This is to certify that the

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PARTIAL CHARACTERIZATION OF EXTENSIN BY SELECTIVE DEGRADATION OF CELL WALLS

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PARTIAL CHARACTERIZATION OF EXTENSIN BY SELECTIVE DEGRADATION OF CELL WALLS

By

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ABSTRACT

PARTIAL CHARACTERIZATION OF EXTENSIN BY SELECTIVE DEGRADATION OF CELL WALLS

By

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Cell walls of plants contain a glycoprotein, extensin, which is thought likely to be cross-linked to wall polysaccharides. Drastic degradative methods are necessary for the release of the protein from the cell wall. Two complementary methods for the selective degradation of cell walls have been developed in order to obtain preparations of extensin fragments for use in structural studies. The first, anhydrous HF solvolysis, cleaves neutral and acidic sugar glycosides with no peptide bond breakage. The second, $NaClO_2$ oxidation, cleaves few peptide bonds, no glycosidic bonds, except to a small extent those of uronic acids, yet solubilizes large amounts of high molecular weight glycopeptides from cell walls.

Treatment of tomato, tobacco, or sycamore maple cell walls with liquid HF did not release extensin from the walls, but did solubilize all the sugars, leaving a cell wall shaped residue of amino acids and some unidentified material. Thus, HF alone cannot be used to isolate intact cell wall protein in a soluble form, but can be used to determine the complete sugar composition of cell walls.

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From experiments with glycoproteins containing the glycopeptide linkages, arabinose-O-hydroxyproline and galactose-O-serine (plant cell wall glycopeptides), N-acetylgalactosamine-O-serine/threonine (pig submaxillary mucin), and N-acetylglucosamine-N-asparagine (fetuin), it became apparent that anhydrous liquid HF, a reagent commonly used by synthetic peptide chemists for the complete removal of protecting groups from synthetic peptides, cleaves the O-glycosidic linkages of neutral sugars in 1 hour at 0 C, and the O-glycosidic linkages of amino sugars in 3 hours at 23 C. The N-glycosidic linkage of N-acetylglucosamine to asparagine is not cleaved by anhydrous HF under any conditions that have been tested. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of HF treated bovine serum albumin reveals no degradation of peptide bonds. Some relatively stable enzymes (lysozyme and ribonuclease) have been shown by others to retain most of their enzymic activity after short treatment (1 hour at 0 C) in HF.

Although the precise chemical nature of the oxidation reaction of $NaClO_2$ with cell walls and proteins is not known, it was determined that $NaClO_2$ releases large quantities of glycopeptides from cell walls and so is of interest. These glycopeptides have been partially characterized by size, charge and buoyant density. Compositional and methylation analyses showed these peptides to be highly glycosylated, containing the short arabinose side chains previously described by Lamport (1) but in addition a single galactose on almost all of the

serine residues and in some cases what appears to be a 3,6 linked galactan linked to a polymer containing galacturonic acid, i.e., pectin.

The results presented give supporting evidence to the model of a primary cell wall put forward by Keegstra et al. (2) and thus place the cell wall protein in a good position to be controlling wall growth.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DMSO	dimethylsulfoxide
GalU	galacturonic acid
GLC	gas liquid chromatography
Hyp	hydroxyproline
Hyp-ara, Hyp-arabinoside	a short chain of arabinose (1-4) on the hydroxyl group of Hyp
NGNA	N-Glycolylneuraminic acid
PSM	pig submaxillary mucin
RNase	ribonuclease
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TMS	trimethylsilyl

INTRODUCTION

To understand the molecular mechanism of cell wall growth we must know which polymers are present in the walls and how they are linked together. There have been a multitude of physiological experiments showing that auxin, gibberellic acid, and even low pH (3) stimulate the elongation of stems, roots, and other organs of a wide variety of plants. There have been theories that these growth promoters are acting by methylation of the pectins, induction of a growth limiting protein, stimulation of glycosidases or activation of a proton pump which will acidify the cell wall and therefore activate glycosidases or cleave some acid labile bonds (4). Of these theories only the proton pump activation mechanism is generally accepted, but neither a linkage susceptible to mildly acidic conditions nor an enzyme activated by low pH capable of labilizing cell wall cross-links has been found.

Until recently, these theories were all based on physiological evidence because a detailed working model of the primary cell wall (the thin, expandable wall of growing cells as opposed to the thickened, lignified, rigid secondary cell wall) was not available.

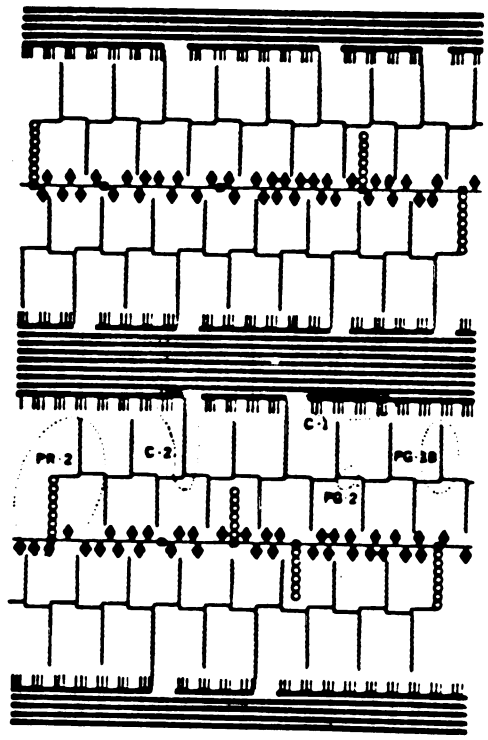
Recently, from their own experiments with degradation of cell walls of sycamore suspension cultures using purified enzymes, the observation of Aspinall et al. (5) that xyloglucan polymers hydrogen bond strongly to cellulose, and the large pool of plant polysaccharide

structures determined over the past century, Keegstra et al. (2) have proposed a model for the cell walls of sycamore suspension cultures, which Albersheim suggests is representative of primary cell walls of all dicots (6).

A diagram of the proposed structure is given in Figure 1. In this model the cellulose microfibrils are cross-linked by the matrix polymers of the wall, the direct link to the fibrils being by hydrogen bonding of a xyloglucan. The xyloglucan is linked at its reducing end to a 4-linked galactan which is then linked to the rhamnogalacturonan of the wall. From the reducing end of the rhamnogalacturonan is a 3,6-linked arabinogalactan which is glycosidically linked to serine residues of the cell wall protein.

The centrally located protein has been characterized almost exclusively by Lamport and his collaborators. While studying the composition of walls prepared from suspension cultures of sycamore maple cells, Lamport found that these walls (7), and later that most plant cell walls (8), contained a protein rich in hydroxyproline (Hyp), an amino acid formed by post translational hydroxylation of proline. This amino acid has never been found in an enzyme, but is frequently found in the structural proteins of connective tissue (throughout the animal kingdom). Thus it is reasonable to propose that the Hyp-containing protein in cell walls is a structural protein, especially as it is by far the major component of some cell walls, such as those of Chlamydomonas (9). Lamport and his colleagues have partially characterized this Hyp-rich protein in the cell walls of tomato cell

Figure 1. A model of the primary cell wall of dicots. Reproduced with permission from Keegstra et al. (2).


PR-2
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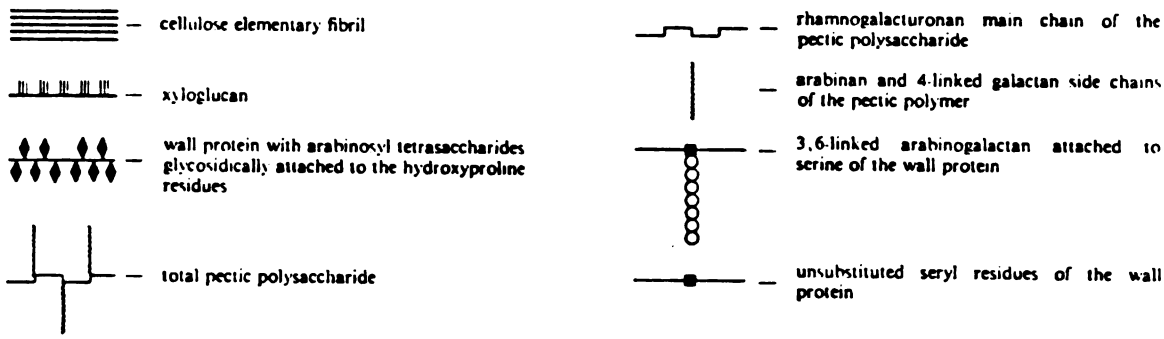
cellulose elementary fibril
xyloglucan
wall protein with arabinoxyl tetrasaccharides glycosidically attached to the hydroxyproline residues
total pectic polysaccharide
rhamnogalacturonan main chain of the pectic polysaccharide
arabinan and 4-linked galactan side chains of the pectic polymer
3,6-linked arabinogalactan attached to serine of the wall protein
unsubstituted seryl residues of the wall protein

Figure 1

suspension cultures. In tomato and other plants in which Hyp has been found there is an oligosaccharide attached glycosidically to the hydroxyl group of most of the Hyp residues (8). These Hyp-oligosaccharides are easily prepared by alkaline hydrolysis of intact cell walls (1). In higher plants the Hyp-oligosaccharides are almost exclusively composed of arabinose and are from 1 to 4 residues long (8).

The protein has not been solubilized intact from walls of cells from suspension cultures but can be partially extracted by crude cellulase, pronase (10) or trypsin (11). For solubilization with trypsin the walls must be pretreated with acid, refluxing at pH 1 for 1 hour, before an appreciable fraction of the Hyp can be released. This acid treatment removes the arabinose residues from the Hyp and a fraction of the pectin, perhaps indicating that the arabinose side chains protect the protein from proteolysis by invading pathogens. The shorter peptides released by a combined pH 1 treatment followed by digestion with trypsin have been sequenced (11) and all contain the remarkable sequence Ser-Hyp-Hyp-Hyp-Hyp, the longer peptides (15 amino acids long) containing the sequence twice. Larger tryptic peptides, although not yet fractionated and sequenced, are also extremely rich in Hyp, indicating that they too probably will contain many repeats of the sequence Ser-Hyp_4. As polyproline and polyhydroxyproline each form a 3 residue-per-turn left-handed helix (12), it is possible that extensin also could take a helical conformation which would cause it to be a rigid rod and thus play a structural role in the wall.

