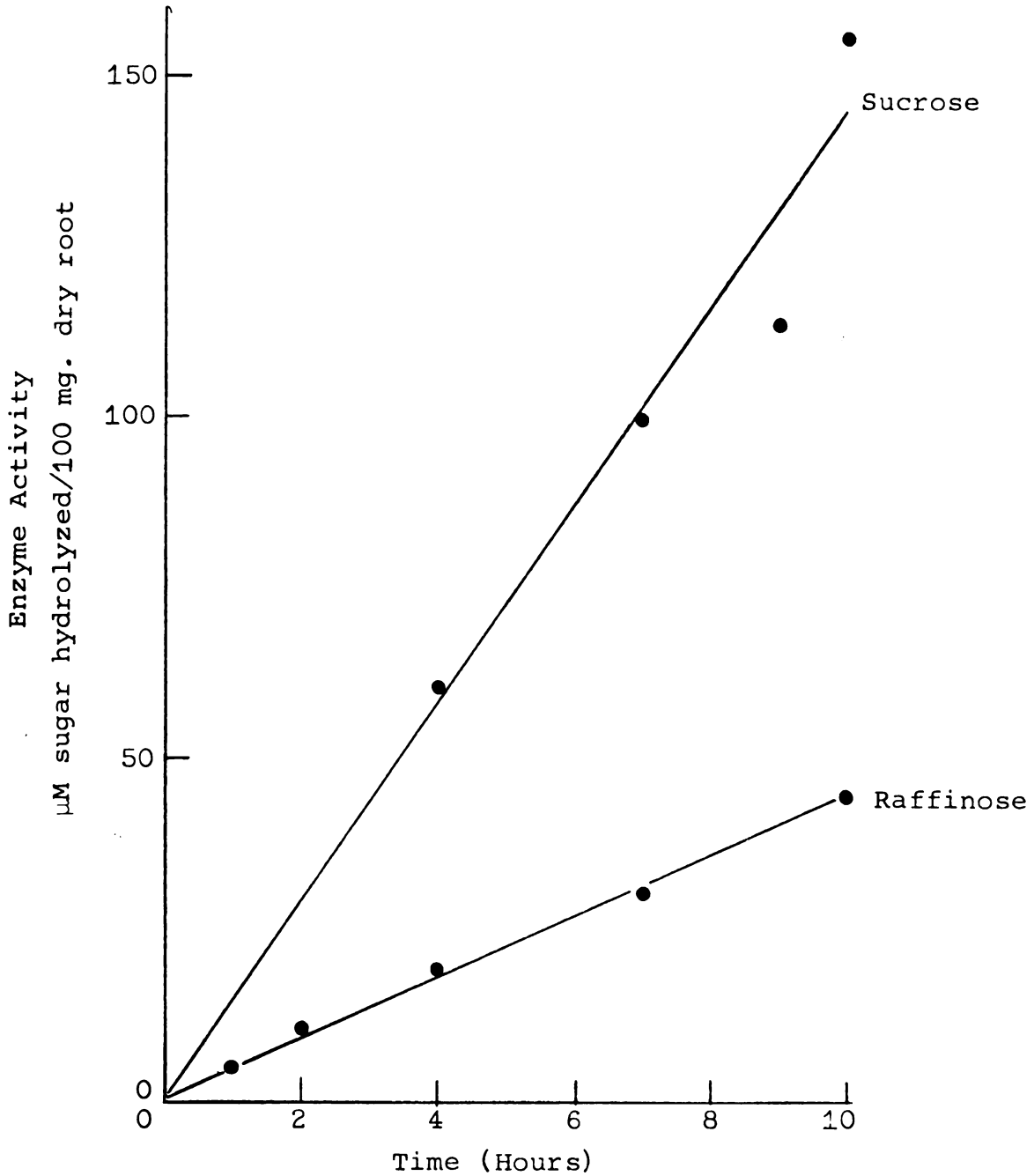

Figure 13. Comparison of invertase and β-fructofuranosidase activities in corn roots.

