POLYSACCHARIDES EXTRACTED WITH SDS FROM WHEAT SEEDLING ROOTS

by

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INTRODUCTION

Electron microscope autoradiography of primary wheat root caps which were treated with a pulse of U-^{14}C-glucose has shown that a radioactive substance is produced in the Golgi bodies. The radioactivity was observed to be transferred subsequently to the Golgi-derived vesicles and finally to the cell wall and the root cap slime (3). The electron microscope and histochemical evidence indicate that this material, a precursor of extracellular substances, is polysaccharide in nature.

Actual chemical proof of the presence of polysaccharides in Golgi body preparations has been provided by workers in this laboratory and by Bowles and Northcote (5). Much of this work has concentrated on materials resembling hemicellulose; that is, they are not soluble in lipid solvents or in water but they are soluble in KOH. Since polysaccharides are often seriously degraded by treatment with KOH it is possible that milder solubilization procedures would provide a more useful polysaccharide extract.

A preliminary study showed that radioactively labeled, high molecular weight carbohydrates could be isolated from Golgi body preparations after solubilization by detergent. This investigation seeks to determine the practicality of isolating this material on a larger scale using the detergent extract of whole roots. A second objective is to make a preliminary investigation into the types of polysaccharides being synthesized in growing wheat seedling roots.

LITERATURE SURVEY

Reviewed here is the body of literature which deals with the synthesis and structure of the cell wall components and precursors. The components with which this thesis is concerned may be classified as cellulosic microfibrils, matrix polysaccharides and the glycoprotein called extensin. Another component present in secondary cell walls is lignin but since it is not present in the primary cell walls dealt with here, discussion of this substance is omitted.

I. Golgi Bodies and Cell Wall Synthesis

In searching for the particular structures within the cells which are responsible for the production of the cell wall, one should probably focus attention primarily on the Golgi bodies. Golgi bodies have been implicated in the synthesis and secretion of polysaccharides and in the preparation of proteins for secretion. Electron microscope autoradiography has provided convincing evidence to this effect in the pancreatic production of zymogen (1), the production of mucopolysaccharide by intestinal goblet cells (2), and the production of root slime by the outer cells of wheat root caps (3). These studies were performed by introducing labeled metabolites into the tissues and examining the distribution of the radioactivity at various time intervals after the pulse. Further confirmation was provided by isolating the various subcellular particulate fractions in the centrifuge and examining the location of the radioactivity (4,5). There have been many other reports of Golgi involvement in secretion from a wide variety of tissues, but since they have been recently reviewed in several places (6–10) there is little point in listing them all here.

One might expect, then, that Golgi bodies, the secretory machinery of

the cell, should have a role in the secretion of cell wall polysaccharides. The actual evidence for such a role is as follows:

1. Golgi bodies have been found in close proximity to cell walls in tissues where active cell wall synthesis is taking place, e.g., in tips of root hairs (11), in pollen tubes (12), in fern rhizoids (13), in secondary thickening of xylem (14,15) and in cell plate formation (16,17).

2. In some cases rapidly developing cell walls are accompanied by hypertrophied Golgi bodies, i.e., swollen for increased production (12,15,16). This is a condition which has also been found in many other rapidly secreting tissues, both animal and plant (1-3,6).

3. Staining reactions indicate a similarity between the cell wall material and the contents of vesicles in the region of the plasmalemma (13,16-18). These vesicles are probably derived from the Golgi bodies for the purpose of transporting the Golgi produced material to the cell wall. O'Brien points out that most of the fixation and staining techniques used are not chemically specific, and similarity in the staining reaction should not be regarded as proof of identity but as concurring evidence only (19).

4. Polysaccharides which have monosaccharide compositions similar to that of the cell wall matrix have been isolated from the Golgi body particulate fraction (5,20,21).

5. Enzymes have been isolated from particulate preparations of plant cells which are required for the synthesis of polysaccharides. For example, an enzyme has been found in corn cob Golgi which is capable of transferring a methyl group from 5-adenosyl-L-methionine to the glucuronic acid group of certain xylans (22).

The Golgi body is composed of a stack of discs called cisternae. Each cisterna is highly fenestrated in the outer regions to the extent that the peripheral portions may be characterized as a network of tubules. The cisternae are believed to be formed on one face of the apparatus (dictyosome) and dissipated on the other face. In actively synthesizing dictyosomes the tubules in the outer region of the cisternae contain swellings which are believed to bleb off to form the vesicles which are often observed in the vicinity. The mature face of the dictyosome is usually smaller in diameter, probably because it has lost material due to blebing (7,23). An array of intercisternal fibrous elements has been found to lie between the central portions of adjacent cisternae (24). They appear to be involved with binding the cisternae together.

With the growing number of electron micrographs of Golgi bodies and other organelles, a general theory of intracellular transport has begun to evolve. This theory in its current form is presented by Northcote in his review, "The Golgi Apparatus" (6). The following is a brief synopsis. Proteins created by the ribosomes on the surface of the rough endoplasmic reticulum are discharged from the ribosome into the lumen or, if lipophylic, into the membrane itself. They are thus transported to the smooth endoplasmic reticulum which is then blebed off into small vesicles. In some cases there may be large amounts of protein destined for excretion such as zymogen in pancreas or mucopolysaccharide in goblet cells; however, a very important portion of the proteins are enzymes and structural proteins to be used in the functioning and transformation of the membrane system. The small vesicles are transported to the forming face of the dictyosomes where they are incorporated. The Golgi apparatus concentrates the product and performs certain

chemical operations on it. The membrane is transformed so that it resembles the plasmalemma in staining properties. Large vesicles are blebed off from the periphery of the stack and from the mature face. These vesicles may perform further modification and concentration of the product as they transport it to the plasmalemma. At the surface of the cell the contents of the vesicles are discharged by reverse pinocytosis, the membrane becoming a new addition to the plasmalemma. In the Golgi apparatus and possibly at other points along the route mono- or oligosaccharides may be bound to form glycoprotein or used for the synthesis of polysaccharide.

The whole transport process is excellently demonstrated in goblet cells (25) where a single large dictyosome extends across the entire breadth of the cell as a cup shaped organ. The endoplasmic reticulum is extensive and very closely juxtaposed to the forming face. The small interval between is seen to be filled with small, lightly stained vesicles. Such a confined space precludes any possibility of the vesicles traveling anywhere except to the opposing membrane surface. The overall direction of transport has been well established in this tissue by autoradiography (2).

The synthetic role of the membrane system is emphasized by Northcote (6) who concludes:

> "...since synthetic activities also occur within the [membrane] system it is comparable more to the production line of an industrial process rather than a pipeline through which preformed material is passed."

Although some have proposed that cellulose as well as matrix polysaccharides may be synthesized in Golgi bodies, this does not now seem likely. It is too difficult to explain how cellulose molecules could become oriented into the microfibrils and how the microfibrils could become oriented in the wall. It is currently believed that clusters of enzymes in the plasmalemma give rise to whole microfibrils (26).

II. Properties of the Cell Wall Materials

A. Microfibrils and Their Organization

The cell wall can usually be divided into three distinct zones: the middle lamella, the primary cell wall and the secondary cell wall. The primary cell wall consists of a gel-like matrix of polysaccharides strengthened with widely spaced cellulose microfibrils (27). While microfibrillar arrangements vary widely from tissue to tissue, they have been classified into six groups by Roelofsen (27).

In the wheat root tissues used in this study the predominant growth type corresponds to Roelofsen's class D, "Tissue Cells with Predominant Growth in Length". In these cells the microfibril orientation is initially circumferential. The cells of the meristem at first grow both in length and girth but later only in length. The growth along the length is quite extreme and it is seen by ^{14}C uptake and by examination of pit field spacings to be evenly distributed along the entire cell (28). As the cell elongates the older microfibrils are drawn first into a "multinet" pattern and eventually into an axial orientation (27). Newly formed microfibrils, however, continue to be laid down in a circumferential direction on the inside surface of the cell wall where they are utilized to resist the radial component of the turgor pressure. A cross section of the wall, therefore, shows a continuous gradation from circumferential to axial alignment (27).

Meristematic cell walls join together to form compartments roughly hexagonal in cross section. The turgor of the cell, however, tends to force the cavity to become circular by squeezing material from the sides into the corners to form thickenings or "ribs" (27). The

microfibrils in these ribs tend to be aligned axially. These and other thickenings of the primary cell wall should not be confused with secondary thickenings which are formed only after growth has ceased.

The secondary cell wall consists of very closely spaced microfibrils packed together to form a thick rigid capsule. In woody tissue the long narrow xylem cells receive most of the secondary thickening forming tracheids or wood fibers. The microfibrils are deposited in three distinct layers each oriented in a different direction to increase strength (29,19).

The microfibril itself is a compact bundle of cellulose molecules with an elliptical cross section measuring about 200 Å by 75 Å (27,30). They are mostly crystalline in nature but they also contain about one third "paracrystalline" material (same as amorphous except that molecules remain roughly parallel). The paracrystalline region occurs mostly in bands every 300-600 Å along the strand (27). In addition the entire surface is covered with an amorphous hair-like layer composed of stray cellulose molecules and matrix glycans which have been bonded to the microfibril surface (27,30). Some authors feel that microfibrils must actually be composed of four or more smaller elementary fibrils (31).

B. Matrix Structure

The matrix of the cell wall is primarily a gelatinous mixture of various neutral and acidic glycans in water. In order to understand the structural roles of these polysaccharides it is necessary to examine their physical properties and the underlying chemical microstructures which cause those properties. Because the properties of a polysaccharide are greatly influenced by its ability or inability to crystallize, the

crystallization phenomenon will be dwelt upon in considerable detail. Discussion of specific chemical structures of these polysaccharides is reserved for a later section.

The propensity of a polysaccharide to crystallize involves two factors. The first is the stability of the crystal formed (32). Thus cellulose, which is able to form into a compact lattice with all three hydroxyls linked by hydrogen bonding, creates a crystal of superior stability. The second factor and probably the more important is the prevention of crystal formation by the insertion of hetero sugars in the backbone or by the attachment of side chains. If crystallization is thus stereochemically prevented, the material will have to remain in viscous solution even though crystallization would be energetically more stable. For this reason also, it's no surprise that cellulose, a long unbranched homoglycan should be so easily crystallized.

Although crystallization is much less common among matrix polysaccharides it does sometimes occur. Homoxylans, for instance, can be easily crystallized (33-35). The crystal structure contains chains with a helical twist requiring three residues for each full turn (33) whereas cellulose requires only two residues (Fig. 1). Because of this twist xylan cannot hydrogen bond its number three carbon hydroxyl within the chain as easily as cellulose (33). Also, since it is only a five carbon sugar it has only two hydroxyls available for hydrogen bonding rather than three. The crystal structure is looser than that of cellulose giving it room to accommodate a row of water molecules which helps to stabilize it (Fig. 2). This space can also be used to accommodate a very limited number of side chain groups (35,32). Other homoglycans which have been found to be capable of crystal formation are mannans (36)

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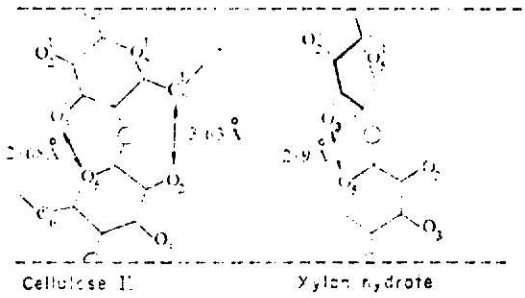


Fig. 1. Configurations of Xylan Hydrate and Cellulose (34).

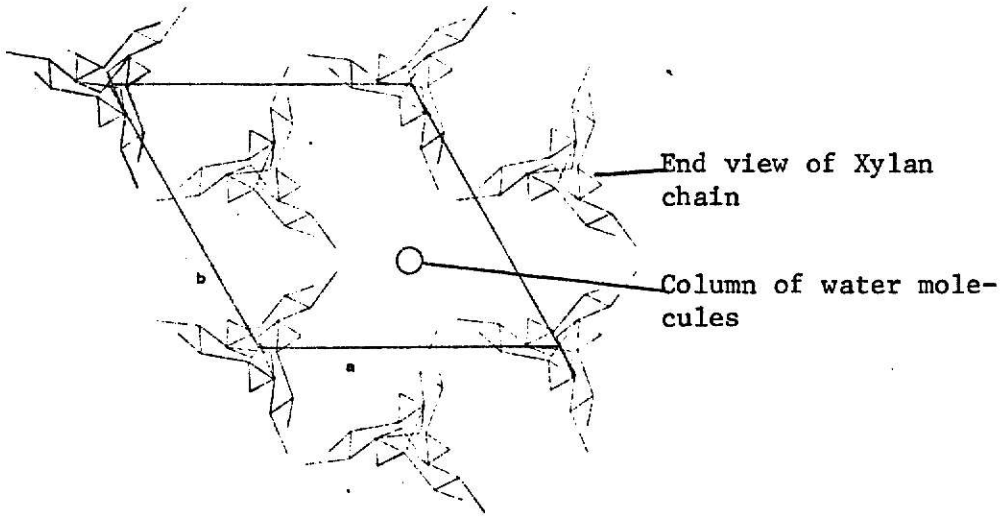


Fig. 2. Unit Cell of Xylan Hydrate (35).

and polyuronides (37).

If a polysaccharide does not have the unbranched homoglycan configuration required for extensive crystallization, it is still possible that the substance can form a limited type of crystal referred to as a crystallite (37). A crystallite is formed whenever two or more parallel chains link together in a regular lattice. Side chains must be oriented outward away from the center of the crystallite. Obviously, the size to which a crystallite can grow is limited by the number, size, and degree of branching of the side chains.

The macroscopic behavior of a polysaccharide is heavily dependent on the degree to which it is able to crystallize. A highly branched polysaccharide tends to act like a sponge holding large volumes of water in a viscous or slimy solution. On the other hand, a polysaccharide which is able to cross-link by forming crystallites will lose some of its capacity to act as a solute and will instead adopt the semi-rigid framework of a gel (37,38) or, if crystallization is more extensive, a precipitate will be formed.

These concepts can be readily applied to the polyuronides and other glycans of the primary cell wall. Since polyuronides contain some rhamnose in the backbone and numerous side chains which serve to restrict the degree of crystallization one would expect the matrix to contain microcrystallites. That this is indeed true was shown in the case of intact celery petioles by x-ray diffraction (39). Rees (37) theorizes that the non-covalent bonds of crystallite gels are the primary source of the strength of the matrix. An alternative theory will be mentioned later.

C. Interaction Between the Matrix and the Microfibrils

Related to the ability of certain glycans to bond together in crystallites is their ability to bind to the surface of the cellulose microfibrils. Some xylans and glucomannans, for example, have been shown to be adsorbed on the surfaces of microfibrils in vitro as a result of hydrogen bonding or van der Waals forces (40). In the adsorbed state these polysaccharides must be oriented with their side chains directed outward away from the surface of the microfibril thus preventing the adsorption of any further layers of polysaccharide (41). The ends of the adsorbed glycans as well as their side chains are dispersed in the matrix creating a moderately firm bond between the matrix and the microfibril (27).

The cell wall is quite analogous to modern high strength composite materials such as fiber glass-resin. Indeed, the stress analysis theories applicable to such materials have been successfully applied to cell wall structures (42). It has been found that when the appropriate variables are measured and inserted into the formula for the tensile strength of a composite, an accurate prediction of the breaking point of wood fiber and other plant substances can be determined.

III. Extensin

In 1960 it was discovered that the hydroxyproline content of plant tissue is almost entirely contained in the cell wall (43,44). Subsequently it was found that the hydroxyproline is part of a glycoprotein which appeared to have a central role in the structure and function of the cell wall. Lamport called this material "extensin" and the name is now widely used. An accurate picture of the structure and function of extensin is not yet in hand and much of what has been discovered remains unconfirmed. It is, therefore, convenient

to treat each significant discovery separately and evaluate the evidence.