Originally Lamport suggested that primary cell wall consists of cellulose fibrils cross-linked to the cell wall protein via the hemicellulose and the side chains of arabinose on the Hyp residues (13). For this model to be plausible one has to postulate that the cross-links from the ends of the arabinose chains to the hemicellulose are alkali labile because nothing has ever been found attached to the arabinose-containing oligosaccharide (prepared by alkaline hydrolysis). Methylation analysis of sycamore walls (a method also involving strong base) showed many terminal arabinoses consistent with there only being short arabinose chains on the Hyp or an alkali labile substituent. Glycosidic linkages to sugars are alkali stable.

In the model of primary cell wall proposed by Keegstra et al. an arabinogalactan cross-links the protein to the pectins of the wall. The protein-arabinogalactan linkage was supported by Lamport et al. (14) who found that most of the serine residues in the cell wall tryptic peptides were galactosylated, but by a single galactose. If the sugar attached to this galactose were a furanose, it would be cleaved during the pH 1 treatment used to allow trypsinization. Thus the galactose could be the start of the arabinogalactan of Keegstra et al. The evidence (2) that the hypothetical polysaccharide is an arabinogalactan is rather indirect. The data are mostly from a protein, found in the medium bathing the cells, which was thought to be similar to the protein in the cell wall. Pope (I5) found later, a polysaccharide in this extracellular protein attached to the hydroxyl group of Hyp. This is most probably the one which was presumed to be linked to serine by

Keegstra et al. (2). In addition, the Hyp-arabinoside composition of the extracellular protein is unlike that of the cell wall, implying that the extracellular protein is not extensin.

The role of the Hyp containing protein extensin in the cell wall still remains to be discovered, and therefore the possibility that it is involved in cell wall loosening, the prerequisite for wall extension or growth, remains reasonable.

At the start of this thesis research I was hoping to determine the size of the polypeptide portion of extensin. As the protein had only been extracted from walls after extensive degradation by acid and trypsin, the longest characterized tryptic peptide consisted of only 15 amino acids. To determine the size of the polypeptide portion of the protein I wished to solubilize the cell wall selectively by cleaving glycosidic linkages, leaving the peptide bonds intact.

Sodium periodate was not very effective in labilizing glycosidic linkages under conditions in which there were minimal effects on the protein. Dr. Roy Vagelos suggested in 1972 that I try using 60% hydrofluoric acid, a reagent he had used to remove phosphopantotheine from acyl carrier protein (16). He had also used anhydrous HF to remove protecting groups from synthetic acyl carrier protein (17). As the apparatus for removing 60% aqueous HF from proteins was not readily available nor was 60% HF (commercial HF is 40%), I tried several of the reagents used by synthetic peptide chemists (18). Later, I found that 60% HF would not have worked. The first I tried was HBr gas in trifluoroacetic acid (TFA). It worked very well on tryptic peptides of

cell wall protein, but on treatment of the entire cell wall there remained an insoluble residue which contained all of the protein. No non-destructive solubilizing agent solubilized the protein. As plants are notorious producers of phenolic compounds, I thought that these could be complexing with the protein to render it insoluble during the HBr solvolysis. In an effort to avoid interference by phenolics after I had used various known methods to reduce phenolic contamination with no better results, I tried to selectively oxidize the phenols with sodium chlorite, a compound often used for delignification of wood by carbohydrate chemists. This reagent bleached the walls from their pale or dark brown color to a brilliant white and then, in some cases, allowed total dissolution of cell walls with HBr in TFA. On analysis of the walls after $NaClO_2$ oxidation I realized that the oxidation itself was partially solubilizing the walls and was especially effective at solubilizing the protein. As $NaClO_2$ oxidation is used by carbohydrate chemists especially for its non reactivity towards sugars, I devoted much time to the study of the protein released by chlorite oxidation hoping that it would still be attached to polysaccharides. Since $NaClO_2$ does react with proteins (it destroys tyrosine) my initial goal to solubilize intact extensin was not achieved, again, but a new approach was opened to the determination of how extensin is linked to the cell wall.

Having been so successful in deglycosylating cell wall peptides and cell walls with HBr in TFA, I extended this chemical method to all glycoproteins using an even more effective deglycosylating agent, anhydrous liquid hydrogen fluoride.

Thus from one problem, as yet unsolved, two others arose. Therefore, this thesis is in two parts: Part I--The use of anhydrous liquid HF to selectively depolymerize polysaccharides thus allowing deglycosylation of glycoproteins or quantitative sugar recoveries from complex polysaccharides, and Part II--Solubilization and characterization of cell wall protein glycopeptides by $NaClO_2$ oxidation.

PART I

THE USE OF ANHYDROUS LIQUID HF TO SELECTIVELY DEPOLYMERIZE POLYSACCHARIDES THUS ALLOWING DEGLYCOSYLATION OF GLYCOPROTEINS OR QUANTITATIVE SUGAR RECOVERIES FROM COMPLEX POLYSACCHARIDES

A. Introduction

In the study of cell wall protein it is often inconvenient to work with glycosylated peptides. Variation in glycosylation can cause heterogeneity of a single peptide species and thus complicate its characterization. Lamport (11) found it necessary to remove the arabinosides from the Hyp residues of cell wall protein to make the protein *in situ* susceptible to trypsin. During this deglycosylation, a pH 1 treatment of 100°C for 1 hour, it is likely that some acid labile peptide bonds, e.g., at aspartic acid, are cleaved and about one-third of the cell wall protein is solubilized in a form which is not susceptible to trypsin. Thus an effective way to deglycosylate glycoproteins and glycopeptides was needed to simplify the characterization of extensin.

If the cell wall protein were linked in the cell wall matrix by only glycosidic linkages (as proposed by Keegstra *et al.* (2)) then a method to break glycosidic linkages specifically should allow extraction of soluble intact cell wall protein and thereby make possible determination of the size of extensin.

Plant cell walls contain only neutral and acidic sugars but many glycoproteins contain amino sugars and so testing the general

applicability of any deglycosylation method should also involve testing of protein containing amino sugar linkages.

The classical techniques of degrading polysaccharides under conditions which do not affect peptide bonds have serious drawbacks. The commonly used Smith degradation (19) involves periodate oxidation which also affects tyrosine residues and terminal serine residues. Usually periodate oxidation must be performed repetitively to remove all the sugars from a glycoprotein. In the case of the cell wall protein, extensin, the reaction with terminal arabinose residues is exceedingly slow. Enzymatic deglycosylation involves isolation of protease-free enzymes capable of hydrolyzing each particular glycosidic linkage present. Therefore, it would be advantageous to have a method which cleaves all glycosidic linkages nonspecifically but leaves peptide bonds intact.

Synthetic chemists often have the need to protect sensitive functional groups during reactions and so many easily removable protecting groups have been developed. In peptide synthesis this approach must be carried to great sophistication. It is necessary to have many different functional groups protected yet to be able to unmask some of them selectively to allow continuation of the addition of amino acids to the peptide undergoing synthesis. At the end of the synthesis all the protecting groups must be removed to give a peptide indistinguishable from the biosynthesized one. HBr in TFA and anhydrous liquid hydrogen fluoride are both examples of reagents which are used to perform the final step of completely removing all the protecting groups of chemically synthesized peptides.

After only partially successful initial attempts to deglycosylate cell wall glycopeptides via periodate oxidation I tested the most promising synthetic peptide deprotecting agent which could be used without specialized equipment, HBr in TFA. This reagent was effective with both cell wall glycopeptides and intact cell walls. Anhydrous liquid hydrogen fluoride, although dangerous, is more convenient to use than HBr, given the proper equipment (see methods), is a better solubilizing agent than TFA, and can be used at lower temperatures thus giving a better possibility of retaining enzymatic activity of glycoproteins to be deglycosylated. Lysozyme and ribonuclease (20) have already been treated with HF with retention of the majority of their enzymatic activity. Consequently, a Kel-F hydrogen fluoride line (approximately $3000.00) was purchased. The effects of HF were first tested with cell walls and the glycoproteins ovomucoid and ovalbumin. Ovomucoid is not a good model glycoprotein as it is not well-characterized and ovalbumin did not redissolve after HF treatment. In later experiments, the well-characterized glycoproteins fetuin and pig submaxillary mucin, the polysaccharides arabinogalactan and chitin, and cell wall glycopeptides were used to determine the specificity and effectiveness of HF in cleaving various glycosidic linkages.

1. The Use of Anhydrous HF in Protein and Carbohydrate Chemistry

The solvating properties of anhydrous liquid HF have been known for many years. As early as 1869 Gore (21) reported that paper, gum arabic, India rubber, sealing wax, many other organic and inorganic

materials dissolve rapidly in liquid HF. Fredenhagen and Cadenbach (22) suggested that the dissolution of the polysaccharides of wood with liquid HF would allow quantitation and recovery of the lignin which is insoluble in HF. Fredenhagen (23) also found that casein, hemoglobin and nucleic acids are soluble in liquid HF. Katz (24) extended the solubility studies of HF to amino acids and a number of proteins. Amino acids are extremely soluble in HF, e.g., 30 gm/100 gm for tryptophan at -78 C, as are globular proteins, e.g., 350 mg/ml for bovine serum albumin at 0 C. After removal of the HF by evaporation under vacuum about half of the proteins tested became water insoluble. Bovine serum albumin, one of the proteins which became insoluble, remained water soluble if aspartic acid, lysine, glycine or hide collagen was added to the HF solution.

Katz suggested that HF could be used as an excellent solvent for protein chemistry due to the wide range of compounds which it can dissolve in high concentration. In subsequent experiments, Koch et al. (25) found that ribonuclease retained its nuclease activity for at least 2 hours in HF at -78 C. At 0 C for 2 hours a variable proportion of the activity was lost (up to 66%). After 2 hours at 30 C ribonuclease had essentially lost its enzymatic activity. Lysozyme under similar conditions lost its activity somewhat more rapidly. Koch et al. estimated the degree of peptide bond breakage under various conditions by the colorimetric determination of amino groups. After 2 hours at 0 C there was no increase in amino groups in ribonuclease and a slight increase (less than one per molecule) for lysozyme. After 2 hours at

30 C there was an increase of approximately 2 in ribonuclease and 1.5 in lysozyme. After 24 hours at 30 C ribonuclease had 6 additional amino groups. No disulfide interchange was found after 24 hours at 30 C. However, peptide bond breakage need not account for the appearance of all the new amino groups.