Table 9. The effect of pH on exocellular corn root phosphatase.

pH	O.D. of ρ-nitrophenol at 410 mμ	
	1½ hrs.	4 hrs.
5.0	1.155	2.579
7.0	1.557	2.697
8.9	0.919	1.932

variations in the weight of the roots used. Since maximum activity was observed in a neutral or slightly acid medium, all subsequent assays were done at pH 7.0. Phosphate was either omitted completely, or added to both the germinating seedlings and the incubation mixtures in order to determine if the enzyme was induced or constitutive. No significant difference was detected and the enzyme appears to be constitutive in nature. This is in variance with the results observed by Ratner and Samoilova (100) who found insignificant phosphatase activity in older corn plants well supplied with inorganic phosphate. Since the present work employs very young seedlings this disagreement may be due to reserve orthophosphate available to the seedling from the corn kernel. Figure 14 shows the phosphatase activity of corn roots incubated at pH 7.0, with and without inorganic phosphate.

Similar data for β-glucosidase activity in the corn roots is presented in Figure 15. It will be noted, as in the other three analyses a reasonably linear relationship is obtained.

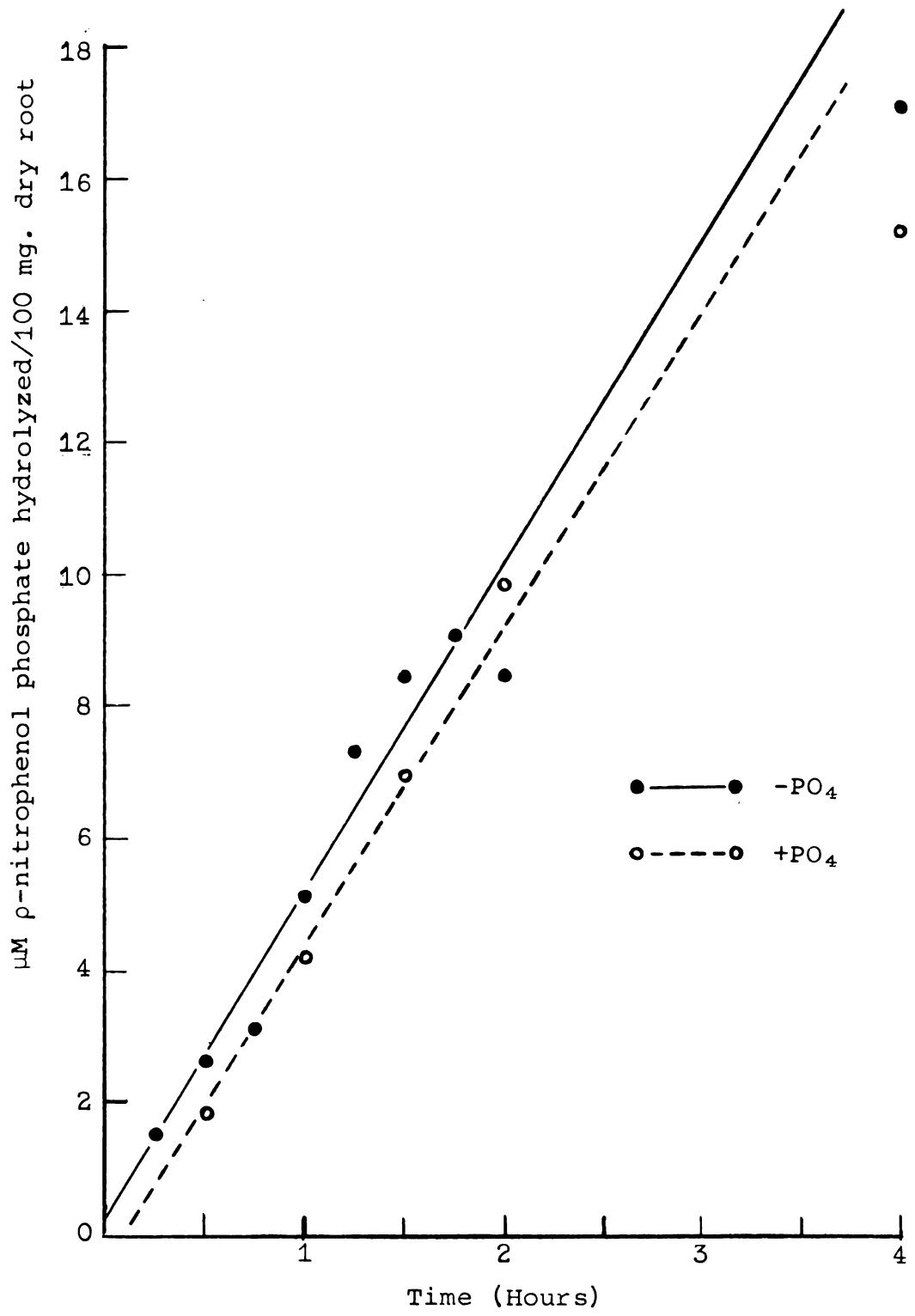

Figure 14. Exocellular acid phosphatase activity in corn roots.