A. Its Structure

The most reliable quantitations of the various components of extensin (45,46) are actually derived from its immediate precursor, a substance which appears to be identical to extensin in every way except that it is not irreversibly bound to the cell wall matrix. Since this precursor can be easily extracted from the cytoplasm or cell walls with non-destructive reagents, it is a natural target for structural investigations. The chief drawback to its use, however, is the low concentrations of this material found in most tissues (45). Also, since the amount of the precursor present will only be significant during a period of rapid synthesis of extensin, the experimental tissue must be carefully chosen.

From Table I it can be seen that both Chrispeels and Lamport report large portions of hydroxyproline and serine in the hydrolyzates. Other hydrophylic amino acids such as glycine, threonine, and aspartic acid are also prominent. Since hydroxyproline and proline account for 10 to 24 percent of the amino acid residues, it is quite probable that regions exist where several such residues occur in succession. This has been confirmed by Lamport (47-49) who has actually isolated tryptic peptides from cell-wall bound extensin of tomato cell suspension cultures. The following peptides have been sequenced (49):

Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Hyp-Val-Tyr-Lys
Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys
Ser-Hyp-Hyp-Hyp-Hyp-Lys

Table I. Composition of the Precursor of Extensin

Component	Plant Source				
	Sycamore Maple*	Tomato*	Sphaerocarpos* (Liverwort)	Ginko*	Carrot**
Hyp	29	31	30	28	10.3
Pro	6	—	—	1	2.8
Asp	11	5	15	25	5.7
Thr	14	12	17	16	3.5
Ser	19	19	20	27	18.8
Glu	8	6	10	20	8.8
Gly	9	9	10	14	12.0
Ala	20	26	27	28	6.7
Val	6	7	15	9	4.2
½ Cys	6	4	4	5	—
Met	0	1	3	2	.3
Ilu	4	2	1	4	1.6
Leu	6	4	8	10	2.2
Tyr	1	.5	1	2	3.6
Phe	3	.5	2	3	.9
Lys	7	4	3	6	12.2
His	1	.5	1	2	5.6
Arg	2	1	1	2	1.0
Gal	++++	740	++++	++++	0
Ara	++++	540	++++	++++	++++
Total carbohydrate	95%				40% (estimated from density)

* Lamport (45); expressed as residues per molecule

** Brysk and Chrispeels (46); expressed as mol %

Although these results were originally published in abstract form only, the sequencing of the second peptide listed above has now been shown in detail (49). The configuration of these hydroxyproline rich regions is severely constrained and very likely resembles that of trans polyproline, an extended left handed helix with three residues per turn (45). Rotation is so hindered that the helix may be considered a rigid rod.

Good estimates of the molecular weight and the percent of carbohydrate are lacking. Chrispeels estimates the molecular weight at over 200,000 by gel chromatography and 50,000 by examination of its sedimentation constant. Lamport estimates the molecular weight at 230,000. The number of amino acid residues present in the peptide chain can be derived from these measurements and others like them.

In searching for the covalent linkage between the protein portion and the carbohydrate, it was found that some 70% of the hydroxyproline in tomato cell walls could be released as oligo-arabinosides upon alkaline hydrolysis (47,50). The following molar ratios were reported in a later paper (51):

Hyp	10%
Hyp-Ara	6%
$Hyp\text{-}Ara_2$	4%
$Hyp\text{-}Ara_3$	28%
$Hyp\text{-}Ara_4$	52%

The applicability of these findings to other plant species has been thoroughly examined. Phylogenetic searches showed that hydroxyproline is present in the cell walls of all land plants (52) and in most algae (53). The red algae and Nitella, however, were prominent exceptions. Most fungi

did not have hydroxyproline (45). The presence of oligo-arabinosides was also investigated in various phyla (51). Generally hyp-ara_4 groups were found in large quantities in higher plants, whereas hyp-ara_2 was more prevalent among the lower plants. Lower plants also had more unglycosylated hydroxyproline. One alga, Chlamydomonas reinhardtii, contained several unusual hydroxyproline glycosides including sequences containing glucose (54).

In addition to arabinose, galactose is also usually found associated with extensin (45,55-57) or with various fragments of it (47,58,59). Lamport (45) suggested that arabinogalactan, a known constituent of cell walls is covalently attached to extensin. Since it is well known that alkaline extraction can remove the arabinogalactan from cell walls, he hypothesized that this link is for some reason base labile. He further hypothesized that the linkage was located at the end of the hydroxyproline tetra-arabinosides. Albersheim, however, suggested that the connection to other polysaccharides was through serine glycosidic linkage (39) which was already known to be base labile by a β elimination reaction. This controversy was apparently settled by Lamport in his recent paper titled "Galactosylserine in Extensin" (49). His proof that galactosylserine links exist rests on the determination of the structure (shown in Fig. 3) of one of the glycopeptides obtained by tryptic digestion of acid-stripped tomato cell walls:

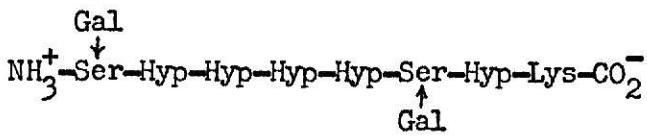


Fig. 3. Structure of a Glycopeptide from Extensin (49)

The acid stripping removed all oligo-arabinosides from the hydroxyproline residues and presumably also cleaved a glycosidic bond between galactosyl-serine and the rest of the arabinogalactan although no proof is offered for this.

Heath and Northcote (60) report a cyclic dipeptide isolated from the cell walls of sycamore tissue-culture cells by hydrazinolysis. In addition to two hydroxyproline residues it also contains about seven arabinose and eleven galactose residues. It is thus implied that the oligo-arabinosides have a number of galactose residues attached to them. Heath and Northcote contend that since hydrazinolysis only cleaves peptide links, the mere fact that the dipeptide was extracted indicates that the oligo-arabinosides are not linked covalently to other polysaccharides.

B. Its Function

Although the "crystallite gel" theory mentioned earlier considerably aids the understanding of some of the properties of the cell wall, Rees's contention (37,38) that it is the sole source of the structural strength of the cell wall matrix has certain drawbacks. Lamport has long argued that non-covalent bonding is insufficient to account for the strength of the matrix (45,52). His theory, which might be called the "covalent gel" theory, attributes to extensin a prominent role in the control of cell wall elongation and hence in plant morphogenesis.

Considerable evidence has recently been presented in favor of the covalent theory. Using sycamore cell suspension cultures, Albersheim's group (59) has found evidence confirming the presence of a covalent link between extensin and a β-(1$\rightarrow$3)-galactan containing arabinose side chains in the cell wall. In addition they have found evidence for several other

covalent links involving virtually all of the polysaccharide types known to be present in the sycamore cell wall matrix. They are as follows (41,59,61):

1. reducing end of galacturonorhamnan→ non-reducing end of β-(1→3)-galactan
2. reducing end of β-(1→4)-galactan→ 4 position of rhamnose in galacturonorhamnan
3. reducing end of arabinan→ 4 position of rhamnose in galacturonorhamnan
4. reducing end of xyloglucan→ non-reducing end of β-(1→4)-galactan

Their proposed structure of the cell wall (59) is shown in Fig. 4. The second and third links listed above confirm the findings of Stoddart and Northcote (62) who concluded that the arabinan and galactan become bound to the galacturonorhamnan after excretion from the cell. It thus appears that Lamport's early tentative hypothesis (45) is being borne out:

> "...the hydroxyproline-rich protein of lower and higher plants is part of the cell wall protein-glycan network (or 'extensin complex') analogous with the peptido-glycan network of bacterial cell walls."

One of the most interesting properties of the primary cell wall is its ability to deform as a slow flowing plastic to accommodate the growing meristem cells. While the motive force for this expansion (the turgor of the cell) is generally constant, the loosening of the cell wall to allow the expansion is under direct cellular control. Thus it has been observed that extension may be increased in oat and corn coleoptiles by natural stimuli such as light or gravity or by artificial stimuli such as carbon dioxide (63), low pH (63), methyl ester of indole acetic acid (64) and in some cases by indole acetic acid itself (64). Since

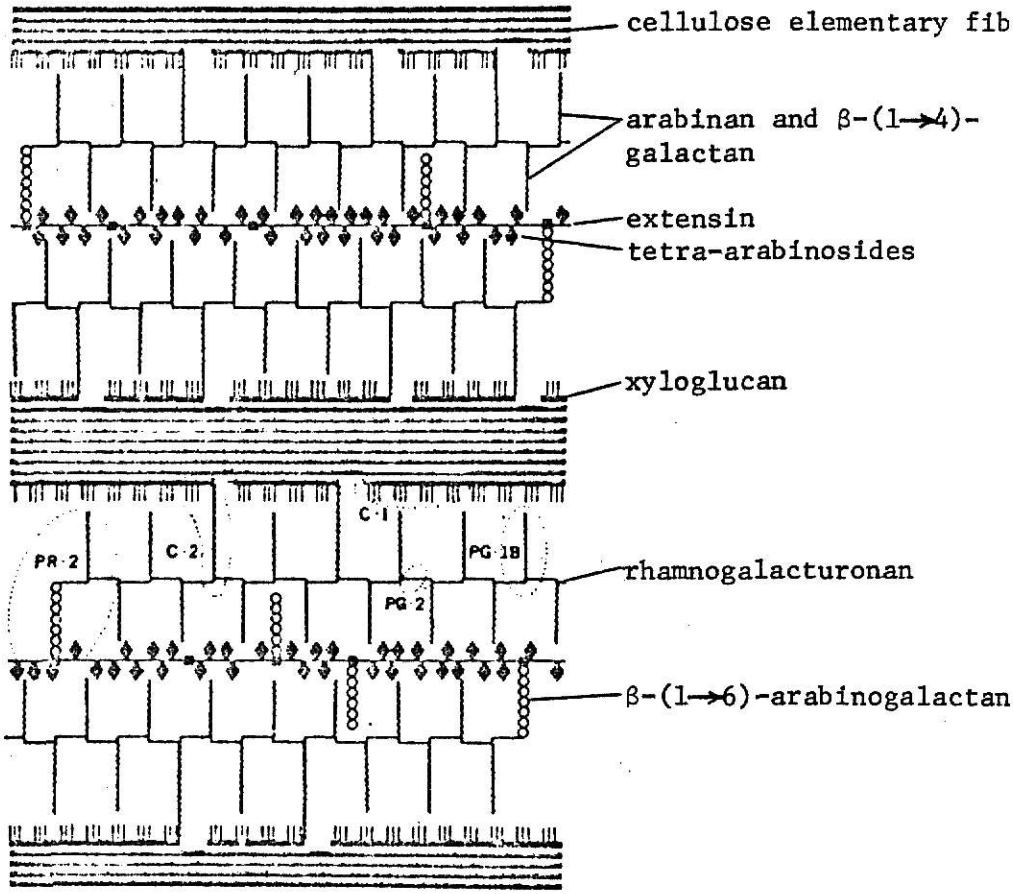


Fig. 4. Model of Sycamore Cell Wall Structure (59).

the reaction requires a time lag of less than 1 minute it can't involve de novo synthesis of mRNA or protein. Rather it is likely that the reaction involves only components already in existence such as particles in the plasmalemma or enzymes, particularly glycosidases present in the cell wall itself (65).

A convenient hypothesis which is gaining popularity is that the plasmalemma contains a hydrogen pump which upon stimulation by auxin, etc., begins to pump protons into the matrix (65,66). The reduced pH then causes the loosening reactions to occur. This makes it somewhat easier to explain a number of observations about wall loosening, such as why respiration inhibitors like KCN should cause loosening to cease almost immediately (67) or why protein synthesis inhibitors cause it to stop after 30 minutes (68).

Since a covalently crosslinked matrix would normally not be capable of plastic-deformation, a mechanism to explain such deformation is necessary to complete the theory. Speculations abound. Lamport (45) proposes that the covalent link between extensin and the matrix polysaccharides is attacked by an enzyme in the matrix gel. The link may be the base labile galactosylserine link which was discussed earlier. Presumably the enzyme would be able to make and break bonds in a sort of equilibrium process without consuming energy. Rayle (66) proposes that the acid pH is directly responsible for breaking certain labile covalent linkages such as arabinosides; however, as Albersheim points out, energy would then be required to reform the links, an unpalatable complication (59). Albersheim proposes that the polysaccharides which adhere to the cellulose microfibrils (xyloglucan in sycamore) may be able to creep slowly along the surface of

the microfibril in a sort of caterpillar fashion, but only under acid conditions.

To attribute the role of expansion control to extensin does not appear to be entirely consistent with physiological observations. In studying indole acetic acid stimulation of pea epicotyls (69,70), it was found that the induced growth could be prevented by the addition of benzimidazole or Ethrel. The effect of these inhibitors is to cause a sharp increase in extensin (hydroxyproline) synthesis. The effect of increased extensin, however, can be counteracted by adding αα'-dipyridyl, an inhibitor of proline hydroxylation. Thus it appears that at least one purpose of extensin is to bring a halt to cell wall elongation at the proper time. It has indeed been found that extensin synthesis is more prevalent in the region where growth is ceasing than in the growing meristem (69). It is not clear whether this contradicts Lamport's proposal or simply requires a modification.

C. Its Synthesis

Although a complete picture of extensin synthesis is not yet available, considerable information has been gathered. The cytoplasmic precursor of extensin has been found by means of radioactive tracers to be concentrated in preparations of membranous organelles (71,72). It is probably safe to conclude that extensin is secreted through a membrane bound transport system just as polysaccharides of the cell wall. The protein portion is synthesized on the ribosomes and released into the lumen of the rough endoplasmic reticulum. It passes to the smooth endoplasmic reticulum and is then transported to the plasmalemma. There is some evidence from ^{14}C proline autoradiography (73) and electron microscopy of organelle preparations (72)

to indicate that it is not routed through the Golgi apparatus but is secreted directly by smooth membraned organelles. This conclusion, however, may not be generally applicable to all tissues.

Hydroxyproline cannot be incorporated directly into proteins because the genetic code has no codon for it. It is, therefore, necessary for the cell to convert proline to hydroxyproline after incorporation into the peptide. An enzyme capable of doing this has been characterized and partially purified (74). It requires O_2, Fe^{++}, ascorbic acid and an α keto acid. It does not react with free proline but it does react rather non-specifically with peptidyl proline including protocollagen. The reaction can be conveniently inhibited by $\alpha\alpha'$-dipyridyl, a chelating agent for Fe^{++} (75). According to information contained in Table I, between 83 and 97 percent of all the proline is hydroxylated.

Kinetic studies have verified the sequence of events leading to extensin secretion. A four minute lag occurs between the incorporation of proline into the peptide and the appearance of radioactivity in hydroxyproline (76). This indicates that hydroxylation occurs after the release of the peptide from the ribosome. The lack of hydroxyproline in the nascent peptides attached to the ribosomes confirms the conclusion (76). A further delay of 4 minutes occurs before glycosylation begins (76). Uncoupling of oxidative phosphorylation causes a depletion of ATP which causes a halt to secretion of extensin. This indicates that energy is required for the secretion of extensin. The effect of the inhibitor is too quick to be accounted for by the inhibition of protein synthesis (77).

D. Extraction Procedures

The chief obstacle to the extraction of extensin from cell walls is

the polysaccharides which anchor it to the matrix. The solution is either to find a hydrolytic catalyst that is specific for one of the links in the polysaccharide or to use the unbound precursor instead.

It appears that NaOH extraction as used for preparation of hemicellulose would be sufficiently basic to cause the cleavage of the serine glycosidic link (49). This may explain why the arabinogalactan appears in the hemicellulose fraction. This treatment does not, however, remove the extensin from the cell wall. Lamport (50) found that more severe alkaline conditions resulted in destruction of the protein chain leaving the resulting peptides still attached to oligosaccharides by glycosidic links. Weak acid hydrolysis can be used to remove the arabinose oligosaccharide side chains with little damage to the protein (55), but stronger conditions cause generally non-selective degradation.