Sakakibara et al. (26) found a slow N to O acyl shift of the peptide bond to serine and threonine residues at room temperature which was about 95% complete in glycyl-DL-serine and glycyl-DL-threonine after 15 days in HF. Lenard and Hess (27) have applied the N to O shift to the specific cleavage of oxidized insulin A chain and α-melanocyte stimulating hormone, since the resulting ester bond can be easily cleaved in mild alkali. The shift was accelerated by using HF at 30 C, thus the N to O shift was 65% complete after only 13 hours. This shift could be totally reversed by allowing the peptide to stand in sodium bicarbonate solution overnight or by the addition of pyridine. During the HF treatment internal ester formation between serine hydroxyl groups and carboxyl groups of insulin prevented the acyl shift from going to completion. This could be overcome by adding 25% methanol to the HF. The methyl esters so formed could be cleaved in aqueous piperidine at 0 C. The inclusion of methanol allowed the shift to go to 85% completion in 12 hours at 30 C with negligible peptide breakage. Paper electrophoresis and amino acid analysis of insulin before and after methanolic HF treatment, piperidine reversal of N to O shift and methyl ester cleavage showed that the treated peptide was indistinguishable from the starting material. To cleave a rearranged peptide, the amino

groups must be acylated to prevent the reversal of the N to O shift during the alkaline treatment which specifically cleaves the amino acid ester link to serine. This is easily accomplished by formylation. After formylation the rearranged peptide is cleaved by an aqueous 1 M piperidine treatment which also cleaves the methyl esters used to allow a high yield of N to O shift. Eighty to 85% cleavage was reported for α-melanocyte stimulating hormone. The removal of the formyl group was not investigated, but has been reported by Sheehan and Yang (28).

The only peptide bond known to be cleaved by anhydrous HF is that of methionine. Lenard et al. (29) found that methionyl-glycine was completely cleaved after 36 hours at 30 C in HF, yet after oxidation of the methionine to its sulfone the dipeptide was completely stable. The cleavage of methionyl bonds in peptides and proteins is much slower.

From the results described above Sakakibara and Shimonishi (30) proposed that HF would be an excellent reagent for the acidolysis of the protecting groups used during peptide synthesis. Results obtained with the fully protected nona-peptide of oxytocin proved them to be correct. After 1 hour in HF at room temperature and oxidation of the cysteine residues in air, the yield of biologically active peptide was 36-40%, a 4-fold improvement over previous deprotection methods. Lenard and Robinson (31) found that HF could also be used to cleave a protected synthetic peptide from the resin used in the Merrifield solid-phase method of peptide synthesis allowing cleavage from the resin and deprotection in one step.

Hydrogen fluoride is now the method of choice for complete deprotection of synthetic peptides and has proved its usefulness in the synthesis of many peptides.

The reactions of polysaccharides in liquid HF have not been investigated to any great extent. Fredenhagen and Cadenbach (22) found that cellulose was depolymerized quantitatively to glucosyl fluoride in HF at room temperature, but that unless the HF was removed rapidly and in the cold, the product was a polyglucan. Helferich et al. (32) obtained similar results with starch and maltose. Thus it appears that the glucosyl fluorides formed in HF reoligomerize to a degree dependent on the concentration of sugar in the HF.

Monosaccharides form glycosyl fluorides when they are dissolved in liquid HF whether or not the hydroxyl groups of the non-reducing carbon atoms are acylated. Penta-O-acetyl or -O-benzoyl glucose gives a high yield of the corresponding 2,3,4,6 tetra-O-acetyl or -O-benzoyl glucosyl fluoride in HF at room temperature for 10 minutes (33). But when the reaction is prolonged to a number of hours there is often inversion at the 2-carbon and ring contraction. The NMR spectrum of cis 1,2-diacetoxycyclohexane in HF indicates that these side reactions may proceed via a cyclic 1,3 dioxolenium ion (34). This cyclic intermediate did not form with trans 1,2 diacetoxycyclohexane. In the absence of ester linkages to the sugars these side reactions should not take place.

The reactions of sugar fluorides differ considerably from those of other glycosyl halides. The fluorides are much less reactive than the bromides or chlorides and so, are not used in glycoside synthesis.

Glucosyl fluoride is even stable in aqueous solution at neutral pH. Glucosyl fluoride can act as a substrate, in place of sucrose for a glycosyltransferase from Streptococcus mutans (35).

When glucosyl fluorides are mixed with aromatic compounds in HF, a Friedel-Crafts reaction takes place with the C-1 of the sugar alkylating the aromatic nucleus (36).

B. Materials and Methods

1. Materials

Ovomucoid, ovalbumin, bovine serum albumin, fetuin, and crab shells were bought from Sigma Chemical Co., St. Louis, Mo. and larch arabinogalactan from K and K Laboratories, Inc., N. Y.

The cell wall tryptic glycopeptide, Ser(Gal)-Hyp-Hyp-Hyp-Hyp-Ser(Gal)-Hyp-Ser(Gal)-Hyp-Hyp-Hyp-Hyp-Unk-Tyr-Lys was prepared by Katona and Lamport (11).

Pig submaxillary mucin, prepared according to the method of de Salegui and Plonska (37) but without separating the major and minor fractions, was a gift from Joseph Sung (Michigan State University).

Tomato cell suspension cultures were grown in a modified White's medium (38) under continuous light on a rotary shaker at 100 RPM and harvested 4 weeks after subculturing.

Walls from suspension cultured tomato cells were prepared by homogenization of the cells in ice with a Super Dispax DS 45 homogenizer (Tekmar Co., Cincinnati, Ohio) for 2 minutes at maximum speed (10,000 RPM) in the presence of a small amount ($\sim$2 g/500 ml) of dithionite to

prevent oxidation of phenols. This was followed by a 2 minute sonication of 150 ml aliquots at full power (300 watts) with a Bronwill Biosonik III (Bronwill Scientific, Rochester, N. Y.). Aliquots of about 200 ml of wall preparation were then extensively washed on a coarse fritted glass funnel with cold water until the filtrate was clear. Two or more times during the washing procedure the cell walls were removed from the funnel and the fritted glass filter unclogged by hot concentrated sulfuric acid. The walls were then washed with 5 volumes of 1M NaCl followed by 10 volumes of distilled water. The combined washed cell wall fractions were then washed extensively with acetone and left to dry overnight in a vacuum desiccator at 60 C.

Sycamore-maple suspension cultures were grown in M_6E medium (39) but with yeast extract which had had most of its macromolecules removed by ethanol precipitation to allow recovery of extracellular sycamore-maple polysaccharides and proteins uncontaminated by yeast polymers.

GLC column support, Gas Chrom Q, and coating materials SE 30 and SP 2100 were bought from Supelco Co., Bellefonte, Pa. The alcohols used in gas chromatography were dried by refluxing over magnesium turnings and iodine as described by Bhatti et al. (40). All other reagents were used without further purification.

2. Handling of Anhydrous Liquid HF

a. Description of HF Line

Anhydrous hydrogen fluoride can be safely handled only in a closed system. The apparatus used for the experiments, purchased from Peninsula Laboratories, Inc. (P. O. Box 205, San Carlos, Ca. 94070) was

originally designed for deprotection of synthetic peptides by HF after their chemical polymerization. It consists of an HF cylinder, a reservoir, two reaction chambers, another reservoir, and a trap filled with calcium oxide to neutralize the HF when it is removed from the reaction vessel. These components are connected together by Kel F tubing and stopcocks. The outlet from the trap is connected to a vacuum pump. A mercury manometer is provided with the apparatus to indicate the course of evacuation or filling of the reaction vessels. A more complete description and diagram of the apparatus can be found in references (18,20,41).

b. HF Solvolysis

HF was dried over cobalt trifluoride in the first Kel F reservoir. The dried sample (5-20 mg) and 1 ml of anisole scavenger,[1] if required, were added to the reaction vessel and the entire HF line, excluding the HF containing reservoir, evacuated. The reaction vessel was cooled in a dry ice-acetone bath and ca. 10 ml HF was allowed to distill over from the reservoir. Both vessels were stirred continuously by Teflon coated magnetic stirrers to ensure smooth distillation of the HF. After introduction of the HF, the reaction vessel was allowed to warm to the desired temperature as indicated by the vapor pressure of HF measured by the mercury manometer. The vapor pressure of HF at 0 C is 364 mm Hg

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[1] HF solvolysis generates fluorinated compounds, e.g., glycosyl fluorides, which readily alkylate aromatic amino acids, the HF acting as a Friedel-Crafts catalyst (36). Addition of a large excess of anisole protects these amino acids, by competing for the alkylating compounds. Thus, when studying sugar recovery, the anisole must be omitted because the adducts involve a C-C linkage which does not permit recovery of the original sugar.

and at 23 C is 850 mm Hg. Timing of the reaction was started when the vapor pressure of the HF was within 5 mm Hg of that at the required temperature, as heat transfer through Kel-F vessels is very slow. An ice bath was used to keep the reaction at 0 C ; no regulation was used for 23 C , room temperature. At the end of the reaction period the HF was evaporated quickly under reduced pressure. The pressure was dropped as rapidly as possible to keep the time for which the reactants were concentrated in HF as short as possible. Fredenhagen and Cadenbach (22) found that removal of the HF from a solution of glucosyl fluoride, the product of cellulose dissolution, led to the formation of oligomers of glucose unless the solution was evaporated rapidly and in the cold. Too rapid evacuation of the reaction vessel resulted in frothing of the HF solution out of the vessel. The line was left evacuated for at least 1 hour after there were no more visible signs of HF (the anisole changed color from light brown through red to colorless) to ensure complete removal of excess HF.[2] The sample was then taken up in 0.1 N acetic acid or 50% acetic acid if the residue was difficult to redissolve and the anisole extracted into ether. The product was then dialyzed or chromatographed on an appropriate Sephadex column.

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[2]The small amount of HF which remains after this 1 hour period should not affect the protein as HF is a weak acid (pK 3.2) at concentrations below 5M. Above this concentration HF is a very strong acid, but rather than form $H_3O^+ + F^-$ as it does at low concentrations, the HF dissociates to $H_3O^+ + HF_2^-$, $H_2F_3^-$, etc. (42).