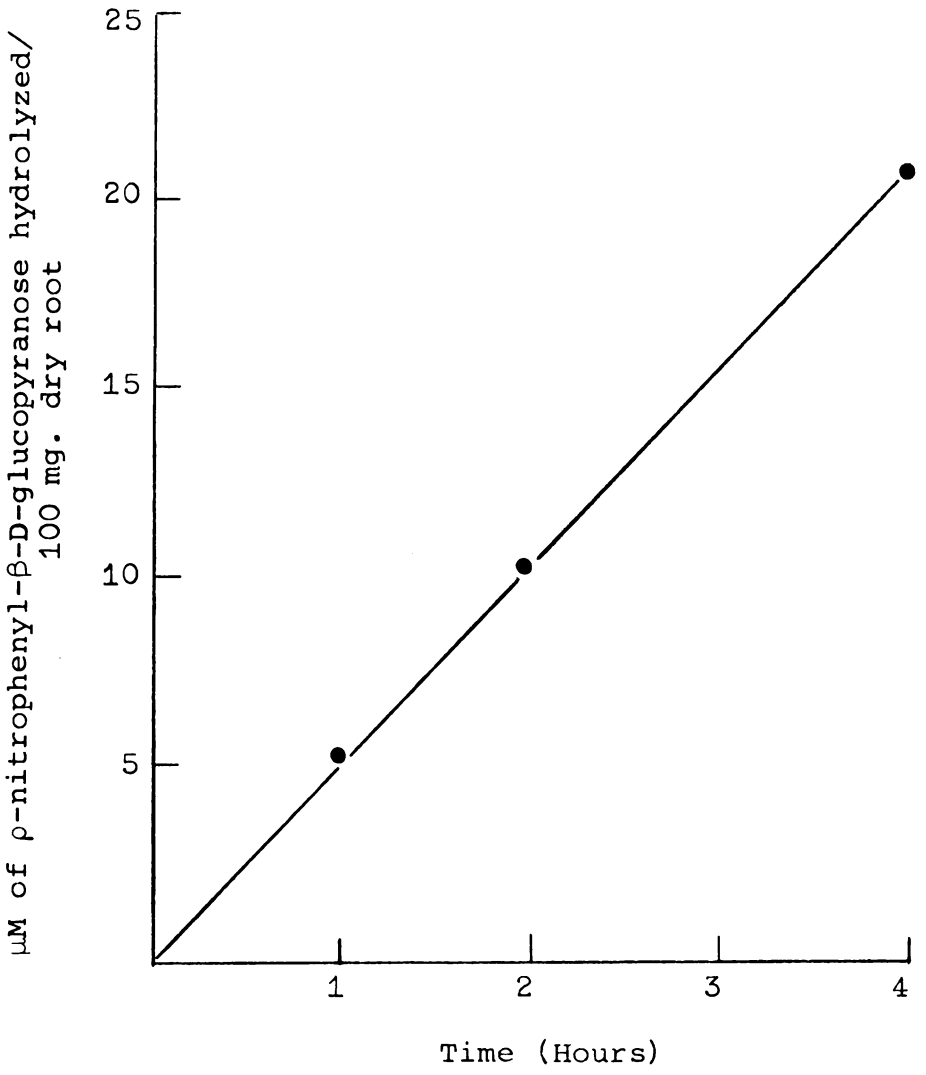

Figure 15. Exocellular β-glucosidase activity of corn roots.

The presence of β-fructofuranosidase and the absence of α-glucosidase and α- and β-galactosidase is corroborated in a recent publication by Göring (44) who used excised corn root tips and coleoptiles.

Effect of Acid Treatment on Enzyme Activity and Root Growth

The treatment of corn roots with acid, even for short periods of time, caused considerable inactivation of the three enzyme systems examined. No significant effect on root growth (elongation) was observed with treatment for as long as 2 minutes. However, acid treatment for 5 minutes resulted in a 51% reduction in elongation. The overall effect of acid treatment on enzyme activity and growth are shown in Figure 16. Activities and growth are expressed as percent of that observed in untreated seedling under the same conditions.