The use of proteases to remove fragments from the cell wall for characterization has been investigated (55,58). None were effective, however, unless the arabinose oligosaccharides were first stripped with weak acid (55). Some glycosidases were effective but the enzyme preparations available were impure, resulting in cleavage of peptide bonds as well.

While extensin is relatively difficult to extract intact, its immediate precursor is readily obtained either from cell walls or from the cytoplasm. The most commonly used method is extraction with cold TCA solutions (46,70,45,56,57). In one case workers report that as much as 30% of the cell wall hydroxyproline can be extracted (56). Material which can be extracted in this way is probably bound ionicly rather than covalently to the cell wall. It appears to be identical in every way to its cytoplasmic equivalent (46). In some cases it can be extracted with

salt solutions (46) but not in others (57). The greatest advantage of TCA is that most proteins remain insoluble and only the glycoproteins are dissolved. For this reason even salt extracted fractions are usually purified by treatment with TCA (46). Cytoplasmic material is theoretically extractable with water but salt solution or TCA is usually preferred.

IV. Polysaccharides of the Matrix

An understanding of the structure and composition of the various polysaccharides of the cell wall matrix will be useful later on for comparison with experimental results. In order to facilitate this end, special emphasis is given to wheat and other gramineae.

A. Classification

The matrix polysaccharides of plant cell walls are traditionally separated into two fractions on the basis of their extractability. Hemicelluloses are those substances which cannot be extracted from plant tissue with water (or sometimes ammonium oxalate solution) even after delignification and lipid removal but can be extracted with alkali. Pectins, or more correctly "pectic substances" (78), on the other hand are extractable with neutral solvents. This distinction, however, is somewhat dependent on the extraction conditions, the age of the tissue, the type of tissue, etc. A glycan which appears normally in the hemicellulose fraction may at times be partially included in the pectic fraction (45).

Because of these difficulties, some authors have redefined the above terms according to the chemical nature of the glycans involved (29,79). Sufficient knowledge of the structure of these materials is now available to warrant grouping them according to their backbone structure. The

matrix polysaccharides can thus be grouped as in Table II (34). Within each group there is a variety of subgroups characterized by different sets of side chains. The number and type of such side chains is often dependent on the species, tissue type, age, growth conditions, etc., of the source. The terms "hemicellulose" and "pectin" refer primarily to the cell wall polysaccharides of land plants (29). Although they are sometimes used to refer to polysaccharides derived from other plant materials, such usage tends to add confusion to an already somewhat vague classification.

Table II. Classification of Matrix Polysaccharides

Group	Backbone	Extractability Group*
Xylans	β-(1→4)-D-xylose	Hemicellulose
Glucans	β-(1→3)(1→4)-D-glucose	"
	β-(1→4)-D-glucose (distinct from cellulose)	"
Glucomannans	β-(1→4)-D-glucose and β-(1→4)-D-mannose	"
Galactans	β-(1→3)-D-galactose	Pectic substances
	β-(1→4)-D-galactose	"
Arabinans	α-(1→5)-L-arabinofuranose	"
Rhamnogalacturonans	α-(1→4)-D-galacturonic acid and (1→2)-L-rhamnose	"

* By definition; even though they may vary in actual extractability.

B. Typical Matrix Polysaccharides of Gramineae

The cell wall matrix of mature gramineae contains chiefly xylans and glucans plus a small amount of those materials classified in Table II as pectic substances. Although a wide range of DP (degree of polymerization)

values have been reported for these materials, most estimates range between 50 and 200 (29).

The xylans consist of a backbone of β-(1→4)-D-xylan to which is usually attached a number of L-arabino-furanosyl residues and D-glucuronosyl residues as in Fig. 5. Other substituent groups are also sometimes found in xylans of gramineae (80). A list of substituent groups is shown in Table III.

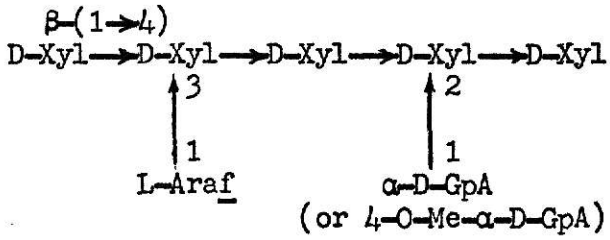


Fig. 5. A Typical Xylan of Gramineae

Table III. Substituent Groups Sometimes Found in Xylans of Gramineae

1. L-Araf-(1→3)-
2. α-D-GpA-(1→2)-
3. 4-O-Me-α-D-GpA-(1→2)-
4. D-Galp-(1→4)-D-Xylp-(1→2)-L-Araf-(1→3)-
5. D-Xylp-(1→2)-L-Araf-(1→3)-
6. Galp-(1→5)-L-Araf-(1→3)-
7. D-GpA-(1→4)-D-Xylp-(1→4)-D-Galp-(1→2)-
8. α-D-Xylp-(1→3)-L-Araf-(1→3)-

A fairly large number of studies are available to support the above generalizations and some of the more outstanding ones should be reviewed. The xylan of Cocksfoot grass was found to contain groups

1 and 3 above. In addition, D-xylopyranose was found to be bound to the backbone by a (1→3) link (81). Perennial ryegrass roots contain a xylan with groups 1, 3 and 4 (82). This is somewhat more complex than the xylan from the leaves which contains only groups 1 and 3 (83). A xylan from corn hulls containing groups 1, 2 and 4 was exceptional in that the D-galactose residue was replaced by L-galactose (84). Spear grass xylan contains groups 1 and 3 (85). The xylan of bamboo is one of the very few with no arabinose. Its only side chain is glucuronic acid attached to the 2 position (86). Esparto grass contains the only known homoxylan (87). The xylan from barley contains groups 1, 3 and 4 (88). Two xylans have been isolated from oats. One contains groups 1 and 3 (89) and the other contains 1, 2, 3, 4, 5, 6 and 7 (90). A good many other studies prior to 1959 (reviewed by Aspinal) (91) are in accord with this view of the xylans of gramineae.

The xylans of the endosperm of cereals appear to be related to the xylans of the rest of the plant although they are usually referred to not as hemicelluloses but as "cereal gums" or more properly as "seed mucilages" (79). They differ, however, in that there are no glucuronic acid residues present and the arabinose residues are attached to the 2 position of xylose as well as to the 3 position. In some cases arabinose is attached to both the 3 and 2 position of the same xylose (91,92).

The glucans of the cell walls of gramineae are a relatively new discovery. Wada and Ray in 1963 reported isolating a glucan from among the constituents of the cell walls of oat coleoptiles (93). The structure of an oat leaf glucan separated from the hemicellulose fraction by repeated ethanol precipitation was subsequently found to resemble lichenan, a

linear β-(1→3)(1→4)-D-glucan. When pure, it is insoluble in water or weak base. Hydrolyzates contain only D-glucose. It comprises 3.3% of the total hemicellulose of oat leaves (94-96). Further studies showed that this glucan was also present in the stems and roots (97) and in the stems of mature barley, rye and wheat (98,88) and corn (99). The ratios of (1→4) to (1→3) links are given in Table IV. The values were observed to increase with maturity (97). The search for cell wall enzymes has led to the discovery of a β-(1→3)(1→4)-D-glucan which can be autolytically solubilized from corn coleoptiles by indigenous enzymes (96). One worker reports evidence for a dextran-like β-(1→6)-D-glucan in oat coleoptiles which can also be autolytically solubilized (100).

Table IV. The (1→4):(1→3) Ratios of Glucans of Gramineae

Tissue	Age	(1→4):(1→3)	Ref
Oat leaf	2½ months	1.65	(94)
Oat leaf (lab grown)	10 days	3.85	(97)
Oat root (lab grown)	10 days	2.38	(97)
Oat coleoptile (lab grown)	10 days	3.23	(97)
Barley stem	mature	4.04	(98)
Rye stem	mature	2.05	(98)
Wheat stem	mature	1.78	(98)
Corn stem	mature	2.0	(99)

Glucans also have their counterparts in the endosperm. The structure of oat flour β-glucan was shown to be identical to that of lichenan. The (1→4):(1→3) ratio was between 2 and 3 (101). Two distinct varieties of β-glucan have been found in barley seed having a (1→4):(1→3) ratio of about 2. They differ in molecular weight by a

factor of several fold. The heavier one is linear while the lighter is slightly branched (102).

The remaining three types of polysaccharides, arabinan, galactan and rhamnogalacturonan are members of the pectic fraction. In gramineae they have not been isolated and studied, probably because they are present in only minute quantities in the mature tissues which are of economic importance. While some evidence for the presence of all three in oat coleoptiles has been presented (103), structural studies are lacking. In order to gain an idea of what these substances may be like, it is profitable to review the general structures obtained from other angiosperms.

Arabinans such as those found in mustard seed and sugar beet consist of a linear backbone of α-(1→5)-L-arabinofuranose with single α-L-arabinofuranosyl-(1→3) side groups at frequent intervals. This structure must be considered tentative (79).

Galactans such as those found in soybean cotyledons are composed of a β-(1→4)-D-galactose backbone with L-arabinofuranosyl-(1→5)-L-arabinofuranosyl-(1→3) side chains (79).

The rhamnogalacturonans have a backbone of α-(1→4)-D-galactopyranuronic acid interspersed with a small portion of (1→2)-L-rhamnopyranose residues. In a few instances the rhamnose residues are absent, making the molecule a simple galacturonan. Most rhamnogalacturonans also have a number of complicating side chains which will not be reviewed here. They have, however, been thoroughly covered in an article by Rees (37).

C. The Matrix Polysaccharides of Wheat

The xylans of wheat straw were investigated by a number of groups during the '50's. For the most part the investigators agree that the structure is that of a typical β-(1→4)-D-xylan such as that shown in Fig. 5.

They are less than unanimous, however, on several details (91,92). D'Appolonia et al. attribute this disagreement to differences in extraction procedure (92). The xylans from the leaves were similar and had no unusual features. The DP was about 34 (104). The xylans from wheat bran were unusual in several respects. Some of the xylose residues bore an arabinofuranosyl group on both position 1 and 2. To this extent it resembled the xylans of endosperm tissue. There was also evidence for more complex side chains linked to the backbone by means of a 3-O-L-arabinofuranosyl residue. The arabinose content was exceptionally high. The DP was about 300 (105).

The glucans from mature wheat stem are typical β-(1→3)(1→4)-D-glucans (98). A cell free extract from the roots of 24 to 40 hr old seedlings has been shown to be able to synthesize β-(1→3)(1→4)-D-glucan from UDP-glucose (106).

D. Monosaccharide Analysis of the Polysaccharides of Wheat Seedling Roots

Aside from the above study (106) there has been little research performed on isolated and purified polysaccharides of wheat roots. However, because of the importance of such tissue to this study, it is pertinent to discuss here the monosaccharides which have been obtained upon hydrolysis of wheat seedling root fractions. Although three of the cited references deal with corn, they are included here because of their obvious implications for wheat tissues.

Wheat seedling roots about 1 cm long were divided by Northcote and Pickett-Heaps into a "tip" section and a meristematic section. The non-cellulosic polysaccharides were extracted with KOH and hydrolyzed. The

results are shown in Table V (3).

The root caps of corn, wheat and other gramineae are known to produce a slime which coats the growing root. The polysaccharide contents of this material must be considered along with the cell wall material when evaluating the composition of root extracts. The slime of corn seedling roots has been collected and hydrolyzed. These results are also presented in Table V (107). Harris and Northcote (108) indicate that the identification of ribose in this work and xylose in the work of

Table V. Composition of Wheat and Corn Root Polysaccharides

	Wheat Root Tip (3)	Wheat Root Meristem (3)	Corn Root Cap Slime (107)
Galactose	15.1	13.0	35
Glucose	42.5	32.0	37
Arabinose	25.5	27.0	—
Xylose	17.5*	28.0*	5
Mannose	Trace	Trace	—
Ribose	—	—	11*
Galacturonic acid	—	—	12

* Includes fucose because of similarity of R_f values.

Northcote and Pickett-Heaps (3) were probably incorrect. They have identified a large percentage of fucose which because of a similarity in R_f values was previously mistaken for ribose and xylose respectively. According to them, the analysis is compatible with the idea that a galacturonan and a galactan are synthesized in the root cap slime. The fucose and glucose could be associated with the galacturonan in a structure

resembling a vegetable gum. It should also be mentioned here that Roberts (109) has confirmed the presence of mannose in the slime and has shown that at least some of the galactose is L-galactose.

MATERIALS AND METHODS

MATERIALS

I. Wheat seeds: Untreated wheat Triticum vulgare, variety Shawnee, was supplied by the Kansas State University Agronomy Farm.

II. Pronase: Calbiochem, San Diego, California, B grade, 45,000 PUK/gr., lot 200,191.

III. SDS-Phosphate Buffer: 10 mM sodium dodecyl sulfate (SDS) in 0.1 M sodium phosphate buffer, pH 7.6.

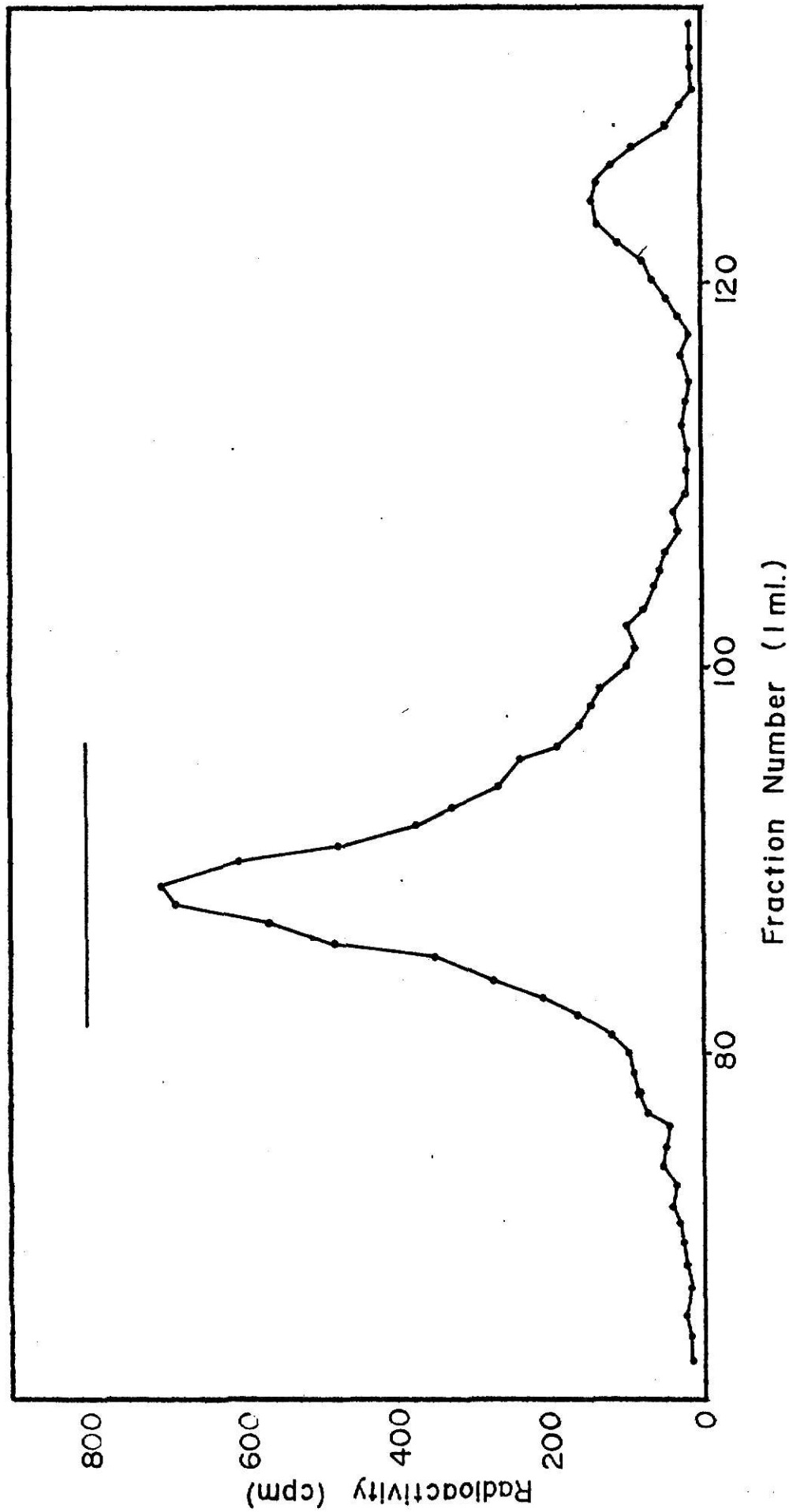
IV. TLC Plates: Brinkmann Pre-Coated Silica Gel G thin layer plates, E. Merk.