3. Analytical Methods

a. Liquid Chromatography

Chromatography of HF treated chitin and glycopeptides which were solubilized from cell walls by chlorite oxidation was performed on a 110 cm x 0.9 cm Biogel P-2 column in 0.1 M acetic acid. Chromatography of extracellular glycoprotein which had been treated with HF was performed on a 2.5 cm x 85 cm Sephadex G-100 column in 0.1 N acetic acid.

Hydroxyproline was determined on an automated hydroxyproline analyzer (8) after partial hydrolysis of the sample in 5 N NaOH at 121 C for 1 hour and neutralization with 5 N HCl.

b. Gas Liquid Chromatography

GLC was performed on a Perkin-Elmer 900 or 910 Gas Chromatograph fitted with dual columns, with the output connected to a Spectra Physics (Mountain View, California) System IV Autolab integrator.

Most amino acid analyses were performed on the heptafluorobutyryl isobutyl ester derivatives as described by MacKenzie and Tenaschuk (43, Figure 2), others were performed by liquid chromatography (10, Figure 3).

Sugar analyses were performed on the trimethyl silylated methyl glycosides as described by Bhatti et al. (40, Figure 4), or by the alditol acetate method of Albersheim et al. (44, Figure 5).

Glucosamine and N-acetylglucosamine were distinguished by formation of their TMS derivatives in 1:1 mixture of bistrimethylsilyltrifluoroacetamide and pyridine at room temperature for at least 2 hours and then chromatographed on a 12 foot x 1/8" I.D. 3% SP2100 column programmed from 165-200 C at 1.5 C per minute.

Figure 2. Gas liquid chromatography of amino acids as their heptafluorobutyryl isobutyl esters.

One hundred nanomoles of each amino acid plus 50 nanomoles of pipicolic acid, as internal standard, were dried in a 1 ml reactivial (Pierce Chemical Co.) in a vacuum desiccator over P_2O_5 for 3 hours. Two hundred µl of 3N HCl in dry isobutanol were added and the mixture was heated to 121 C for 20 minutes. After the vial had cooled to room temperature the isobutanol was evaporated under a stream of dry nitrogen at room temperature. Twenty-five µl of heptafluorobutyric anhydride and 25 µl of ethyl acetate were then added and the vial was heated to 150 C for 10 minutes. The product was evaporated to dryness after it had cooled to room temperature and dissolved in 25 µl of ethyl acetate. Two µl of this solution were injected onto a 12 foot column packed with 3% SE 30 or 3% SP 2100 on Gas ChromQ held at an initial temperature of 90 C for 4 minutes and heated at 1 C per minute to 240 C.

Histidine does not give a reproducible peak by this procedure. Arginine tends to decay and so, the injection of the sample should be made immediately after the completion of the derivatization.

Identification of peaks:

1) Ala, 2) Gly, 3) Val, 4) Thr, 5) Ser, 6) Leu, 7) Ile, 8) Pro, 9) Pipicolic Acid, 10) Hyp, 11) Met, 12) Asp, 13) Phe, 14) Glu, 15) Lys, 16) Tyr, 17) Arg, 18) His, 19) Cys.

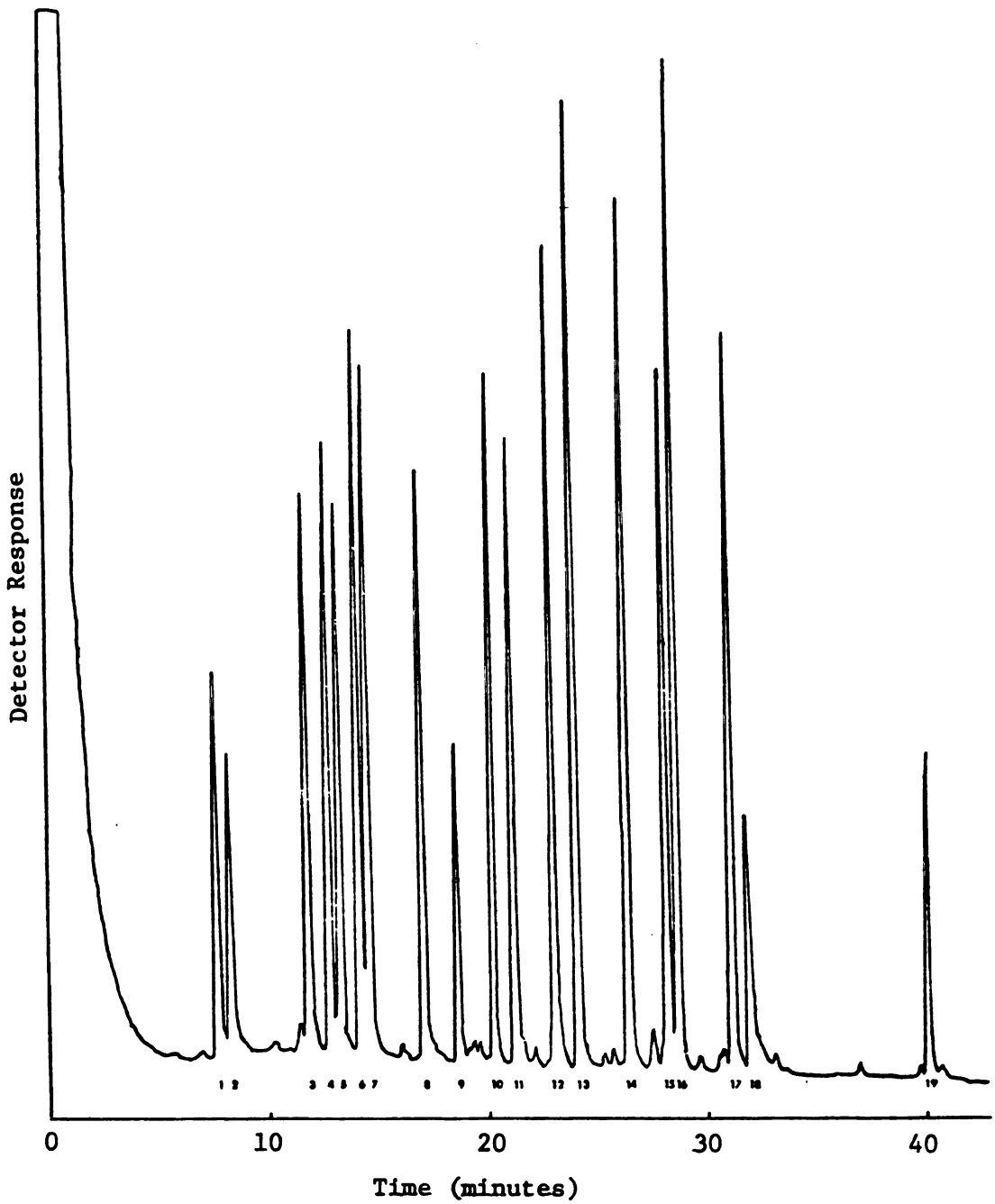

Detector Response
1 2
3 4 5 6 7
8
9
10 11
12 13
14
15 16
17 18
19
0
10
20
30
40
Time (minutes)

Figure 2

Figure 3. Amino acid analysis by liquid chromatography.

Fifty nanomoles of each amino acid, 20 μg of Hyp and 100 nanomoles of norleucine were placed on top of a Chromobeads C column and eluted with a combination pH, ionic strength gradient as described by Lamport (10). The eluent from the column was fed into an automated ninhydrin analyzer and the peak areas calculated by a System IV Autolab integrator. Hyp and Pro were detected by their absorbance at 440 nm, the other amino acids at 570 nm.

Identification of peaks:

1) Hyp, 2) Asp, 3) Thr, 4) Ser, 5) Glu, 6) Pro, 7) Gly, 8) Ala, 9) Val, 10) Cys, 11) Met, 12) Ile, 13) Leu, 14) Norleucine, 15) Tyr, 16) Phe, 17) NH_4^+, 18) Lys, 19) His, 20) Arg.

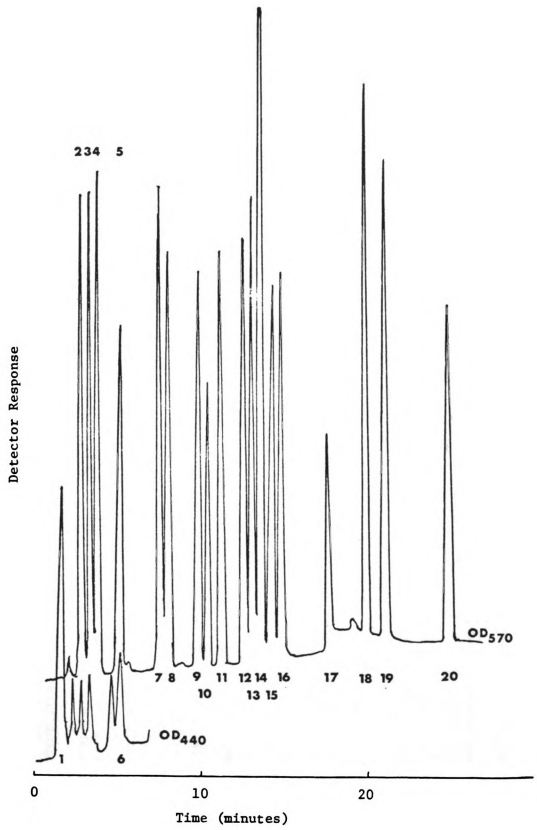

Detector Response
234 5
7 8 9 11 12 14 16 17 18 19 20
10 13 15
1 6
OD570
OD440
0
10
20
Time (minutes)

Figure 3

Figure 4. Gas liquid chromatography of sugars as their trimethylsilyl methyl glycosides.

One hundred nanomoles of each sugar plus 100 nanomoles of mannitol as internal standard were dried in a 1 dram vial (Kimble 60910-L) in a vacuum desiccator over P_2O_5 for at least 3 hours. The paper disk in the cap of the vial was removed and replaced by a Teflon liner (Teflon rubber laminated disk 12412 Pierce Chemical Co.) which had previously been conditioned by heating to 80 C for 1 hour on an empty vial and been tightened while still hot. If this precaution was not taken the vials often leaked leading to concentration of the HCl and caramelization of the sugars. No other vials were consistently free of leaks.