A few observations are immediately obvious although the complexity of the responses precludes a comprehensive assessment at the present time. There appears to be no direct evidence to correlate enzyme activities to root growth. Reduction of enzyme activities of 30 to 70% correspond to only a minor decrease in root growth. The possibility remains, however, that the enzymes are present in an amount far in excess of some low threshold value actually required for normal root growth, and thus, normal growth could continue even though a substantial fraction of a necessary exocellular enzyme had been inactivated.

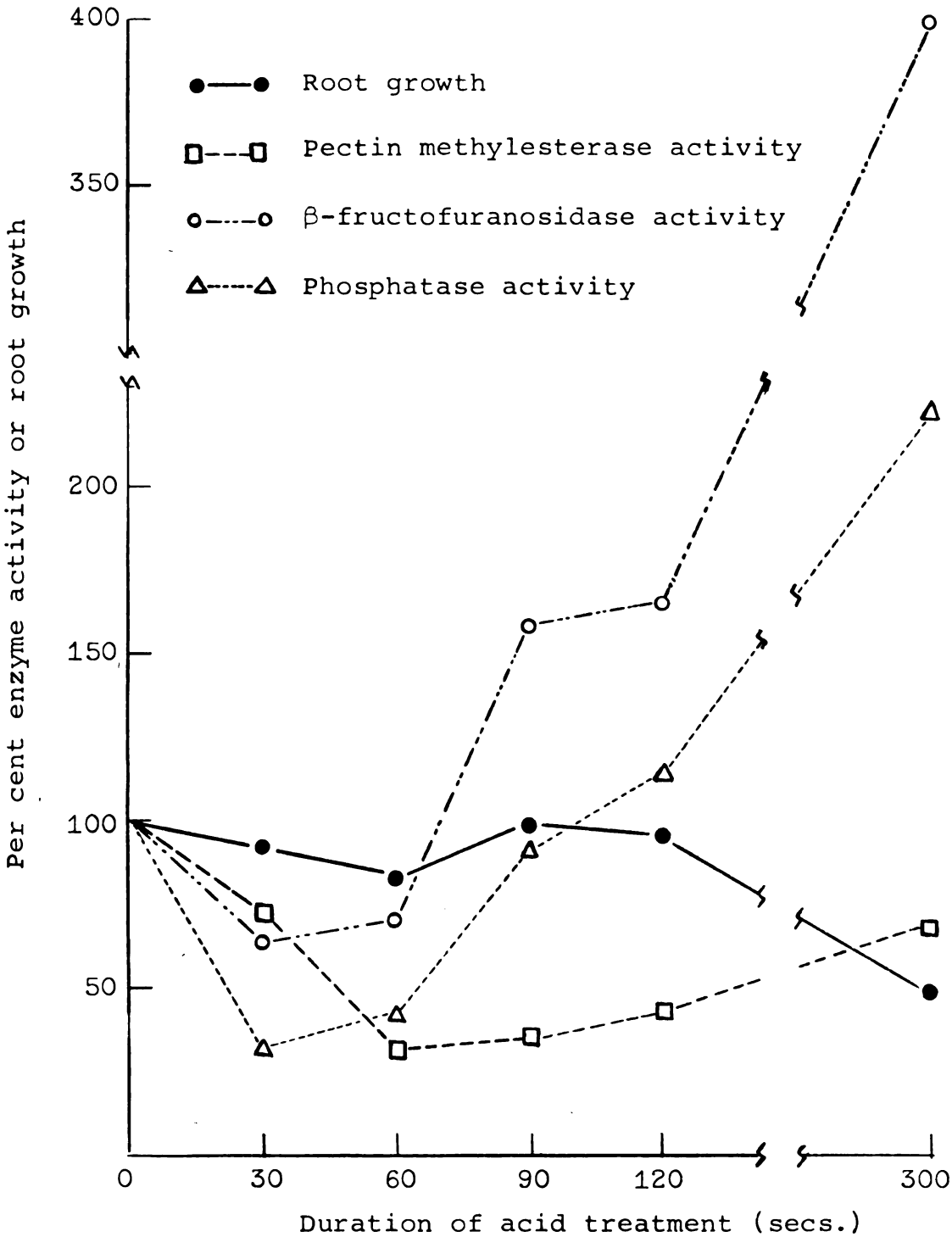

Figure 16. Effect of acid treatment on root growth (elongation) and exocellular enzyme activity.