V. Origin of the Golgi-Derived Material: This material was available from a preliminary experiment done in this laboratory by Moore and Nordin (110). Wheat seedlings were grown in the usual manner and the roots were exposed to U-^{14}C-glucose. They were harvested and homogenized and a crude Golgi body fraction was separated by a series of centrifugations according to the method of Jilka, Brown and Nordin (21). The washed crude Golgi were homogenized in SDS-phosphate buffer and centrifuged. The supernatant was placed on a Sepharose 6B column which had been equilibrated with SDS-phosphate buffer. Besides the material which was eluted at V_o and $V_{glucose}$, a single major peak was eluted at $V_e/V_o = 1.95$. While treatment with pronase was successful in eliminating certain small side peaks, it appeared to have no effect on the major peak (Fig. 6). The sample resulting from this experiment was then stored in counting vials under toluene based fluor.

Fig. 6

Fractionation of Golgi-Derived Material
on Sepharose 6B (110)

A crude Golgi preparation from wheat seedling roots which had been exposed to U-^{14}C-glucose was homogenized in SDS-phosphate buffer (10 mM sodium dodecyl sulfate in 0.1 M sodium phosphate buffer, pH 7.6) and digested with pronase. It was then eluted through a Sepharose 6B column (85 cm x 1.4 cm) with SDS-phosphate buffer. The void volume peak, V_o, is not shown. The small peak to the right corresponds to the glucose elution volume. The large peak has a V_e/V_o of 1.95. The fractions recovered for further study are indicated.


Radioactivity (cpm)
800
600
400
200
0
80
100
120
Fraction Number (1 ml.)

The discs and vials were rinsed with fresh toluene to remove the fluor and dried. They were then extracted in SDS-phosphate buffer for a total recovery of 6500 cpm or about 86% of the total estimated to be present in the sample.

METHODS

I. Wheat Seedlings

Wheat seedlings were prepared according to the method of Brown (9). The seeds were soaked for three hours in distilled water and placed between two layers of wire screen. After 60 hours the primary roots growing down through the lower screen in response to gravity could be harvested by scraping them off with a razor blade. Each tray of growing wheat seedlings was covered with aluminum foil to prevent drying and to insure dark growing conditions. A total of 725 g of wheat was normally used to fill six trays. This produced a yield of .56 g of lyophilized extract after homogenization, centrifugation, and dialysis.

II. Homogenization

The wheat roots were cut into short lengths and homogenized in a Sorvall Omni-mixer cooled in an ice bath at maximum speed for 60 seconds, using about 60 ml of SDS-phosphate buffer. The homogenate was strained through cheese cloth, rinsed twice with about 10 ml of buffer and centrifuged at 10,000 rpm for 30 min. Since SDS precipitates from the buffer in the cold, the straining and centrifugation were performed at room temperature relying on the detergent effect of the SDS to prevent bacterial and enzymatic degradation.

III. Dialysis and Lyophilization

Material to be dialyzed was placed in dialysis tubing two inches in circumference and dialyzed 36 hours or more during which time the bath was changed 3 times. Each rinse was in a bath of distilled water about 40 times the volume of the sample. Normally the procedure was performed at room temperature using $CHCl_3$ in the bath as a preservative. The dialyzed material

was lyophilized on a Vir-Tis Freeze Mobile lyophilizer. The product contained 41% protein as measured by the micro Kjeldahl technique (111).

IV. Molecular Sieve Fractionation

A column of Sepharose 6B (85 cm x 1.4 cm) was prepared and equilibrated in SDS-phosphate buffer. The sample was dissolved in 3 ml or less of elution buffer. An LKB Ultrorac type 7000 fraction collector was used to collect 150 1 ml fractions over a period of about 15 hours. In some cases an LKB Uvicord Type 4701A ultraviolet monitor with a 3 mm diameter tubular cell was used to monitor the effluent at 254 nm. The percent transmittance of the UV monitor was recorded on log paper thereby converting the data to absorbance values. When log paper was not available the transmittance data were converted to absorbance mathematically. No attempt was made to convert monitor readings to mgs of protein.

V. Anion Exchange Fractionation

Dry beads of DEAE Sephadex A-25 (8 g) were soaked in 2 M K_2HPO_4 for two days to convert them to the phosphate form. They were then equilibrated with the sodium phosphate elution buffer and packed into a column 1.2 cm by about 46 cm. Since the buffer varied in concentration and pH, these details are given in each experiment. A new column was prepared for each run using fresh beads.

The column was set up in a cold room at 4°C with the effluent passing first through the ultraviolet monitor and then into the fraction collector where 1 ml fractions were collected. The samples applied were no larger than 2 ml and were adjusted to the conditions of the elution buffer by adding 3 ml of buffer and evaporating the mixture to 3 ml. The flow rate was about 5 drops/min. All salt gradients used to elute the column were linear.

VI. Cation Exchange Fractionation

Dry beads of CM Sephadex C-25 (8 g) were soaked in the elution buffer for 2 days and then eluted with several liters of buffer until the pH reached equilibrium. They were then packed into a column 1.2 cm by about 50 cm.

The operation of the column was the same as that of the DEAE Sephadex column. The elution buffer was 0.2 M NaAc, pH 5.2 preserved with pHix Buffer Preservative as directed on the container. This amounts to about 1 mg/l of pentachlorophenol. All salt gradients used to elute the column were linear.

VII. Carbohydrate Analysis

The phenol sulfuric acid assay (112) with a xylose standard was used to determine carbohydrate concentration. Since the response of the sugars was significantly below normal when SDS-phosphate buffer was used, a second standard curve was prepared for use with such buffer solutions.

The presence of NaCl in some of the fractions also created a problem. The rapid introduction of H_2SO_4 into such a sample caused a somewhat violent evolution of gas. Tests showed that up to a concentration of 0.4 M NaCl the assay was quite reliable but above this concentration the readings became progressively lowered and unreliable.

VIII. Protein Analysis of Fractions

Protein content of eluted fractions was estimated from the absorbance at 280 nm in a Beckman spectrophotometer. An unidentified absorbance maximum at about 210 nm was also present contributing a large absorbance at 280 nm. To determine the absorbance contributed by protein, a base line for the 280 nm peak was estimated by interpolating the slope of the unidentified peak and subtracted from the total absorbance at 280 nm. This operation was individually performed on the UV spectrum of each fraction. Since many proteins have an absorbance of about 1.0 when in a solution of 1 mg/ml and viewed

through a 1 cm cuvette (113), this value was used to estimate the concentration of protein in the fraction. Because of the various assumptions made, the resulting values are very approximate but are adequate for comparing samples.

IX. Scintillation Counting

Counting vials were filled with 5 ml of a fluor containing 0.5% 2,5-diphenyloxazole (PPO) in toluene. The background was counted on a Beckman LS-200B liquid scintillation counter for 5 minutes per vial using a ^{14}C window.

Sample fractions of 1 ml each were transferred from the test tubes in which they were collected to counting vials where they were frozen and lyophilized in a vacuum desiccator. The lyophilized material was redissolved in 0.2 ml of distilled water and a glass fiber disc was introduced to absorb the solution. The discs and the vials containing them were dried under a heat lamp. The vials were filled with 5 ml of scintillation cocktail and counted for 20 minutes each under the same conditions as the background. The background count was subtracted individually for each sample.

X. Activity of Pronase

In order to verify the activity of the pronase, 0.5 mg of denatured bovine serum albumin in 2 ml of potassium phosphate buffer, .001 M, pH 8 containing .02% NaN_3 were incubated with 1 mg of pronase at 37°C for 100 hours. Aliquots of 10 µl were removed at various times from 72 sec to 100 hours and spotted on a thin layer plate. The plate was not eluted with solvent but was sprayed immediately with ninhydrin (reference 114, page 178). The amount of material responding to ninhydrin increased for about 40 hours after which no further increase was observed.

XI. Hydrolysis

For the purpose of monosaccharide analysis carbohydrates were hydrolyzed

in 1.0 N HCl for 1 hour at 100°C. The hydrolysis mixture containing about 10 μg of carbohydrate in 0.2 ml of acid was placed in a test tube fitted with a "cold finger" condenser and immersed in a boiling water bath. The hydrolyzate was aspirated to dryness and then desiccated in a vacuum over KOH pellets for a day to remove HCl. The residue was dissolved in 40 μl of 70% EtOH and spotted.

The samples for the paper chromatography experiment were hydrolyzed in 0.3 N H_2SO_4 and neutralized with solid $CaCO_3$. They were lyophilized and redissolved in 0.4 ml of 70% EtOH in a Superelco heavy glass vial. The solid material was pelletized in the tip by centrifuging at 1000 rpm for 15 min. The supernatant was applied to the chromatogram.

Amino acid analysis required more severe conditions. A sample containing protein was placed into a soft glass ampoule in 0.4 ml of 6 N HCl and the mixture was frozen in acetone and dry ice. After sealing the ampoule under vacuum and incubating it at 110°C for 1 hour, the hydrolyzate was aspirated to dryness. It was then desiccated in a vacuum over KOH pellets for three days to remove HCl. The residue was dissolved in 40 μl of water and spotted on the chromatogram.

XII. Thin Layer Chromatography (TLC)

Silica gel thin layer plates were spotted with 30 μg samples of hydrolyzed carbohydrates along with knowns. They were developed in n-butanol - acetone - 0.1 M sodium phosphate buffer, pH 5 (40:50:10) for two ascents. The aniline diphenylamine spray (reference 114, page 856) was used to visualize the spots.

For quantitative measurements, a method developed in this laboratory (115) was used based on the triphenyl tetrazolium chloride (TTC) reagent and

densitometry. The plate, spotted as before, was developed in n-butanol - acetone (spectroanalyzed grade) - water (40:50:10) for two ascents. It was dipped in a TTC solution of the following composition:

1 g TTC
50 ml dioxane
10 ml methanol or sufficient to dissolve the TTC

The plate was dried, exposed to ammonia fumes for 6 min, and then heated in the oven at 80°C for 10 min. In order to insure a uniform exposure to heat and ammonia, the plate was incubated in a specially constructed aluminum chamber filled with ammonia fumes. After cooling, the plate was immediately scanned on the Photovolt Densicord densitometer.

Amino acid chromatography was performed in two dimensions on silica gel thin layer plates using about 140 µg of protein hydrolyzate per spot. The first dimension was developed in 96% EtOH - 34% NH_4OH (70:30) for two ascents. The second dimension was developed in 96% EtOH - water (70:30) for one ascent. The plates were visualized by the ninhydrin spray reagent (reference 114, page 178). The R_{Hyp} values for some of the amino acids are shown in Table VI.

XIII. Paper Chromatography

Samples were spotted on the narrow side of a piece of Whatman 1-MM chromatography paper 28½ cm x 23 cm. The paper was stapled to form a cylinder and eluted with n-butanol - ethanol - water (40:11:19). After three ascents the chromatogram was dried and then developed with $AgNO_3$ according to the method of Trevelyan, Proctor, and Harrison (116) except that the chromatogram was dipped in the NaOH solution rather than sprayed, and the fixer was 5% sodium thiosulfate solution.

Table VI. R_{Hyp} Values of Amino Acids.

	96% Ethanol – Water (70:30) R_{Hyp}	96% Ethanol – 3½% Ammonia (70:30)(2 ascents) R_{Hyp}
Gly	.80	1.00
Glu	1.08	1.11
Ser	.79	1.04
Pro	.92	.77
Hyp	1.00	1.00
Ala	1.00	1.08
Thr	1.00	1.21
Val	1.15	1.24
Asp	.97	.95
Hydrophobic amino acid	Approx 1.30	Approx 1.25

RESULTS

I. Sepharose Fractionation

Before attempting to determine which of the components of a detergent extract of wheat roots are identical to the components of the Golgi-derived material, it is necessary to devise a suitable method of separating those components so that they can be identified. Since the elution profile of the Golgi-derived material on Sepharose 6B was already known from a preliminary experiment, Sepharose chromatography was selected as the first step in the fractionation of the crude extract.

Wheat roots were homogenized, centrifuged, dialyzed and concentrated. A small portion of the extract was applied to the Sepharose 6B column with the results shown in Fig. 7. Of the four peaks the first corresponds to V_o and the fourth to $V_{glucose}$. The four peaks were hydrolyzed (0.3 N H_2SO_4 at 100°C for 1 hr) and chromatographed on paper. The results are shown in Table VII along with estimations for the total carbohydrate and protein.

Table VII. Composition of the Sepharose Peaks

Peak	Carbohydrate (as xylose) (mg)	Protein* (mg)	Carbohydrate / Protein	Gal	Ara	Xyl	G
I	1.13	3	.4	++	+++	+	+++
II	.58	3	.19	++	++++	+	0
III	.49	1.0	.5	++	++++	+	++
IV	.54	1.4	.4	++	+++	++	++

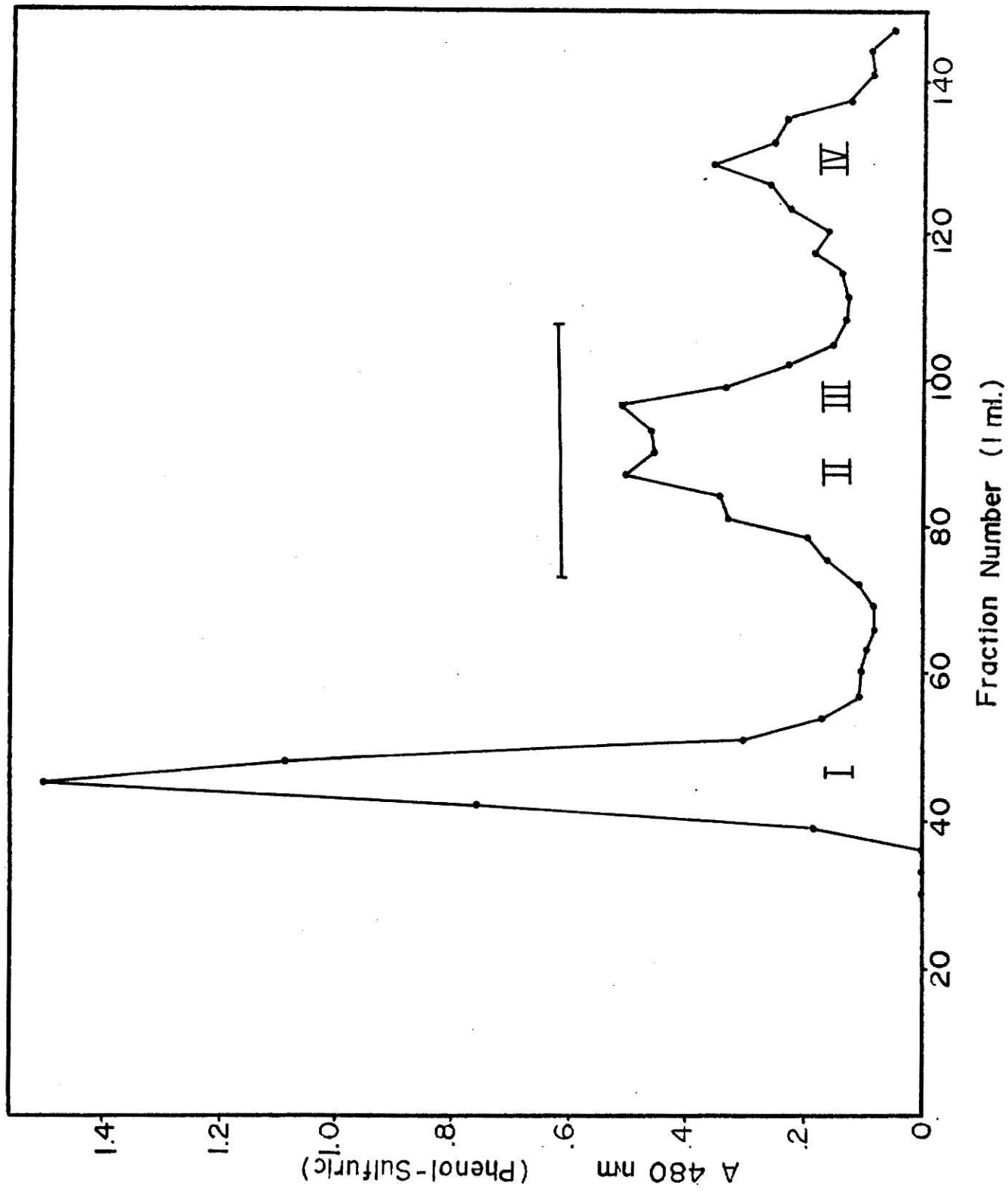
* See "Methods" for calculation

It can be seen that all the peaks contained both carbohydrate and protein. When larger, preparative-sized samples were placed on Sepharose the

Fig. 7

Fractionation of Whole-Cell Extract
on Sepharose 6B

The lyophilized extract from about 100 g of wheat seeds (dry weight) was applied to a column of Sepharose 6B (85 cm x 1.4 cm) and eluted with SDS-phosphate buffer (10 mM sodium dodecyl sulfate in 0.1 M sodium phosphate buffer, pH 7.6). Fractions of 1 ml were collected and assayed with the phenol sulfuric test. Peak I corresponds to the void volume, V_o. Peak IV corresponds to the glucose elution volume. The fractions pooled for further use are indicated.