One ml of 1.5 M HCl in dry methanol was added and the vials heated to 80 C for 12 hours. Silver carbonate was added to the vials until no more CO_2 was released and then 100 µl of acetic anhydride added. After 6 hours the silver carbonate/chloride was removed by centrifugation and washed twice with dry methanol. The supernatants were combined and placed in a vacuum desiccator over P_2O_5 for at least 3 hours and then trimethylsilylated by adding 25 µl of a 1:1:5 (v/v) mixture of hexamethyldisilazane-trimethylchlorosilane-pyridine. Half an hour was allowed for the derivatization.

Two µl of the solution was injected on the same column as described for the heptafluorobutyryl derivatives of amino acids, but with an initial temperature of 140 C for 4 minutes followed by a temperature program of 1 C per minute to 215 C. Reproducibility of these derivatives was very good so long as standards were made the same day as the samples. Duplicates were usually within 3-5% of each other. Arabinose gives rise to three peaks as opposed to the two reported (40). The third peak represents ~10% of the total and from its mass spectrum was identified as the trimethylsilyl glycoside of a furanose form of arabinose (45).

Identification of peaks:

1) Ara, 2) Ara, 3) Rha, 4) Fuc, 5) Fuc, 6) Xyl, 7) Xyl, 8) GalU, 9) Man, 10) Gal, 11) Man, 12) Gal, 13) GalU, 14) Gal, 15) Glc, 16) Glc, 17) Mannitol, 18) GlcNAc, 19) GalNAc, 20) Mannitol, 21) GlcNAc, 22) GalNAc, 23) GlcNAc, 24) GlcNAc, 25) NANA.

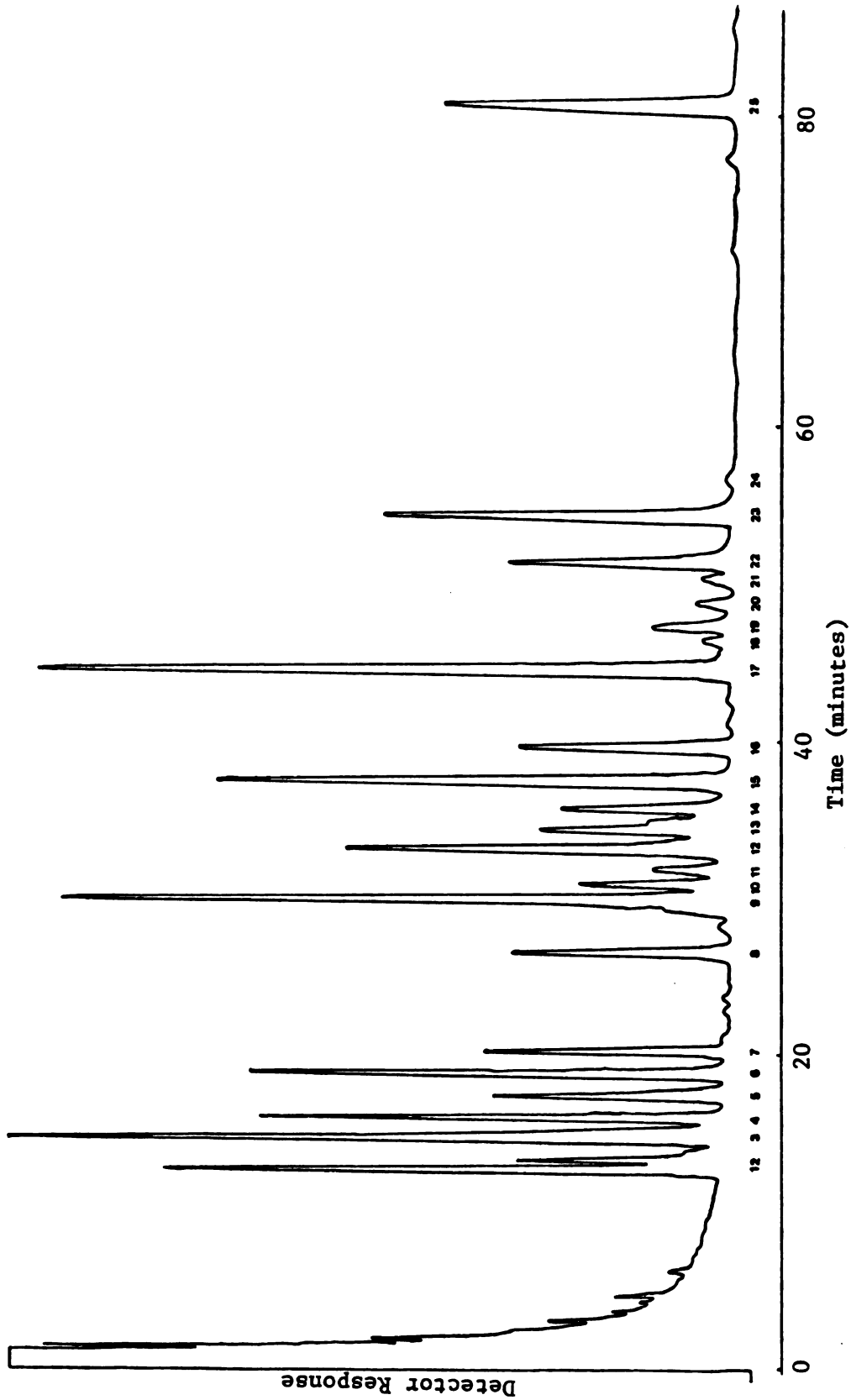


Figure 4

Figure 5. Gas liquid chromatography of sugars as their alditol acetates.

One hundred nanomoles of each sugar was dried in a 1 ml reactivial (Pierce Chemical Co.) under a stream of N_2 at 50 C. Fifty µl of 2 N TFA containing 100 nanomoles of inositol as an internal standard was added and then the mixture was heated at 121 C for 1 hour. The TFA was evaporated, and 50 µl of a 20 mg/ml solution of $NaBH_4$ in 3N NaOH was added. After 1 hour this was neutralized with 2-3 drops of glacial acetic acid and then evaporated to dryness. The excess borate was removed by repeated evaporation of methanol from the sample. The sample was dried in a vacuum desiccator over P_2O_5 for 3 hours and then derivatized by heating to 121 C in 50 µl of acetic anhydride for 1 hour. One to two µl were injected on a column of 0.2% poly(ethylene glycol adipate), 0.2% poly (ethylene glycol succinate) and 0.4% XF-1150 at 140 C for 6 minutes programmed to 180 C at 1 C per minute.

Identification of peaks:

1) Rha, 2) Fuc, 3) Ara, 4) Xyl, 5) Man, 6) Gal, 7) Glc, 8) Inositol.

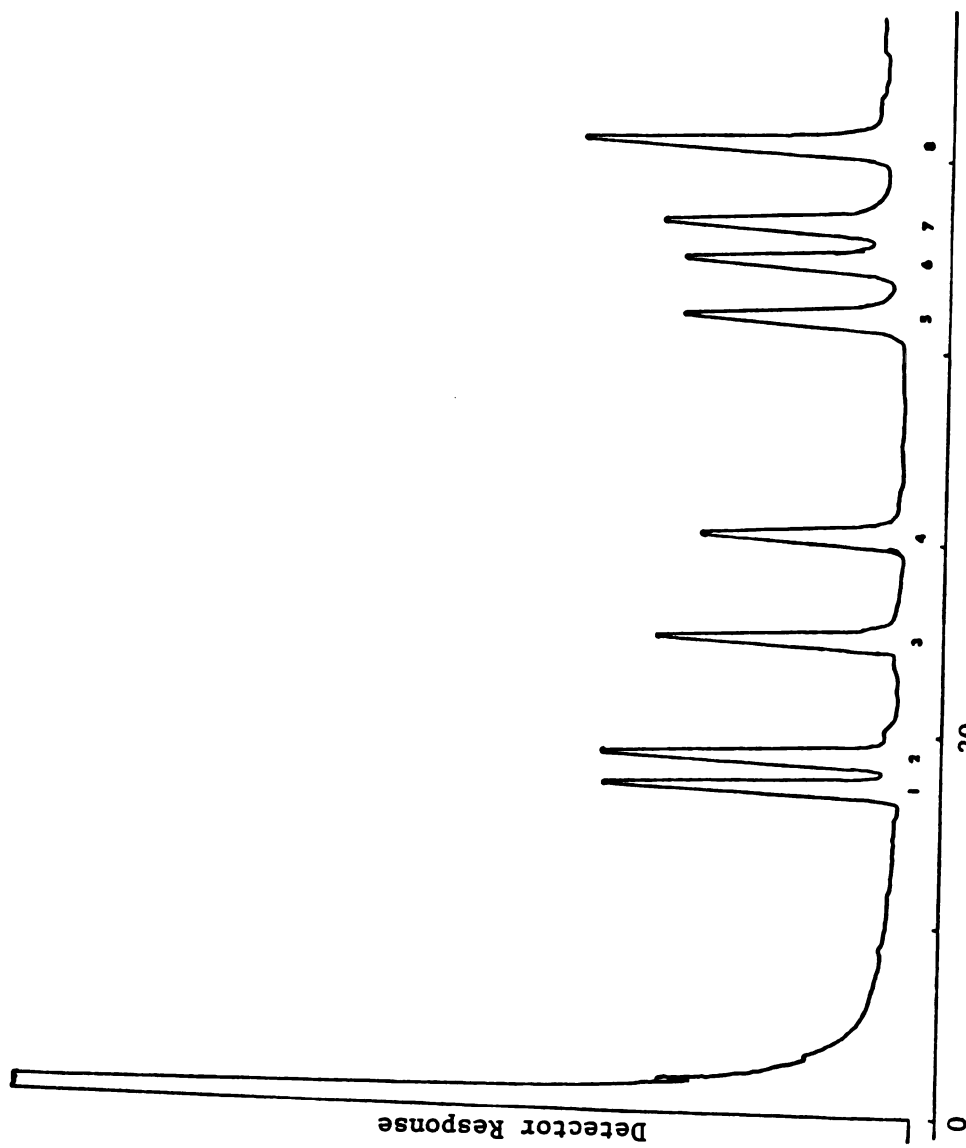


Figure 5

c. Mathematical Calculations

To allow rapid calculation and recalculation of sugar and amino acid compositions I wrote a small FORTRAN IV program for a Hewlett Packard 2100 MX computer. This program is capable of accepting any number of peaks corresponding to a particular amino acid or sugar and applying appropriate correction factors to calculate amount present in the sample by comparison with a standard. The program can also normalize to a particular number of residues of an amino acid to allow easy estimation of an empirical formula for a protein or peptide.