The response of enzyme activities to acid treatment is clearly a two phase phenomenon. Short periods of acid exposure are associated with decreasing activities which probably reflects the inactivation of surface enzymes as suggested by Mandels (71). In response to prolonged acid treatment there is an abrupt rise in enzyme activities until 2 and 4 fold rates are achieved with 5 minute exposure. It seems quite likely that this increase is attributable to permanent damage of the membrane allowing leakage of intracellular enzymes. Assuming this to be the case, the term "apparent enzyme activity" should be adopted since the possibility is strong that non-enzymatic, cellular components are contributing to the determinations. This is especially true in relation to any reducing materials from within cell that might contribute to the total reducing power, and therefore appear to be a result of β-fructofuranosidase activity.

An interesting facet of enzyme inactivation can be observed in the differential rates to which they are effected. Thus 28% pectin methylesterase, 36% β-fructofuranosidase, and 69% phosphatase inactivation with 30 second acid treatment might be explained by different susceptibilities of each enzyme to acid, or compartmentalization of the enzymes within the wall in such a way that they are exposed differently to the medium.

A final observation can be made in regard to the apparent increase in growth with $1\frac{1}{2}$ and 2 minute acid treatment over

½ and 1 minute treatment. If this is a real response, then, the increase in enzyme activities might indeed prove significant.

DISCUSSION

A survey of enzymes associated with the cell wall of corn roots was performed with the intention of relating their presence and activities directly to the overall root growth. Willstäter and Lowry (124) and Mandels (71) have demonstrated that short term acid treatment inhibits surface enzymes without damaging the integrity of the tissue. Utilizing this knowledge, it was proposed to demonstrate that an inactivation of one or a group of wall enzymes would cause a concomitant reduction in root growth. Thus, it was intended to show which enzymes hold the key roles in cell wall metabolism and root growth.

It has been demonstrated in the present work that intact corn roots possess the ability to hydrolyze the sucrose moiety of raffinose, organically bound phosphate, and a β-glucan linkage, and to demethylate pectin when they are incubated in a nutrient solution enriched with the appropriate substrate. In each assay a linear rate of reaction was observed with time. Average hydrolytic rates observed were 4.8 (raffinose→ fructose + melibiose), 5.3 (ρ-nitrophenyl-β-D-glucopyranoside→ glucose + nitrophenol), 5.1 (ρ-nitrophenyl-phosphate→ P_i + nitrophenol), and 2.1 (pectin→ pectic acid + OCH_3) μM of product/hour/100 mg. dry root.

The possibility that the observed enzyme activity was a result of microbial or fungal contamination is negligible due to preliminary sterilization of the corn kernels and the semi-aseptic conditions under which the experiments were conducted. In addition, when aliquots of nutrient solution, in which corn roots had been grown, were removed and incubated with pectin, no demethylation activity was observed. Control samples of substrates were always run during experiments and no indication of breakdown was ever observed, therefore the activity was a result of root action. Furthermore, a wider range of enzyme reactions might be anticipated, particularly pectinase, if microbial or fungal activity was involved. Tests for exocellular root lactase, maltase, amylase, pectinase and hemicellulase proved negative under the same physiological condition which allowed the four systems listed above.

Criticism that sloughing of dead cells from the root cap or root breakage produced the activities may be approached with similar reasoning. One would expect a larger number of enzyme activities, particularly amylase since the corn root is known to possess a substantial amount of intracellular starch. Chang's report (24) of no protease activity reinforces this argument against breakages as a prime factor in enzyme activity. Since the reports by Hellebust and Forward (49) and Mertz (76) indicate maximum enzyme activities of invertase and ascorbic acid oxidase are associated with the

elongation zone of roots, little consideration need be given to the root cap hypothesis.

That these enzymes are exocellular is substantiated by the results of acid treatment. It was observed that bathing the roots in 0.1 N HCl for short periods of time inactivated the enzymes without causing serious damage to the cell membrane as evidenced by near-normal root growth. This phenomenon could only occur if the enzymes were located external to the membranes. The tremendous increase in enzyme activity observed with extended periods of acid treatment probably coincides with membrane disorganization and leakage of cytoplasmic enzymes into the medium. Significantly, pectin methylesterase, whose function is almost entirely related to the cell wall and therefore would not be expected to be found in the cytoplasm, does not reflect this enormous upsurge.