A 480 nm (Phenol-Sulfuric)
0
.2
.4
.6
.8
1.0
1.2
1.4
20
40
60
80
100
120
140
Fraction Number (1 ml.)
I
II
III
IV

peaks were somewhat broader and separation was, therefore, less distinct.

II. DEAE Sephadex Fractionation

A lyophilized extract was prepared as shown in Fig. 8. It was passed through Sepharose 6B, and peaks II and III were pooled. This material was dialyzed, concentrated, and applied to a column of DEAE Sephadex A-25 equilibrated with .04 M sodium phosphate elution buffer, pH 7.2. The influent was regulated as follows:

buffer	50 ml
gradient (100 ml buffer to 100 ml 0.3 M NaCl in buffer)	200 ml
gradient (100 ml 0.3 M NaCl in buffer to 100 ml 2.0 M NaCl in buffer)	200 ml
2.0 M NaCl in 0.1 M NaAc buffer, pH 4.8	50 ml

Since the accuracy of the phenol sulfuric acid assay is impaired in samples with a high content of NaCl, the effluent from the column was passed through a Biomed flow-through dialysis cell and then into the ultraviolet monitor. The dialysis bath consisted of elution buffer diluted 1:10. Its flow rate was about 20 times the flow rate of the effluent from the column. The resulting three peaks are shown in Fig. 9 and Fig. 10.

Results from a second crop of seedlings are shown in Fig. 11. Five new peaks were revealed on the ultraviolet monitor. In addition, peak 2 was apparently shifted so as to form a shoulder on peak 1.

The procedure of the second run differed from that of the first in several ways. The elution buffer was changed to 0.2 M, pH 7.4. The influent was regulated as follows:

buffer	40 ml
gradient (100 ml buffer to 100 ml 0.4 M NaCl in buffer)	200 ml
2.0 M NaCl in 0.1 M NaAc buffer, pH 4.7	50 ml

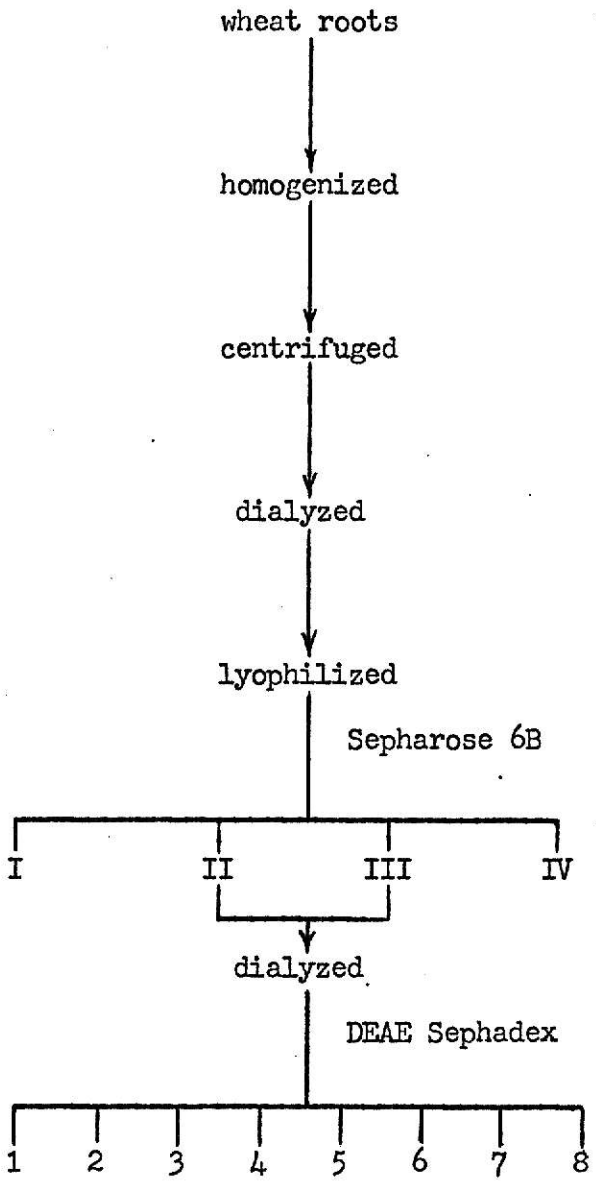


Fig. 8. Extraction and Fractionation Scheme

Fig. 9

DEAE Sephadex Fractionation
of Peaks II and III

Peaks II and III from the Sepharose fractionation of the lyophilized extract were applied to a column of DEAE Sephadex A-25 (46 cm x 1.2 cm) and eluted with .04 M sodium phosphate buffer, pH 7.2 and a salt gradient (as shown) for a total of 450 ml. A 0.1 M NaAc buffer, pH 4.8 was then applied. The effluent was passed through a flow-through dialysis cell to remove NaCl.

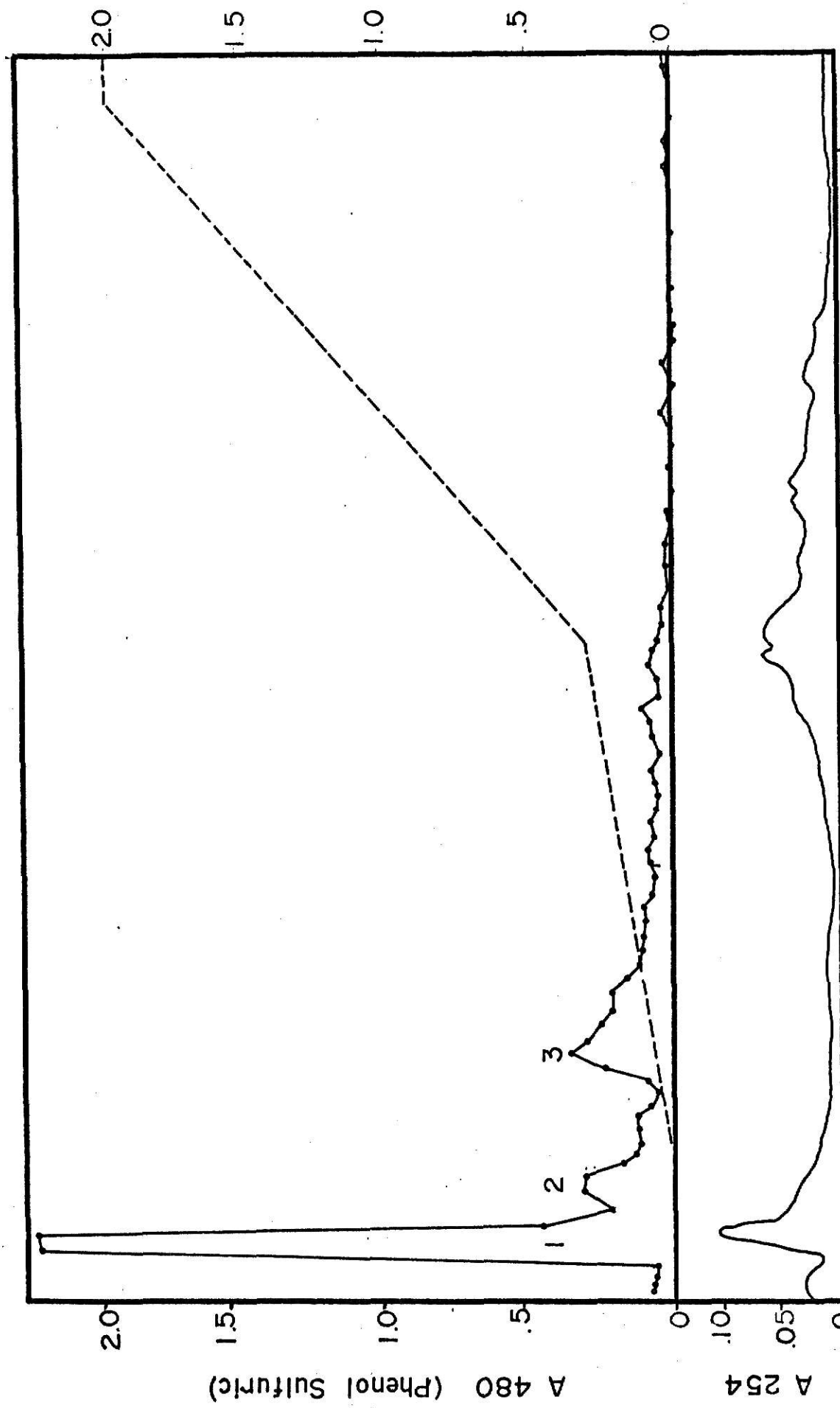

Molarity of NaCl
2.0
1.5
1.0
.5
0
A 480 (Phenol Sulfuric)
A 254
.10
.05
0
1
2
3
100
200
300
400
Fraction Number (1 ml.)

Fig. 10

Expanded Scale of Elution Profile
Shown in Fig. 9

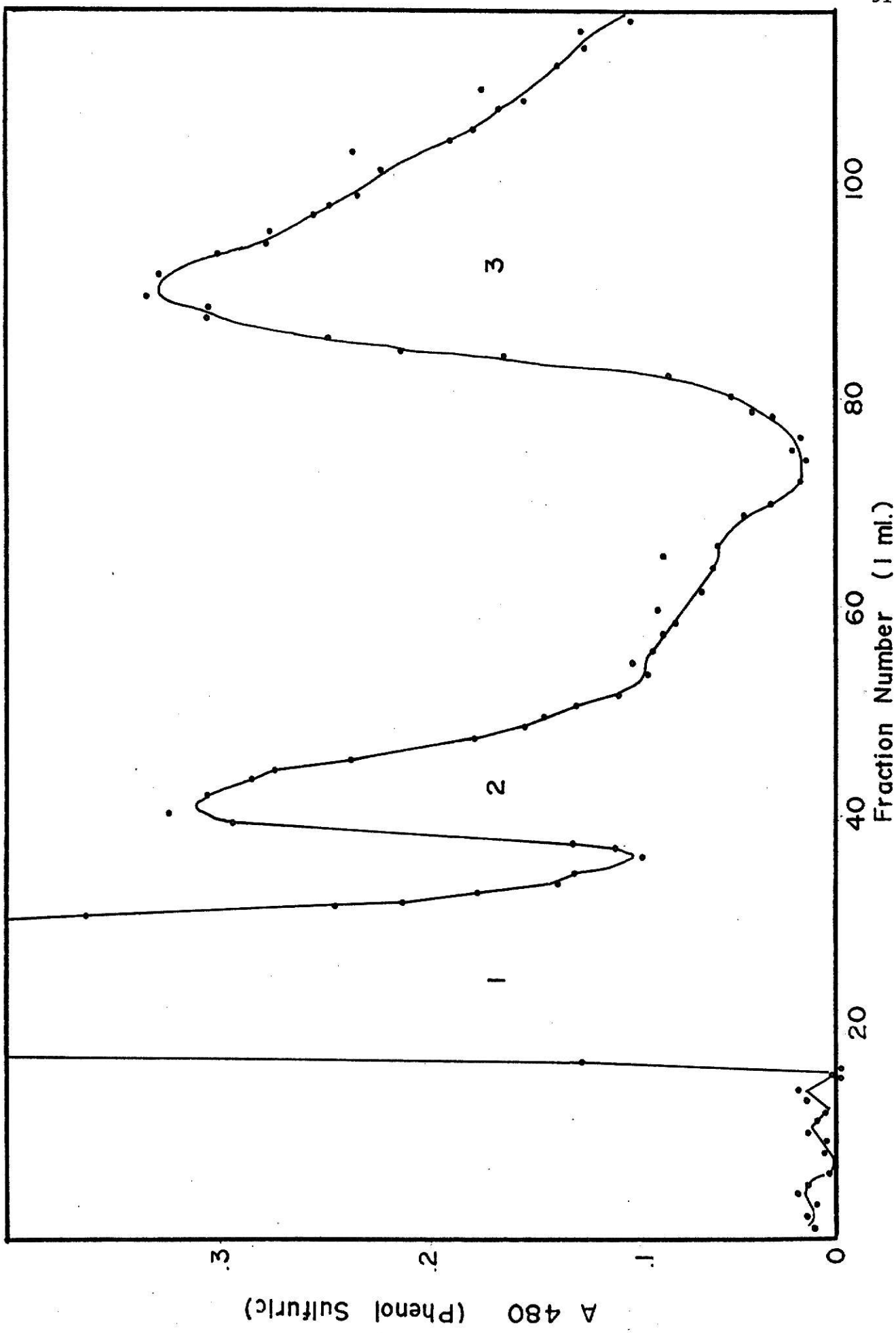
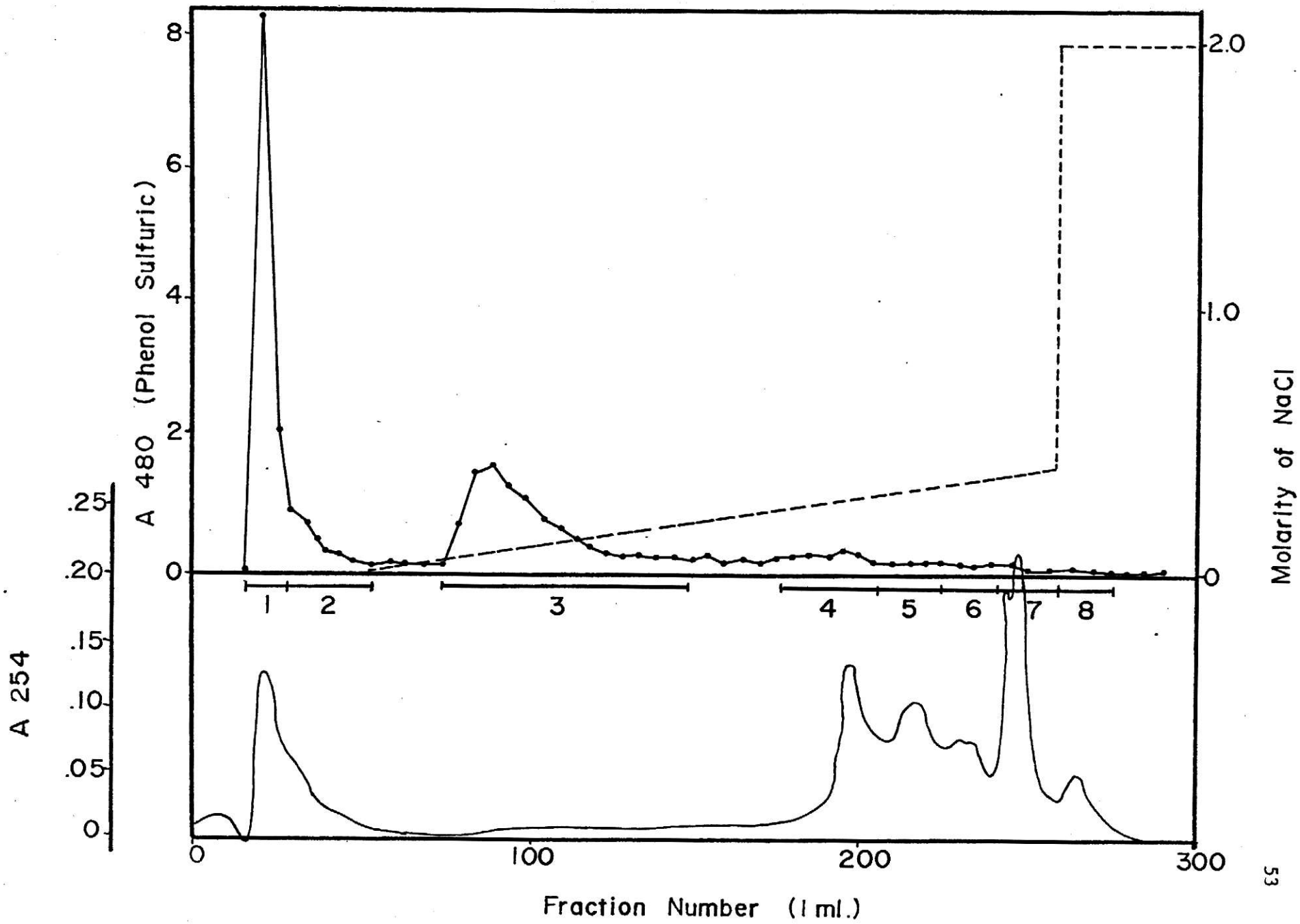