Amounts of polypeptide were calculated from the sum of the number of micrograms of each amino acid present in the hydrolysate of an aliquot of the protein after 18 hours at 110 C in constant boiling HCl. As histidine, cysteine and methionine could not be determined reliably by the gas chromatographic method of MacKenzie and Tenaschuk (43), they were not included, and, consequently the result was not a true polypeptide weight. Amounts of sugar in glycoproteins were determined by summing the number of micrograms of each sugar in an aliquot of the protein after 12 hours of methanolysis as determined by GLC of the TMS sugar derivatives (40). Both the amount of polypeptide and of sugars were corrected to exclude water added to each residue of amino acid or sugar during the hydrolysis.

Carbohydrate contents of the glycoproteins fetuin and PSM are expressed as mg sugar residue per 100 mg polypeptide. Thus,

$$\% \text{ deglycosylation} = 1 - \frac{\text{final mg sugar/100 mg deglycosylated polypeptide}}{\text{initial mg sugar/100 mg untreated polypeptide}}$$

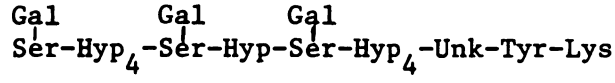
where weights of polypeptide exclude sugar. Note that while sugar contents are often expressed as a weight percent of the total, i.e., sugars + polypeptide, I prefer to use the formula given above because a weight loss of sugars expressed on the basis of polypeptide weight is directly proportional to percent deglycosylation.

C. Results

1. Initial Deglycosylation Experiments

a. Deglycosylation of Glycopeptides from Cell Walls by Periodate and by HBr in TFA

When the cell wall tryptic peptide:



was treated with periodate (0.05M) for 3 hours, half of the galactose residues of this glycopeptide were removed but all of the unknown amino acid had disappeared. The unknown amino acid is present in some of the tryptic peptides described by Lamport (11) and is probably a derivative of tyrosine. It was detected by the appearance of a peak which was broader than normal on the amino acid analyzer. There may have been some conversion of the unknown amino acid into tyrosine as the molar ratio of tyrosine per peptide rose above one.

When this peptide was treated with HBr gas in TFA in the presence of anisole for 3 hours and then chromatographed on a Biogel P2 column it contained only 5% of the original galactose as determined by GLC analysis of its TMS derivative and no bound serine-O-glycoside by the

hydrazinolysis method of Lamport (14). The unknown amino acid and all other amino acids were undegraded. The peptide moved slightly further than the untreated peptide on paper electrophoresis at pH 1.9 as would be expected. No smaller peptides were detected, indicating no peptide bond cleavage.

b. Attempted Solubilization of Intact Extensin by HBr in TFA

When tomato cell walls were treated with HBr in TFA for 3 hours most of the cell wall was soluble in water, but a residue accounting for about 10% of the initial weight of the walls remained which was insoluble in TFA, formic acid, 8 M urea, 2% SDS or water. This residue contained about 98% of the Hyp of the starting material and by amino acid analysis about 40% of its mass was polypeptide. The other 60% has not been identified. Only a trace of sugars was present in the residue as tested by the anthrone assay. When viewed under a microscope the residue had the appearance of very thin cell walls.

c. Deglycosylation of Ovomucoid by HF

Although the structure of the oligosaccharides of ovomucoid are not known and the degree of glycosylation is variable, it is a highly glycosylated protein and does have an easily assayable biological activity:trypsin inhibition. After 1 hour in HF at 0 C the glucosamine content fell by 50-75%, but the treated ovomucoid was still ~80% as effective as the starting material in inhibiting trypsin (Table 1). The elution profile of the treated ovomucoid on a Sephadex G-50 column shows that HF treatment decreased the size of the ovomucoid (Figure 6).

Table 1. The Effect of HF on the Inhibitory Activity of Ovomucoid

Reaction Mixture	Rate (ΔOD/Min)	Percent Inhibition	Concentration of Inhibitor	Activity of Inhibitor
50 µl trypsin[a]	0.380	0.0	-	-
100 µl trypsin + 25 µl native ovomucoid	0.191	75.0	1 mg/ml	100
100 µl trypsin + 25 µl HF treated ovomucoid, fraction 50	0.190	75.0	1.3 mg/ml	77
100 µl trypsin + 25 µl pooled HF treated ovomucoid	0.264	65.0	1 mg/ml	87

[a]Trypsin 0.45 mg/ml.

Note: Trypsin activity was assayed as described by Waheed and Salahuddin (47) using α-N-benzyl-DL-arginine p-nitroanilide as a chromogenic substrate. The enzyme and inhibitor were incubated together in phosphate buffer, pH 7.0, for at least 5 minutes to allow the enzyme inhibitor complex to form. An appropriate aliquot of the mixture was added to 1.0 ml of a 1 mg/ml solution of the chromogenic substrate and the volume of the reaction mixture made up to 2.7 ml with phosphate buffer. The course of the reaction was monitored as a change in optical density at 420 nm using a Gilford 2000 recording spectrophotometer. The inhibitor concentrations used were adjusted to be close to that which would cause 50% inhibition so that the effectiveness of the untreated and treated inhibitor would be linear with concentration.

The assay system described by Waheed and Salahuddin (47) was used rather than the more commonly used one described by Rhodes et al. (48) using p-toluenesulfonylarginine methyl ester because the latter was not linear with respect to time. This non-linearity had been noted previously (49) in studies of trypsin inhibitors and proposed to be due to the displacement of the inhibitor by the substrate. It is likely, therefore, that the Km of α-N-benzyl-DL-arginine p-nitroanilide is higher and so this substrate does not displace the inhibitor.

The inhibitory activities of the material in fraction 50 of the Sephadex G-50 column (see Figure 6) and the pooled peak fractions were determined.

Figure 6. The elution profile of ovomucoid on Sephadex G-50 before and after a 1 hour HF treatment at 0 C.

Approximately 15 mg of ovomucoid, which had been previously separated from a lower molecular weight contaminant by G-50 chromatography, was treated in 10 ml of HF in the presence of 1 ml anisole at 0 C for 1 hour. After the treatment the HF was rapidly evaporated and the anisole was extracted into ether. The product was then chromatographed on Sephadex G-50 and each tube assayed for protein by the method of Lowry (46).

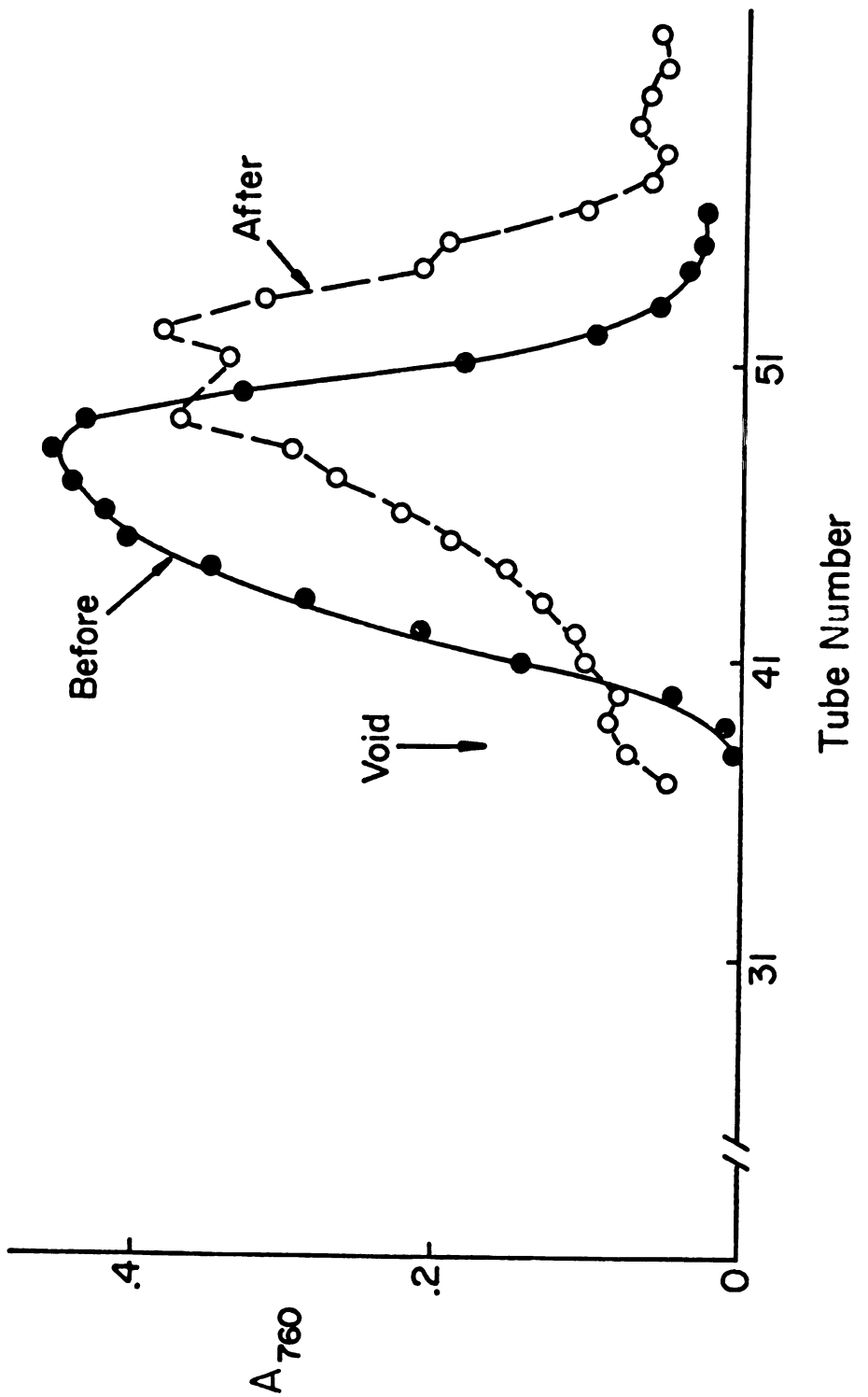


Figure 6

2. Use of HF for the Depolymerization of Polysaccharides for the Quantitative Recovery of Monosaccharides

Some polysaccharides such as cellulose are notoriously difficult to hydrolyze without considerable destruction of the sugar. However, anhydrous hydrogen fluoride readily depolymerizes such polysaccharides at low temperature which raised the possibility of using this as a method to obtain good quantitative sugar analyses of "difficult" polysaccharides. Thus, I compared the sugar recovery after HF solvolysis of (a) larch arabinogalactan and (b) tomato cell walls, with the sugar recovery after TFA hydrolysis only.

a. Arabinogalactan

Larch arabinogalactan (19.7 mg) was dissolved in 1 ml H_2O containing 1 mg/ml mannitol. Aliquots of this solution were hydrolyzed in 2 N TFA and then analyzed as alditol acetates, or methanolyzed and then analyzed as the TMS-O-methyl glycosides.