It is impossible to establish what relationship exists between the individual enzymes and root growth, because normal growth (elongation) continued even after 50% of the various enzymes was inactivated. The possibility remains, of course, that only a very small percentage of the enzymes is required for normal metabolism and that the current method of examination is not effective enough to show this.

The results are interpreted as indicating phosphatase, β-glucosidase, β-fructofuranosidase, and pectin methylesterase activities associated with the cell wall of the corn

root. The use of large (pectin) and non-metabolic (substituted nitrophenols) is further evidence that these enzymes exist and function externally to the permeability barrier of the cell.

The presence of exocellular phosphatase is reported extensively throughout the literature. It is generally referred to as an acid phosphatase. The present report of considerable phosphatase activity when roots were incubated in a medium at pH 8.9 suggests that there may be at least two species, an acid and an alkaline phosphatase. The presence of these enzymes in the cell wall, and therefore, in close contact with the environment, would be of ecological as well as metabolic importance to the plant. This situation could make organically bound phosphate available to the plant in the event that the inorganic supply was limited. Therefore, in addition to exocellular enzymes involved directly in growth, there appear to be some that may play an important role in overall nutrition. A significant supply of phosphate in the cell wall becomes increasingly essential in light of reported nuclease activity and the inference of *in situ* wall synthesis.

The presence of exocellular β-fructofuranosidase or invertase is puzzling since fructose is not reported in cell wall tissue. It appears likely that this sugar is utilized in intracellular metabolism. Although it is generally accepted that invertase has differential affinities for both

sucrose and fructose-containing oligosaccharides, Edelman and Jefford (34) reported the isolation from sunflower of an invertase and two fructofuranosidases, the latter having little activity against sucrose. Thus, the possibility exists that sucrose and raffinose hydrolysis by corn roots occurs through two separate enzyme reactions.

The presence of substantial pectin methylesterase and β-glucosidase activities lends support to the idea that in situ modifications of the wall occur during growth. The proposed function of the esterase in controlling wall plasticity is well known. The presence of β-glucosidase indicates an alternate path by which material can be added to wall cellulose. Not only is it possible for extension of cellulose molecules by end synthesis and by intussusception of new molecules between existing ones, but also by cleavage and insertion of glucose residues along the length of the chain.

The differential rates of inactivation exhibited by the enzymes in response to acid treatment is an interesting phenomenon that can only be treated with speculation at this time. It most probably is a simple reflection of differential sensitivity to acid. A possibility to be considered, however, is that compartmentalization of the enzymes within the wall leads to differing degrees of exposure to the surface. The accessibility of a relatively large molecule (pectin) to the esterase, the enzyme least affected by short term acid treatment, would tend to invalidate this consideration.

The presence of β-glucosidase and the absence of hemicellulase and pectinase activities suggest basic differences in the metabolism of the cellulose, and the hemicellulose and pectin wall fractions. Mechanisms of cellulose metabolism have already been considered. In the absence of rigid organization, as exhibited by cellulose, hydrolytic action may not be required for expansion to occur in the other fractions. Mechanisms of cellulose metabolism have already been considered. In the absence of rigid organization, as exhibited by cellulose, hydrolytic action may not be required for expansion to occur in the other fractions. Thus, addition to pectin and hemicellulose may be a random insertion of new chains between the existing ones with ionic or hydrogen bonds being involved rather than coralent structures.

SUMMARY

1. Phosphatase, β-fructofuranosidase, β-glucosidase, and pectin methylesterase activities have been found associated with the roots of intact corn seedlings.

2. The inactivation of these enzymes without significant damage to the cell membrane or in the overall root growth is taken as evidence that these enzymes are located external to the cell membrane.

3. The evidence suggests that there may be two species of phosphatase, an acid and an alkaline, associated with the wall. The presence of phosphatases in the cell wall has ecological as well as metabolic implications.

4. The presence of pectin methylesterase substantiates its function in cell wall plasticity. The β-glucosidase is presumably essential in developmental changes of the cellulose wall fraction.

5. The absence of pectinase and hemicellulose activity suggests that the metabolism of these fractions differs basically from that of the cellulose.

6. It is not possible to draw correlations concerning the relative importance of these enzymes to the overall growth of the root since growth, measured as total elongation, continues after 50% inactivation of the enzymes by acid treatment.

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