A 480 (Phenol Sulfuric)
0
1
2
3
20
40
60
80
100
Fraction Number (1 ml.)
1
2
3

Fig. 11

DEAE Sephadex Fractionation of
Peaks II and III from a Second Sample of Seedlings

Peaks II and III from the Sepharose fractionation of a new sample of lyophilized extract were applied to a fresh column of DEAE Sephadex A-25 (46 cm x 1.2 cm) and eluted with 0.2 M sodium phosphate buffer, pH 7.4 and a salt gradient (as shown) for a total of 450 ml. A 0.1 M NaAc buffer, pH 4.8 was then applied. The effluent was passed through a flow-through dialysis cell to remove NaCl. The fractions pooled for further work are indicated.


A 480 (Phenol Sulfuric)
8
6
4
2
0
2.0
1.0
0
Molarity of NaCl
1
2
3
4
5
6
7
8
A 254
.25
.20
.15
.10
.05
0
0
100
200
300
Fraction Number (1 ml.)

A quantity of material which normally precipitates from the homogenate during dialysis was centrifuged out of the first sample and discarded. In the second sample and in all succeeding work, however, this material was retained. It was re-suspended when the lyophilized sample was dissolved in SDS-phosphate buffer. Some undissolved material was later filtered from the solution during DEAE Sephadex chromatography forming a brownish precipitate on the top of the bed.

Material from each of the eight peaks was hydrolyzed (1.0 N HCl at 100°C for 1 hr) and analyzed for monosaccharide by TLC. The results are shown in Table VIII along with estimations for the total carbohydrate. Monosaccharides were identified both by their R_f values and by their characteristic colors. A series of standards was also chromatographed along with the samples including rhamnose, galacturonic acid and glucurono lactone. While rhamnose (a unique green color) was found to be present to a small extent in all fractions, galacturonic acid and glucurono lactone were not observed. Galacturonic acid remains nearly at the origin in this solvent system and it is, therefore, very difficult to detect galacturonic acid even when present. The chromatography

Table VIII. Composition of DEAE Sephadex Peaks

Peak	Carbohydrate (as xylose) (μg/ml)	Gal	G	Ara	Xyl + Fuc	Rha
1	394	++	+	++	+	+
2	14			(no data)		
3	68.6	++	+	+	0	+
4	14.1	+++	++++	+	+	+
5	5.19	+++	++	*	*	+
6	5.98			(no data)		
7	8.28	+++	++	*	*	+
8	4.14	++++	++	+	+	+

* Chromatogram obscured by a yellow colored material

of peak 3 was repeated using 10 times as much sample. The results confirmed the prior analysis (particularly the presence of rhamnose) and also revealed a trace of glucuronolactone.

From Figs. 9 and 11 it can be seen that peaks 4 - 8 are probably protein or perhaps glycoprotein in which the carbohydrate portion is small. Peak 3, on the other hand, is a polysaccharide peak. Since peak 1 corresponds to V_o, it probably contains a mixture of proteins and neutral polysaccharides.

Since no peaks were found above an ionic strength of about .45, the flow-through dialysis cell was not used in further experiments.

III. Fractionation of the Golgi-Derived Material on DEAE Sephadex

In a similar manner the Golgi-derived material available from previous work done in this laboratory was fractionated on DEAE Sephadex A-25. In order to identify the various peaks using either the phenol sulfuric test or the UV monitor some material from the previous experiment was added to the sample. The sample composition was as follows:

Peak 1 10% of total available from previous experiment
Peak 3 25% of total available from previous experiment
Peak 5 50% of total available from previous experiment
Radioactive Golgi-derived material 5900 cpm

The sample was reduced to about 3 ml and placed on a column of DEAE Sephadex. The elution buffer was 0.2 M sodium phosphate, pH 7.4. The elution sequence was as follows:

buffer	40 ml
gradient (100 ml buffer to 100 ml 0.4 M NaCl in buffer)	200 ml
2.0 M NaCl in 0.1 M NaAc buffer, pH 4.7	50 ml

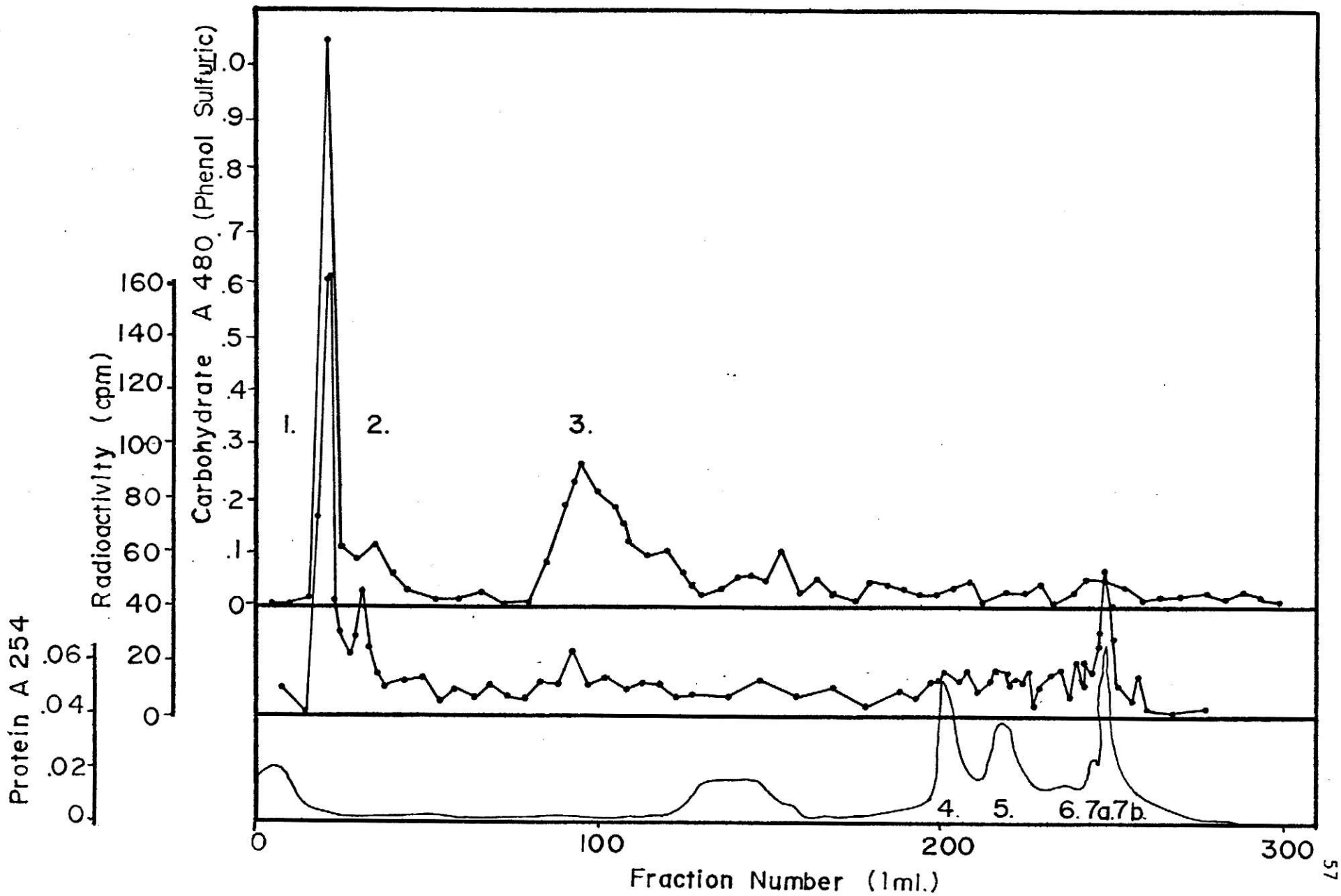
The results are shown in Fig. 12. The counts per minute (cpm) trace represents only the Golgi-derived material whereas the phenol sulfuric trace and the UV monitor trace represent only the added whole-cell-extracted

Fig. 12

DEAE Sephadex Fractionation of
the Golgi-Derived Material

The radioactive Golgi-derived material recovered from the experiment shown in Fig. 6 was applied to a column of DEAE Sephadex A-25 (46 cm x 1.2 cm) and eluted with 0.2 M sodium phosphate buffer, pH 7.4 and a salt gradient. In order to locate the exact positions of the peaks previously obtained from the whole-cell extract, some portions of peaks 1, 3 and 5 were mixed with the sample. The elution sequence was as follows:

buffer	40 ml
gradient (100 ml buffer to 100 ml 0.4 NaCl in buffer)	200 ml
2.0 M NaCl in 0.1 M NaAc buffer, pH 4.7	50 ml


Carbohydrate A 480 (Phenol Sulfuric)
.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
Radioactivity (cpm)
160
140
120
100
80
60
40
20
0
Protein A 254
.06
.04
.02
0
1.
2.
3.
4.
5.
6.7a.7b.
0
100
200
300
Fraction Number (1ml.)

material. (The radioactive material is presumed to be comparatively small in weight.) It was not intended that the entire elution profile of the whole-cell extract be reproduced. The addition of peak 1, 3 and 5 material was simply to act as a marker so that the radioactive peaks could be identified. As it turned out, all of the peaks except peak 8 appeared in the phenol sulfuric or UV traces. This is presumably because the added material contained some overlap from the adjacent peaks.

Activity was found in peaks 1, 2 and 7b. There also appeared to be a slight correlation between the cpm trace and the UV trace in the region of peaks 4, 5 and 6; however, this may not be significant. The data in this region are presented on an expanded scale in Fig. 13. There was no apparent activity in peak 3.

IV. Effect of Pronase on Fractions

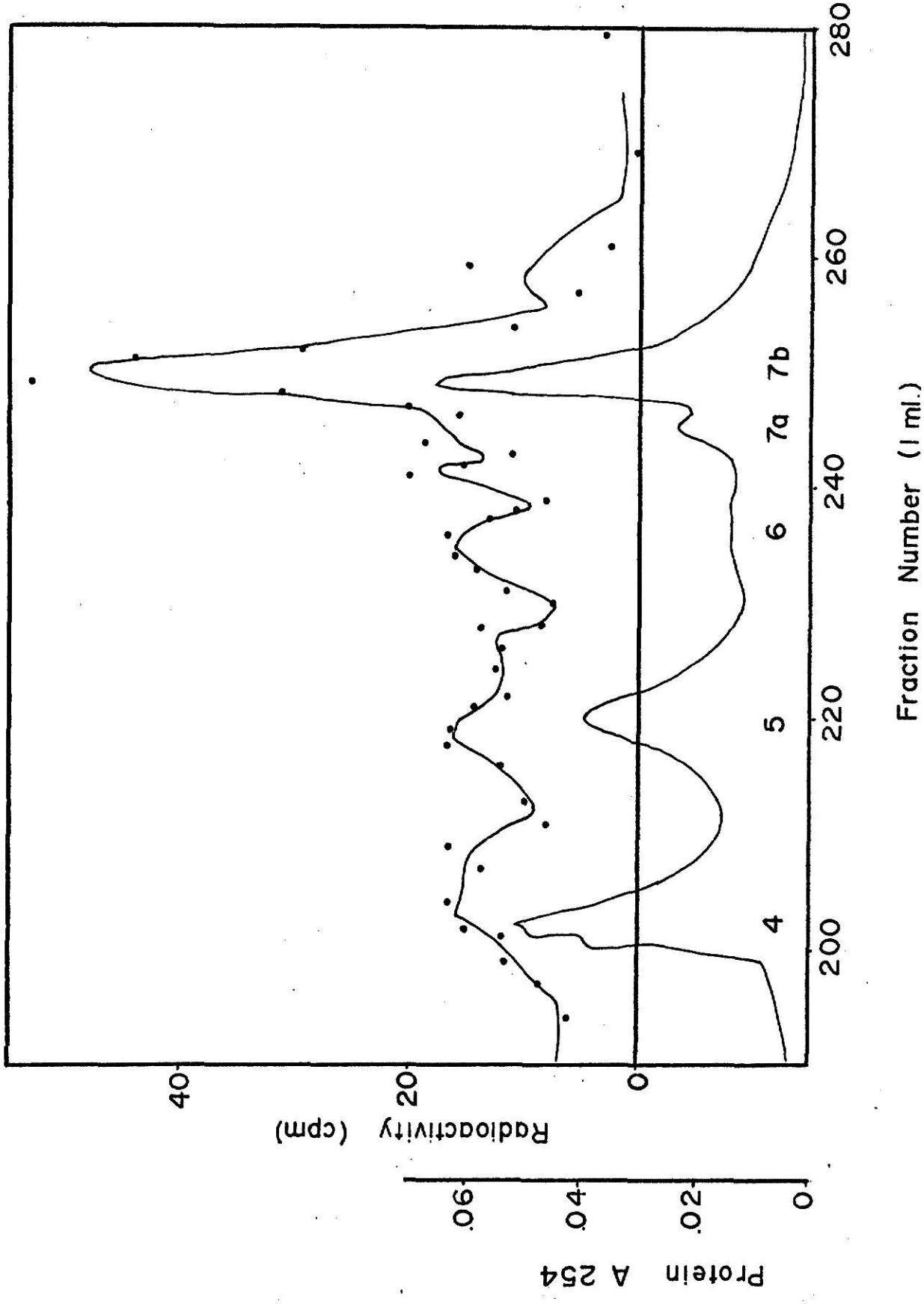
Since the radioactive Golgi-derived material had been treated with pronase it was possible that the distribution of its components on DEAE Sephadex was altered. It was, therefore, necessary to establish what effect pronase has on the whole-cell extract.