The remaining solution of arabinogalactan was dried in the HF reaction vessel, treated with HF for 1 hour at 0 C in the absence of scavenger, and then analyzed by the two methods mentioned above. The results in Table 2 show that HF does not destroy sugar residues.

b. Tomato Cell Walls

Plant cell walls are difficult materials to analyze quantitatively. Cellulose is difficult to hydrolyze, polygalacturonic acid is prone to degradation during hydrolysis, protein tends to react with sugars and sugar degradation products, for example via the Maillard (50) reaction. Because of these and other problems, cell wall analyses usually involve

Table 2. Recovery of Sugars from 19.7 mg Larch Arabinogalactan: A Comparison of HF Solvolysis, Methanolysis and 2N TFA Hydrolysis

	2N TFA[a] 1 hour 121 C.		Percent Recovery	Methanolysis[b] 1.5 M HCl in Methanol 8 hours 85 C.		Percent Recovery
	mg Gal	Mg Ara		mg Gal	mg Ara	
No HF Solvolysis	15.3	3.1	93	12.4	2.3	75
Initial HF Solvolysis	16.2	3.5	100	16.3	3.1	98

[a]Analyzed as alditol acetates.

[b]Analyzed as TMS-O-methyl glycosides.

at least four separate assays: (i) cellulose, (ii) uronic acids, (iii) neutral sugars, and (iv) protein.

About 90% of a cell wall preparation obtained from tomato cell suspension cultures was rendered water-soluble by treatment with anhydrous HF for 1 hour at 0 C , in the absence of scavenger. Lamport and I determined the sugar recovery by analyzing the water soluble products as alditol acetates and as TMS-O-methyl glycosides (Table 3). For maximum sugar recovery it was necessary to hydrolyze the water soluble HF solvolysis products in 2 N TFA or to methanolyze them with 1.5 M HCl in methanol. It seems likely that these treatments were required because of the presence of glycosyl fluoride monomers and polymers (Fredenhagen and Cadenbach (22)) stable in water. The 10% of the HF treated cell wall preparation which remained insoluble in water or buffers appeared microscopically as very thin walls, and consisted of protein plus an unidentified component in the proportion of roughly 1:1 (Figure 7).

Using the HF solvolysis we were able to account for 70-75% of the wall as sugars. The protein content was ca. 5%, the amino acid composition of the untreated cell walls was almost the same as that of the insoluble residue which remained after HF treatment (Table 4).

The sugar recoveries from cell walls after HF treatment, apart from the dramatically increased yield of glucose from the solubilization of the cellulose, were slightly lower than those of 2 N TFA hydrolysis alone. Since the recoveries from arabinogalactan were slightly higher after HF treatment I surmised that something in the walls was trapping

Table 3. Sugar Recoveries from Tomato Cell Walls: Comparison of TFA Hydrolysis and HF Solvolysis[a]

	TFA Hydrolysis		HF Solvolysis Followed by TFA Hydrolysis	
	Nanomoles	µg Residues	Nanomoles	µg Residues
Rha	39	5.7	33	5.1
Fuc	-	-	-	-
Ara	144	19.0	102	13.5
Xyl	77	10.2	60	7.9
Man	16	2.6	(16	2.6)[b]
Gal	100	16.2	86	18.9
Glc	33	5.4	350	56.7
Total Neutral Sugars		59.1 g		97.1 µg
Galacturonic		n.d.		47.0 µg
Percent Cell Wall Recovered as Sugars (Weight basis)				72.1%
Percent Cell Wall Recovered as Amino Acids (Weight basis)				4.2%

[a]10 mg cell walls were used for the TFA hydrolysis (2N, 1 hour, 121 C) and 100 mg cell walls for the HF solvolysis (1 hour, 0 C).

After each treatment, the solvent was removed, water was added, and the solution centrifuged. For GC analyses, a volume of the supernatant equivalent to 200 µg cell wall was used. Neutral sugars were analyzed as alditol acetates and galacturonic acid as its TMS-O-methyl glycoside.

[b]Both mannitol and inositol were used as internal standards in this experiment. Therefore, the added mannitol prevented quantitation of mannose by the alditol acetate method.

Figure 7. Cell walls of suspension cultured cells before and after HF treatment.

Aliquots of cell walls (between 20 and 100 mg) were treated in 10 to 20 ml of HF at 0 C in the absence of scavenger for 1 hour. The residue was resuspended in distilled water and washed two times. Microscopically the residue appeared cell wall-shaped. No loss of cell wall shape was observed after suspension of the residue in 90% formic acid, 8 M urea, or 6 M guanidine hydrochloride but the shape was destroyed by trypsin which also solubilized 50% of the Hyp.

A Sycamore cell walls with no treatment.

B Sycamore cell walls after HF treatment.

C Tobacco cell walls with no treatment.

D Tobacco cell walls after HF treatment.

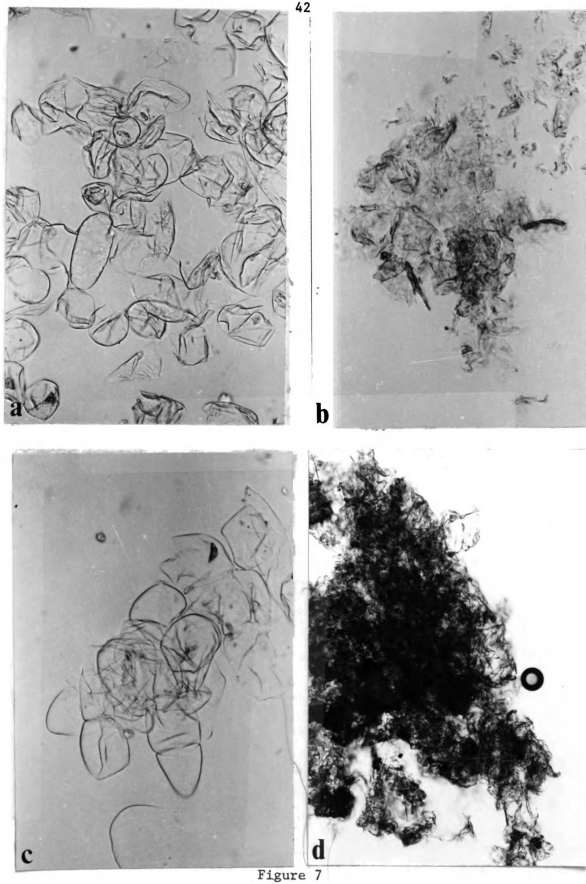


Figure 7

Table 4. Amino Acid Composition[a] of Tomato Cell Walls Before and After HF Treatment

	Before	After
Hyp	30	30
Asp	4.4	3.9
Thr	2.5	2.8
Ser	10.1	9.5
Glu	3.0	3.1
Pro	n.d.	3.6
Gly	3.4	4.0
Ala	2.7	2.6
Val	4.3	4.7
Cys	0	0
Met	0	0.3
Ile	1.5	1.7
Leu	2.7	3.0
Tyr	2.6	2.3
Phe	1.3	1.6
Lys	8.4	8.9
His	1.9	1.7
Arg	1.3	1.3

[a]Normalized to 30 Hyp residues. For experimental details, see Table 3 and Methods.

the sugars in a form from which they could not be recovered as monosaccharides by acid hydrolysis. The amino acid composition of the walls did not show a great loss of aromatic amino acids, as one would expect if the sugars had been alkylating these amino acids by the reaction discussed previously (cf. footnote 1 pg. 19). There remains, however, the unidentified material in the wall residue and quite possibly more of this material in the soluble fraction. If this material were phenolic or easy to alkylate, it could have been trapping the sugars.

To avoid the reoligomerization of sugars in HF reported by Fredenhagen and Cadenbach (22) and perhaps the alkylation of any phenolic or other reactive compounds which might be present in cell walls, I tried including methanol in the reaction mixture with the HF. Lenard (27) had used methanol to prevent inter- or intra-molecular ester formation in his experiments with α-melanocyte stimulating hormone by therefore causing only methyl esters to be formed (c.f. Introduction). I hoped that with a large excess of methanol present, the glycosyl fluorides formed in HF would react only with the methanol.

Sugar compositions of tomato cell walls were determined by treating walls (2-10 mg) in 10 ml of HF in the presence of 1 ml of dry methanol, followed by hydrolysis and derivatization discussed above. Somewhat better yields of sugar, especially pentoses, were obtained from tomato walls so treated (Table 5). The presence of methanol in the HF reaction mixture prevented browning which occurred during the reaction in the absence of methanol.

Table 5. Sugar Recoveries from Tomato Cell Walls: Comparison of Methanolysis and HF Solvolysis in the Presence of Methanol

	Weight Percent of Wall	
	Methanolysis	HF Solvolysis Followed by Methanolysis
Ara	5.99	6.23
Rha	2.33	1.8
Xyl	5.22	7.8
GalU	16.63	18.95
Man	1.05	2.48
Gal	4.82	6.01
Glc	1.97	34.03
	38.2%	77.4%

For methanolysis alone 1.7 mg of tomato cell walls + 500 nanomoles of mannitol were heated in 2 ml 1.5 N HCl in methanol for 18 hours at 80 C and then derivatized as described in the Methods.