A new crop of wheat roots was homogenized and dialyzed as usual. To this was added 80 ml of buffer (.02% NaN_3 in .001 M potassium phosphate buffer, pH 8) and the sample was reduced to 80 ml on the rotary evaporator. Half of the sample was treated with pronase and half served as control as summarized in Table IX. A blank containing only the pronase and the buffer was also prepared. After 26 hours an additional quantity of pronase was added. The 3 samples were incubated in a water bath at 37°C for 50 hours. A preliminary study had shown that the pronase was sufficient to digest a .5 mg sample of bovine serum albumin under these conditions. Each sample

Fig. 13

Expanded Scale of Elution Profile
Shown in Fig. 12

The correspondence between the radioactivity profile and the UV monitor trace in the regions of peaks 4,5,6,7a and 7b is shown in detail.


Protein A 254
0
.02
.04
.06
Radioactivity (cpm)
0
20
40
200
220
240
260
280
4
5
6
7a
7b
Fraction Number (1 ml.)

dialyzed, lyophilized and dissolved in 6 ml of SDS-phosphate elution buffer. Each was passed through the Sepharose 6B column and assayed on the UV monitor but not by the phenol sulfuric test. The fractions corresponding to peaks II and III were pooled for each sample, dialyzed, and lyophilized. They were

Table IX. Protocol for the Pronase Experiment

Sample	Extract in Buffer (ml)	Buffer (ml)	Initial Pronase in Buffer (ml)	After 26 hrs Pronase (ml)
Pronase treated	40	—	40 (8 mg)	1 (8 mg)
Control	40	40	—	—
Blank	—	40	40 (8 mg)	1 (8 mg)

each dissolved in 2 ml of elution buffer and applied to a column of DEAE Sephadex. The elution sequence using 0.2 M sodium phosphate buffer, pH 7.2, was as follows:

buffer	60 ml
0.2 M NaCl in buffer	80 ml
gradient (120 ml 0.2 M NaCl in buffer to 120 ml 0.4 M NaCl in buffer)	240 ml
2.0 M NaCl in 0.1 M NaAc buffer, pH 4.7	40 ml

The resulting elution profiles are shown in Figs. 14 and 15. The results of the blank are not shown since there was no response either in the UV or the phenol sulfuric assay of any consequence. The pronase treated sample shows an unusually high spike on the UV monitor at fraction 203. It is not clear what the meaning of this peak is or whether the proteins which normally

Fig. 14

Control Sample for the Determination
of the Effects of Pronase

A sample of lyophilized extract was divided into two equal portions. One portion was treated with pronase and the other, the control, was treated similarly but without the pronase. After passing the samples through Sepharose 6B, peaks II and III were applied to columns of DEAE Sephadex A-25 (46 cm x 1.2 cm) and eluted with 0.2 M sodium phosphate buffer, pH 7.2 and a salt gradient as shown. The elution profile of the control portion shown here should be compared with that of the pronase treated portion shown in Fig. 15.

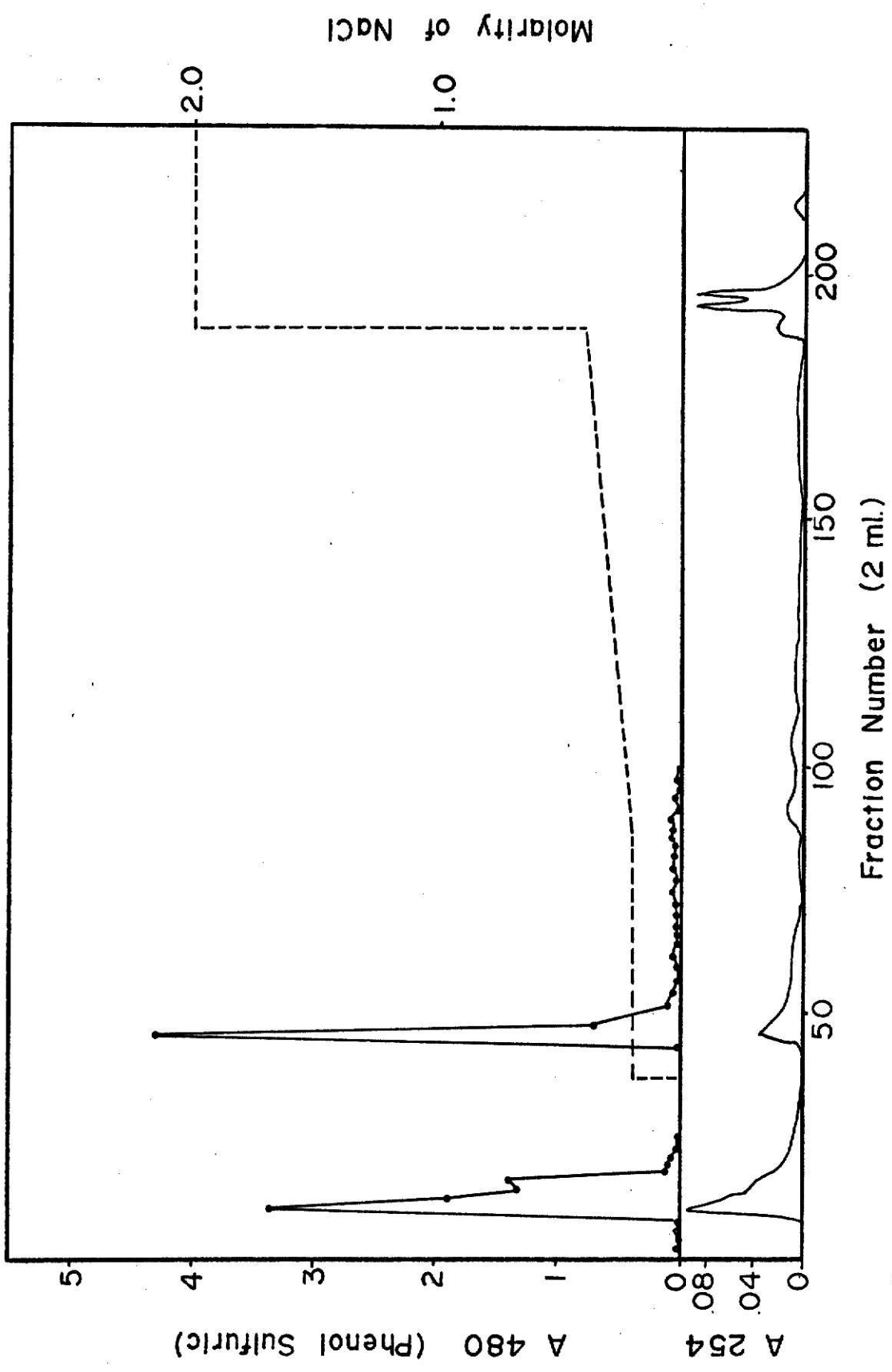
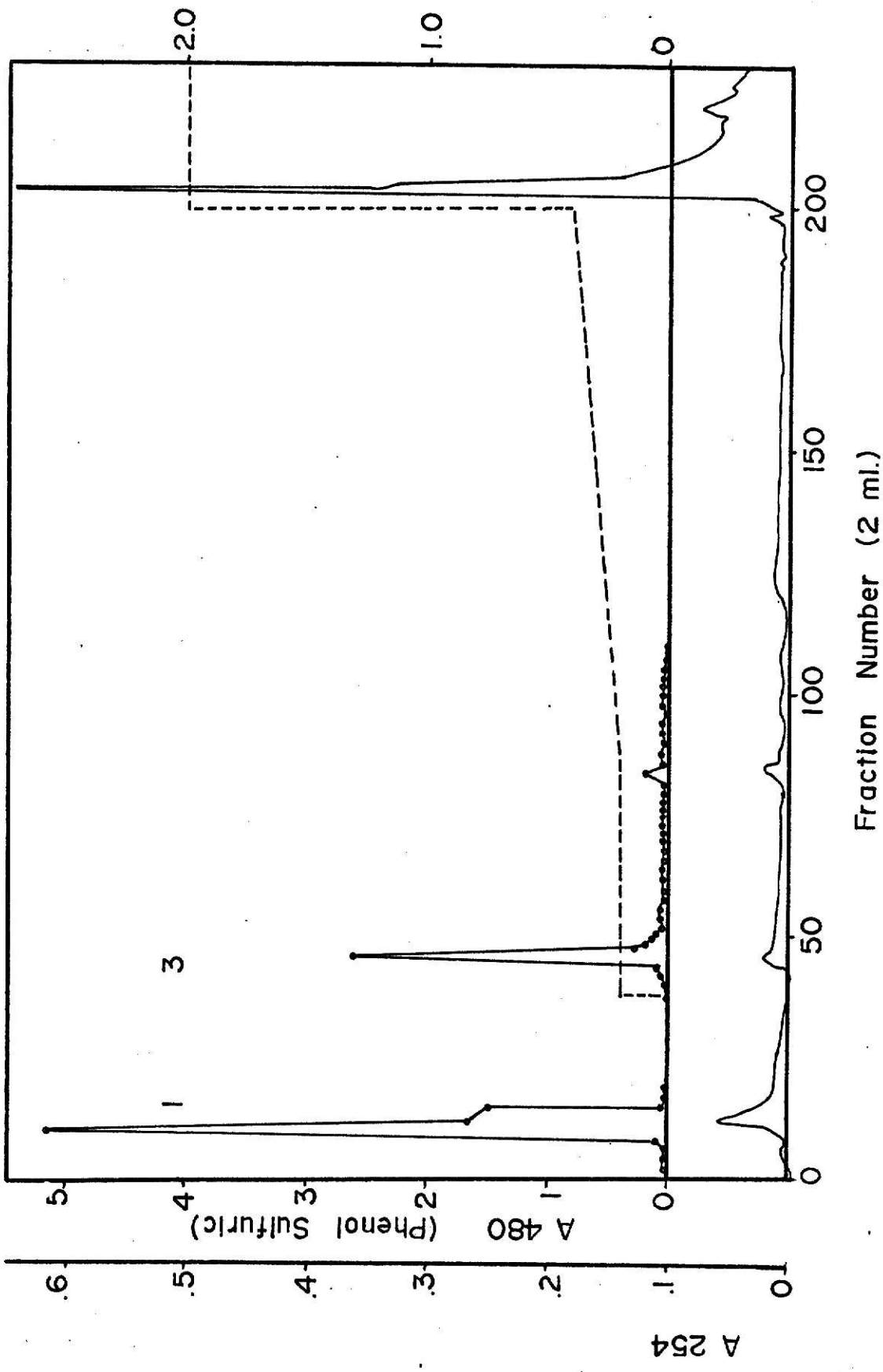

Molarity of NaCl
2.0
1.0
A 254
A 480 (Phenol Sulfuric)
0
.04
.08
0
1
2
3
4
5
50
100
150
200
Fraction Number (2 ml.)

Fig. 15

The Effects of Pronase on the DEAE Sephadex Elution Profile of the Whole-Cell Extract

Compare the elution profile of the pronase treated sample shown here with that of the control sample shown in Fig. 14. The elution procedure was the same as that given for Fig. 14.


Molarity of NaCl
2.0
1.0
0
A 254
A 480 (Phenol Sulfuric)
1
3
Fraction Number (2 ml.)
0
50
100
150
200

are found in this region have been degraded. One might argue that this material is possibly pronase or a degradation product of pronase. This is not believed to be the case, however, because pronase is of lower molecular weight than the Sepharose 6B fractions II and III being investigated and is, therefore, eliminated. In any case, if this material were derived from the pronase then a similar peak should have appeared in the blank.

Peaks 1 and 3 were collected and each was evaluated for carbohydrate and protein. Peak 2 was included in peak 1 since it was not adequately separated. The results of the control and the pronase treated samples respectively are as follows:

Peak 1	Carbohydrate	1.93 mg and 2.25 mg
Peak 3	Carbohydrate	1.36 mg and 1.12 mg
Peak 1	Protein	2.8 mg and .96 mg
Peak 3	Protein	1.9 mg and .86 mg

From these data it can be seen that protein was significantly but not entirely degraded in these peaks. Carbohydrate, however, was not significantly degraded. The experiment demonstrates that pronase treatment could not have caused any major distortions of the carbohydrate portions of the radioactive Golgi-derived material. Specifically, it doesn't account for the absence of peak 3 (Fig. 12).

V. Partial Purification of the Major Carbohydrate Components

Peak 3 material was re-applied to DEAE Sephadex. The UV monitor and phenol sulfuric test revealed that some extraneous carbohydrates and proteins were eliminated. The purified sample, "purified peak 3" was frozen and stored for further use.

In an attempt to better separate peak 2 from peak 1 the appropriate fractions were pooled and applied to a double length column (100 cm x 1.2 cm) containing 16 g (dry) of DEAE Sephadex. The results are shown in Fig. 16. Peaks 1 and 2 were nicely separated; however, both peaks were considerably reduced in size, peak 2 severely so. A large portion of the missing polysaccharide, perhaps all of it, was eluted at an ionic strength somewhat higher than that of peak 3. It was designated peak 3'. The explanation for this unusual behavior is not immediately obvious.

Since peak 1 corresponds to V_o it is possible that it may contain some cationic and isoelectric proteins. To investigate this possibility and to further purify the material, the peak 1 from the previous experiment was chromatographed on CM Sephadex at a reduced pH where most proteins should be cationic. The elution sequence using 0.2 M NaAc buffer, pH 5.2, with preservative (see "Methods") was as follows:

buffer	50 ml
gradient (50 ml buffer to 50 ml 0.8 M NaCl in buffer)	100 ml
NaCl in buffer (saturated)	50 ml

No peaks other than the V_o peak were observed. Since the UV absorbance of the applied sample was very low, it was difficult to determine whether the quantity of protein was reduced. The purified peak 1 material was designated as "purified peak 1".

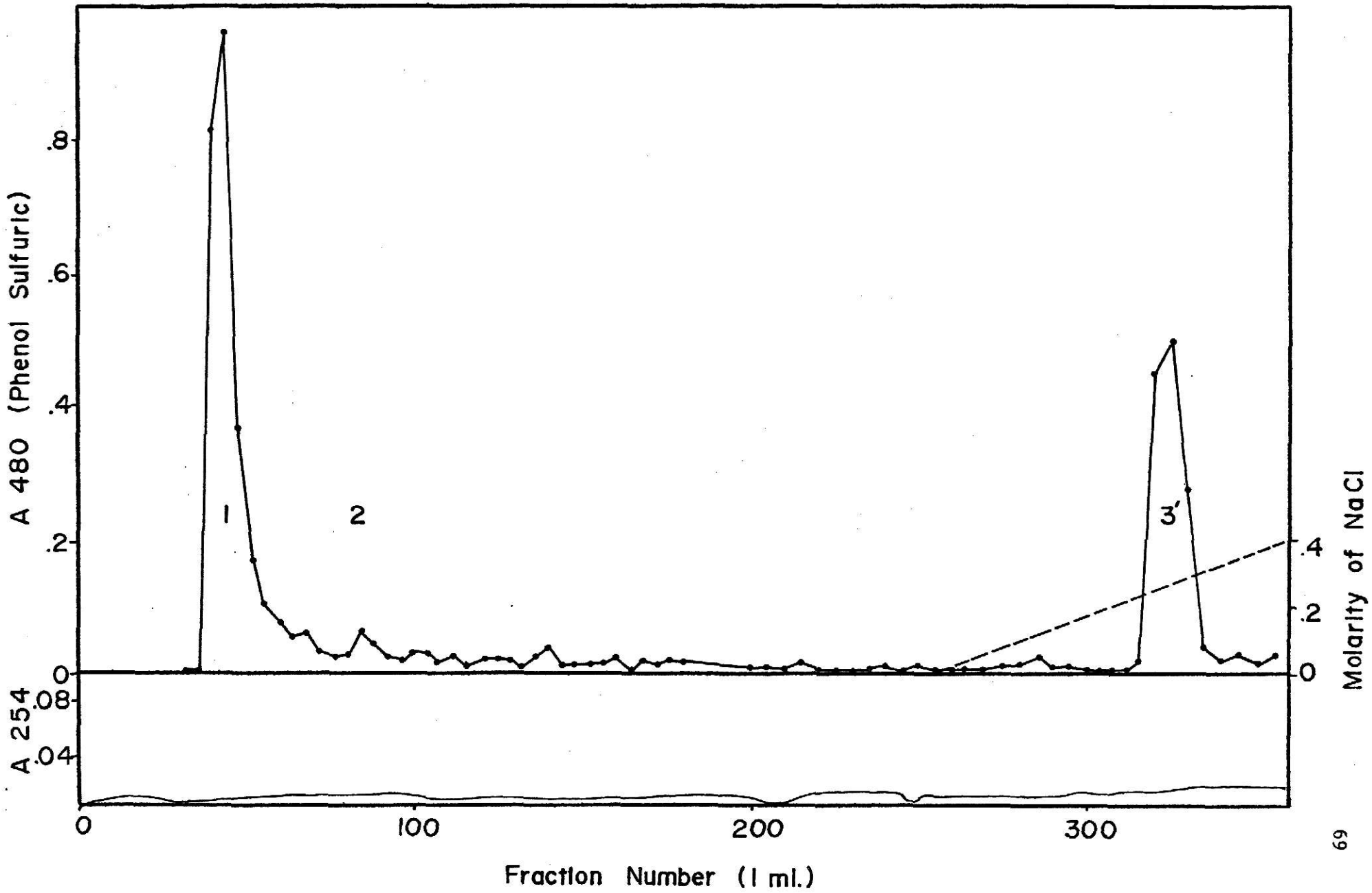
VI. Quantitation of Monosaccharides in Purified Peaks 1 and 3 and Peak 3'

Purified peaks 1 and 3 and peak 3' were dialyzed and 30 µg from each were hydrolyzed. The samples were spotted on thin layer plates and chromatographed. The monosaccharide spots were visualized with triphenyl

Fig. 16

Separation of Peaks 1 and 2 on
DEAE Sephadex

The material from the control portion (shown in Fig. 14) which corresponds to peaks 1 and 2 was applied to a double length column of DEAE Sephadex (100 cm x 1.2 cm) and eluted with 0.2 M sodium phosphate buffer, pH 7.2 and a salt gradient as shown. A new peak was observed and labeled "peak 3'".


A 480 (Phenol Sulfuric)
.8
.6
.4
.2
0
1
2
3'
.4
.2
0
Molarity of NaCl
A 254
.08
.04
0
100
200
300
Fraction Number (1 ml.)

tetrazolium chloride reagent and scanned on the densitometer. The results are shown in Table X.

Table X. Monosaccharides of Purified Peaks 1 and 3 and Peak 3' (mol ratio)

	Purified Peak 1	Purified Peak 3	Peak 3'
Xylose	.13	0	0
Arabinose	.93	.78	.47
Glucose	.10	0	.02
Galactose	1.00	1.00	1.00

While no other sugars were detected in this experiment, this is not to say that no other sugars are present. Galacturonic acid in particular is suspected since it is not adequately detected by this method. The evidence indicated that peak 3' was very similar to the purified peak 3 since both contained only galactose and arabinose. It should be noted, however, that the ratios of these two monosaccharides differed significantly between the two peaks. Also, as shown in Table XI, there was much more protein in peak 3'.

Table XI. Carbohydrate and Protein Content of Purified Peaks 1 and 3 and Peak 3'.

	Carbohydrate µg/ml	Protein* µg/ml	Carbohydrate / Protein
Purified Peak 1	87	62	1.1
Purified Peak 3	570	40	14
Peak 3'	104	80	1.3

* See "Methods" for calculation

VII. Amino Acid Content

The current interest in extensin in the literature suggested that it

would be important to identify this glycoprotein in the whole-cell extract, if possible. Since peak 7 from the Golgi-derived material showed an unusual amount of radioactivity indicating that it may be a glycoprotein, a search for the telltale hydroxyproline was initiated.

Colorimetric assays are available for hydroxyproline; however, it was felt that this method did not offer sufficient verification in the event of negative results. A two dimensional chromatography procedure, therefore, seemed to offer better promise. Although the solvent systems selected (see "Methods") were inadequate for separating the hydrophobic amino acids, they were excellent for the hydrophilic ones. Moreover, they were quick and easy to handle. A test run using the following sample showed that the procedure was adequate:

200 μg bovine serum albumin
20 μg proline
4 μg hydroxyproline

Peak 7 material was dialyzed and a 120 μg sample and a 15 μg sample were hydrolyzed. After drying they were redissolved and spotted at the origins of two thin layer plates. At the extremities of the x and y axes a series of standard spots were placed in order to insure identification of the unknowns. The plates were developed and then visualized with ninhydrin.

The results are shown in Table XII. Although most of the expected amino acids were found, including a significant amount of proline, there was not even a trace of hydroxyproline. Since the ninhydrin spray reagent gives a characteristic yellow spot which is easily distinguishable from most other amino acid spots, the identification of hydroxyproline would have been

unquestionable had it been present. It must, therefore, be concluded that peak 7 does not contain extensin.

Table XII. Amino Acid Composition of Peak 7

Gly -	++++	Lys -	++
Glu -	++++	Arg -	+++
Ala -	+++	Thr -	+
Val -	++	Ser -	none (or less than .05 μg)
Asp -	++++	Hyp -	none (or less than .10 μg)
Pro -	++	Hydrophobic -	++++

DISCUSSION

It appears that the detergent extract of whole cells can be used effectively as a source of membranous organelle polysaccharides and glycoproteins for future metabolic and structural studies. It was shown in Figs. 6, 7 and 12 that all radioactive Golgi-derived fractions obtained on Sepharose and DEAE Sephadex correspond to peaks obtained by the extraction of whole cells. Thus, within the limits of the chromatographic fractions obtained here, the whole cell extract is shown to contain all polysaccharides and glycoproteins contained in the membranous organelles. It should be noted that the crude Golgi extract which was used here actually contains a variety of other membranous organelles in addition to the Golgi bodies. Use of the general term, membranous organelles, is therefore more precise.

However, in order for the whole cell extract to be useful as a source of membranous organelle polysaccharides and glycoproteins, that portion of the extract originating from other sources must be identified and separated. In these experiments it was shown that, in so far as the fractions obtained here are concerned, the Golgi-derived material contains all polysaccharides and glycoproteins contained in the whole cell extract except for peak 3.

It remains likely, however, that there are some non-Golgi substances present in the whole cell extract that are undetectable in the elution profiles obtained here either because they are very low in concentration or because they are eluted coincidentally with other peaks. Consideration of the macromolecules likely to be present in the centrifuged and dialyzed extract shows that the non-Golgi components should not detract from its usefulness. These macromolecules may be classed as follows:

a. Golgi-derived polysaccharides destined for the matrix and their

precursors.

b. Golgi-derived glycoproteins including the precursor of extensin.

c. All other cellular proteins.

d. All cellular nucleic acids.

e. Extracellular root cap slime.

f. Possibly some of the extracellular pectic substances.

The substances of interest for the study of the Golgi apparatus are of course a and b above. While many of the unwanted substances may be eliminated during Sepharose fractionation others undoubtedly remain and, therefore, must be dealt with.

The proteins (now known to comprise 41% of the extract) are probably the largest class of undesired substances present. In future work these should probably be separated from the polysaccharides by precipitating them with cold TCA solution or by some other suitable means.

In this study no attempt was made to identify nucleic acids and none were observed. If, however, they should become a problem in future work, they could be eliminated enzymatically and the products dialyzed.

The root cap slime was not positively identified in any of the fractions isolated here; however, its presence in the extract is very likely. According to Harris and Northcote (108) there is a substantial fucose component in the root cap slime of corn which is not found elsewhere in the root. If this is also true of wheat root cap slime, then the identification of the slime should be quite easy. No fucose has been found in any of the chromatograms produced here. Although fucose runs very close to xylose in the solvent system used, its characteristic green color with aniline diphenylamine spray reagent should readily distinguish it from xylose which is navy

blue.

It is possible that a small portion of the extracellular pectic substances could dissolve in the detergent homogenization medium. There appears to be no positive way to determine from these experiments the degree to which these substances are present in the extract since they are probably identical or very similar to their counterparts in the Golgi apparatus. Workers have found, however, that the pectic substances of the cell wall require heating or ammonium oxalate to break up the gel structure. Thus it is not likely that a great deal of pectic substance could be loosened from the cell wall by a detergent solution.

A second objective in this work was to fractionate the major polysaccharide components being synthesized by growing wheat seedling roots and to perform preliminary characterizations of the fractions with a view toward identifying the polysaccharide types to which they belong. The following types of matrix polysaccharides are considered to be possible components of wheat seedling roots either because they are known to exist in other wheat tissues or because they are known to be components of the pectic substances of nearly all primary cell walls of angiosperms:

a. β-(1→3)(1→4)-D-glucan (98),

b. Xylans containing arabinose and glucuronic acid (104),

c. Arabinans (79),

d. β-(1→4)-D-galactan with arabinose side chains (arabinogalactan)(79),

e. Rhamnogalacturonans with a variety of possible side chain monosaccharides (37).

By matching these polysaccharide classes to the monosaccharides in the hydrolyzates of the peak obtained, tentative identifications may be assigned.

The retention of various fractions on DEAE Sephadex is an additional aid in understanding their composition. Since the carboxyl groups of uronic acids are the only charged groups likely to be present and since they are all roughly equal to each other in binding strength, the degree of retention may be used as an indicator of the prevalence of charged carboxyls.

Since peak 1 (V_o) contains a large quantity of galactose and arabinose it probably is mostly composed of neutral arabinogalactan and arabinan. Traces of glucan and xylan may account for the glucose and xylose observed in Table X. Alternatively, a highly methylated and, therefore, neutral rhamnogalacturonan may contribute these substances. This would account for the traces of rhamnose observed in peak 1 in Table VIII.

Peak 3, according to the experiment shown in Table X, has a composition typical of arabinogalactans; however, its acidic nature belies this simple interpretation. Arabinogalactans are usually described as neutral polysaccharides in the literature (34). Perhaps the arabinogalactan in this fraction is actually covalently linked to a rhamnogalacturonan. Such complexes have been reported by a number of authors. Stoddart and Northcote (62) have observed neutral arabinogalactans in the culture medium of sycamore cells which, over a period of several hours, became bound to an acidic, partially methylated galacturonan (rhamnogalacturonan) to form a less acidic "pectinic acid" complex. Talmadge, Keegstra, Bauer and Albersheim (61) have shown that the reducing end of a galactan is linked to the 4 position of rhamnose in the galacturonan. While galacturonic acid was not observed in peak 3 material because the hydrolysis conditions were insufficient to release it, rhamnose was observed as shown in Table VII.

If peak 3 is in fact a pectinic acid complex such as Stoddart and

Northcote describe, then the final reaction leading to its production would be expected to occur in the cell wall after excretion from the cell. This explains the absence of peak 3 in the Golgi-derived material. In order to make this hypothesis acceptable, however, the metabolic precursors of the complex, i.e., arabinogalactan and rhamnogalacturonan should be located. Peak 1 has already been shown to contain the monosaccharides characteristic of an arabinogalactan providing a ready source for this precursor. The acidic, partially methylated rhamnogalacturonan on the other hand would be expected to form a peak to the right of peak 3 in Fig. 12. A probable reason for the absence of such a peak is that its molecular weight is so low that it is located in Sepharose fraction IV instead of fractions II and III.

From the foregoing discussion of its probable polysaccharide content it is clear that the monosaccharides found are typical of pectic substances as opposed to hemicelluloses. Thus, the fractions obtained appear to be parts of a pectic complex similar to the structure proposed by Keegstra et al. (59)(see Fig. 3). The presence of pronase resistant protein (Fig. 15) may indicate that a glycoprotein is present in peaks 1 and 3. Conceivably this material may also be covalently linked to the pectic complex in a structure similar to that of fraction "PR-2" reported by Keegstra et al. (59). One research project for the immediate future should be to determine the quantity of galacturonic acid present in each peak. This would provide confirmation of the presence of the rhamnogalacturonans tentatively identified here.

In the attempted purification of peaks 1 and 2, the unexpected transfer of a large portion of peak 1 material to the vicinity of peak 3 is a phenomenon requiring further explanation. The reaction could have been caused by a variety of factors since the sample was stored for about two weeks in a refrigerator in unbuffered solution. But regardless of what caused the

change, the phenomenon indicates that both peak 1 and peak 2 can be altered to a more acidic state. Because of this instability it is recommended that in all future work, these materials be stored in the lyophilized state. Possible reactions which may have caused the increase in charged carboxyl groups include air oxidation or demethylation of uronic acids. Both reactions are known to be mediated by alkaline conditions. Demethylation may also have been effected by an SDS resistant enzyme known to be present in many tissues (117).

For extracting the cytoplasmic precursors of matrix polysaccharides and glycoproteins the most promising extraction medium of the several choices available appears to be SDS. Water or buffer should be capable of extracting all unbound polysaccharides provided the tissue is ground with sufficient vigor. However, there is also likely to be a number of protein and lipid bound polysaccharides present which would remain insoluble. Protein denaturants such as 8 M urea and non-ionic detergents such as Triton X-100 are rejected simply on the grounds that they are not as effective as SDS in dispersing protein and lipid structures and because they may in some cases result in the precipitation of proteins.

During the course of this work it became of interest to determine whether any of the protein present in the peaks obtained belonged to the precursor of the cell wall glycoprotein extensin. Although etiolated and actively elongating tissue were not found to be rich in hydroxyproline (69), this may not necessarily be reflected in the contents of the cytoplasm. It is quite possible that the isolation procedure used may have concentrated a fairly large amount of the precursor in the fractions observed. The peak of highest protein concentration, however, did not contain hydroxyproline.

The tracing of ^{14}C proline uptake as described by Chrispeels (46,71) in carrots could be a valuable aid in future research with wheat roots.

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POLYSACCHARIDES EXTRACTED WITH SDS FROM WHEAT SEEDLING ROOTS

by

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B. S., Lehigh University, 1967

AN ABSTRACT OF A THESIS

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ABSTRACT

Soluble polysaccharides from wheat seedling roots were extracted with phosphate buffer, pH 7.6, containing 0.01 mM sodium dodecyl sulfate (SDS). The extract was dialyzed and fractionated on Sepharose 6B into four fractions. Fractions II and III were pooled and separated on DEAE Sephadex revealing 3 polysaccharide peaks and five protein peaks. This procedure was also used to separate a sample of ^{14}C labeled polysaccharides obtained from a crude Golgi body preparation. With the exception of peak 3, missing in the Golgi-derived material, there was a high degree of correlation between the two elution profiles indicating that the whole-cell-extracted polysaccharides were, for the most part, derived from the membranous organelles.

Comparison of the DEAE Sephadex elution profile of a sample which was treated with pronase with that of an untreated sample revealed that pronase had no major effect on the polysaccharides although the proteins were partially degraded.

After hydrolysis, monosaccharides from peaks 1 and 3 were chromatographed on silica gel thin layer plates. Both peaks were found to contain galactose, glucose, arabinose, xylose, and rhamnose. Peak 3 also contained a trace of glucuronolactone. Molar ratios estimated by densitometric scanning of plates visualized by triphenyl tetrazolium chloride were: peak 1, galactose 1.00, arabinose .93, xylose .13, glucose .10 and peak 3, galactose 1.00, arabinose .78. The composition of peak 1 suggested that it contains a neutral arabino-galactan and a neutral rhamnogalacturonan. The composition of peak 3 suggested an acidic rhamnogalacturonan complex containing covalently bound arabino-galactan.

When a sample of peaks 1 and 2 which had been stored in the refrigerator

in buffered solution for about two weeks was chromatographed on DEAE Sephadex, it was found to be unstable under these storage conditions. A large portion of peak 1 and nearly all of peak 2 were converted to a more acidic substance, peak 3'.

One prominent protein containing peak, number 7, was found by means of two dimensional chromatography of the hydrolyzate to contain no hydroxyproline although proline and other amino acids were shown to be present. Thus the possibility that this protein might be a precursor of the cell wall protein, extensin, is precluded.