For HF treatment 8.9 mg of cell walls + 4000 nanomoles of mannitol were treated with ~10 ml of HF and 1 ml of dry methanol. After complete removal of the HF and methanol the sample was dissolved in water and aliquots taken for methanolysis and subsequent analysis.

c. Chitin

HF at 0 C for 1 hour partially solubilized crab shells, a complex of chitin, calcium carbonate, protein and pigments. The solubilized material contains polymeric N-acetylglucosamine and protein which elute in the void volume of a Biogel P2 column. The more rigorous HF treatment of the crab shells for 3 hours at 23 C (in the absence of scavenger) solubilized nearly all the N-acetylglucosamine, and left a water insoluble residue which was considerably enriched in amino acids. The water soluble material, when chromatographed on a Biogel P2 column (Figure 8) gave three fractions: a small void peak containing protein plus a little N-acetylglucosamine, another small peak containing N-acetylglucosamine together with an unidentified compound, and a large peak containing only monomeric N-acetylglucosamine which, after derivatization, co-chromatographed as TMS-N-acetylglucosamine.

3. Use of HF for Deglycosylation of Glycoproteins and Glycopeptides

Various glycoproteins and glycopeptides were used to characterize the reaction of HF with sugars bound to amino acids. Tomato cell wall glycopeptides were used as examples containing neutral sugar-O-glycosidic linkages to hydroxyamino acids. Although not completely characterized, cell wall peptides provide an excellent tool for the study of glycopeptide linkages as they contain many short sugar chains, thus allowing easy quantitation of the cleavage of glycopeptide linkages. Fetuin and pig submaxillary mucin are examples of glycoproteins containing N- and O-glycopeptide linkages of amino sugars. The extracellular

Figure 8. Elution profile on Biogel P-2 of the fraction of HF treated crab shells which was soluble in water.

Twenty-eight mg of crab shells were treated in HF at room temperature for 3.5 hours. The water soluble fraction was then chromatographed on a Biogel P-2 column. The eluant from the column was assayed by taking an aliquot from each fraction and hydrolyzing it in 5 N NaOH for 1 hour in an autoclave. This treatment removes the N-acetyl group from the amino sugars making the glucosamine ninhydrin reactive. The hydrolyzed aliquots were neutralized with 5 N HCl and then fed into the ninhydrin analyzer for quantitation.

The material in the first two peaks was further analyzed by hydrolysis of an aliquot in constant boiling HCl for 4 hours at 105 C and then derivatization and chromatography as described for gas liquid chromatography of amino acids. Glucosamine gives rise to two peaks between phenylalanine and glutamic acid. The large peak was also analyzed by this method but in addition was directly trimethylsilylated and chromatographed as described in the Methods to show that it contained exclusively monomeric N-acetyl glucosamine.

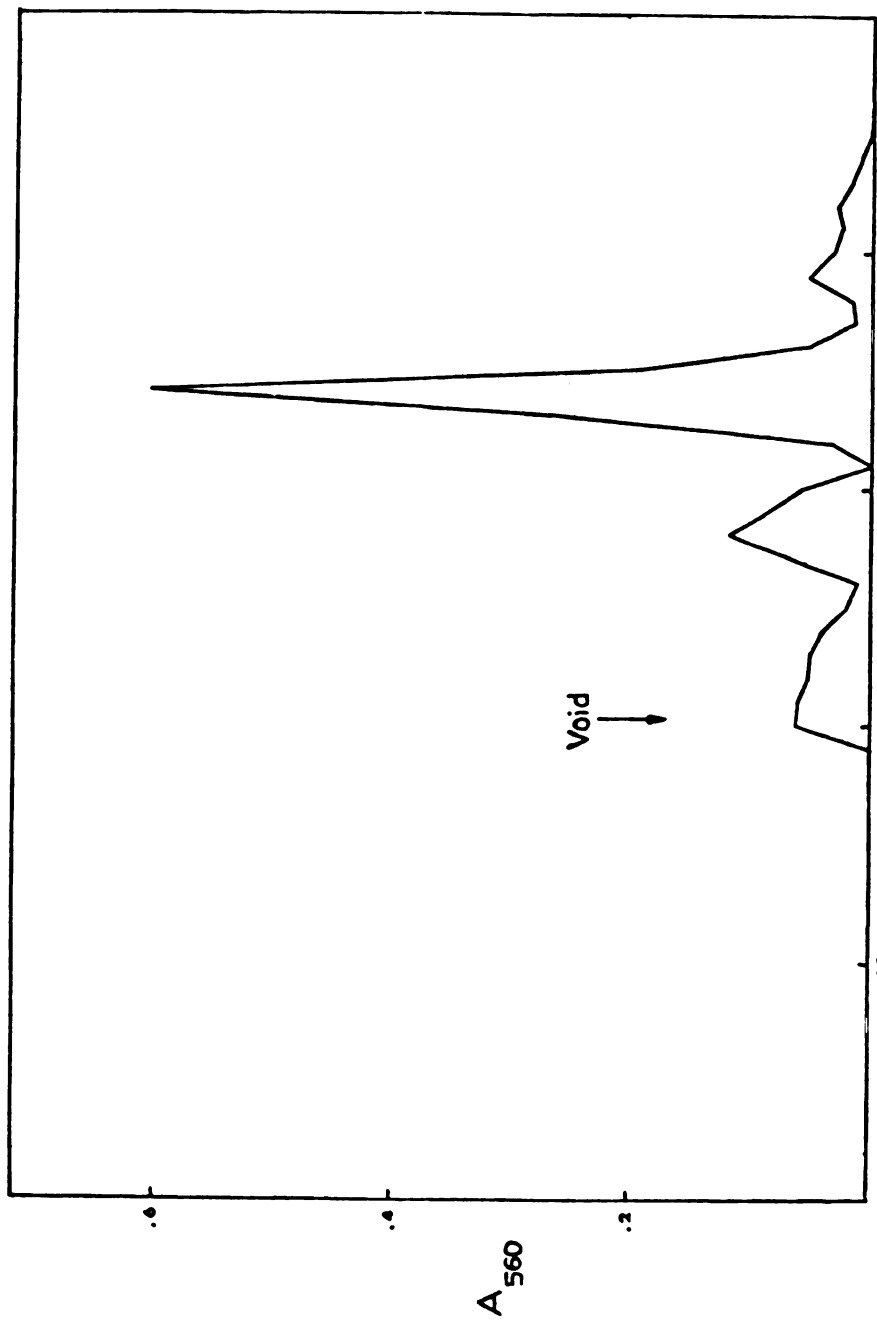


Figure 8

glycoprotein from sycamore-maple suspension cultures is an example of a highly glycosylated protein, which can be easily purified by deglycosylation.

a. Cell Wall Glycopeptides

Because HF cleaves the glycosidic linkages of neutral sugars, I investigated the extent to which HF would deglycosylate some glycopeptides from cell walls of suspension cultured tomato cells. These peptides were released from the walls by oxidation for 30 minutes at 75 C in a solution of 1% acetic acid and 1% sodium chlorite, a slight modification of the procedure described by Mort and Lamport (51). Hyp is over 50 mole percent of the amino acid residues in these peptides, and attached to each hydroxyl group is a short chain of 1 to 4 arabinose residues, predominantly 4, average 3.06 (8). In addition, there are numerous serine residues many of which are attached to a galactose residue (or perhaps polysaccharide) via an O-galactosyl linkage (14).

Treatment with HF for 1 hour at 0 C deglycosylated cell wall glycopeptides obtained by chlorite oxidation (Table 6). The sugars cleaved from these glycopeptides were monomers, judging from their elution in the included position on a Biogel P2 column.

The 2-3% of sugars remaining linked to the peptide were not cleaved possibly because they were in a part of the reaction vial not in contact with the liquid HF. Kel F is very prone to develop a charge of static electricity; because of this it is difficult to ensure that all the sample is in the bottom of the reaction vessel.

Table 6. Sugar Composition[a] of Cell Wall Glycopeptides Before and After HF Solvolysis at 0 C for 1 Hour

	Before	After	Percent Sugar Remaining
Ara/Hyp	5.04	0.14	3
Gal/Ser	4.2	0.09	2
Galacturonic/Ser	7.0	0.04	1.3
Rha/Ser	1.7	0.02	1.2
Glc/Ser	0.84	0.05	6

[a]Data expressed as molar ratios. Sugars were determined as their TMS-O-methyl glycosides, and amino acids as their N-heptafluorobutyryl isobutyl esters.

b. Soluble Extracellular Hyp-Rich Glycoprotein

Suspension cultures of plant cells secrete a mixture of soluble polysaccharides, proteins, and glycoproteins into their growth medium. At least one of these components is a Hyp-rich glycoprotein in which 50% of the Hyp residues are glycosylated with arabinogalactan side chains through an O-galactosyl Hyp linkage (15). Such a highly glycosylated glycoprotein must be deglycosylated before the amino acid sequence can be determined. In addition, deglycosylation allows an appreciable purification of this protein as the effective size of a highly glycosylated protein is drastically reduced after its sugars have been removed. Thus a separation by size before and after HF deglycosylation should select only for those proteins whose size changes drastically. Lamport, Caughey, and Clark partially purified the crude, soluble, Hyp-rich glycoprotein of sycamore-maple cells by ultrafiltration of the culture medium through an Amicon XM100A membrane, followed by desalting on Sephadex G-25 and freeze drying. The bulk of this crude material (both sugars and Hyp) appeared in the void volume of a Sephadex G-100 column. However, after treatment with anhydrous HF (1 hour 0 C) and extraction with 0.1 M acetic acid, soluble Hyp-rich macromolecular material was considerably retarded on a Sephadex G-100 column (Figure 9). The results of amino acid and sugar analyses (Table 7) showed a considerable purification of this HF-deglycosylated material. For example, before deglycosylation there were ca. 120 sugar residues for each Hyp residue; after deglycosylation there were only ca. 3.6. A comparison of amino acid analyses before and after deglycosylation (Table 7) showed a

Figure 9. Gel filtration of a hydroxyproline-rich glycoprotein after HF solvolysis: Sephadex G-100 elution profile.

Eight hundred mg crude glycoprotein were treated with 40 ml HF for 1 hour at 0 C as described in Methods. The residue was dissolved in 20 ml 0.1 M acetic acid and fractionated by two separate applications on a 2.5 x 85 cm Sephadex G-100 column equilibrated with 0.1 M acetic acid. Five hundred microliter aliquots of each fraction were assayed for Hyp. The major peak contained 827 micrograms of Hyp and accounted for 51% of the Hyp (1624 micrograms) in the crude glycoprotein before HF solvolysis. (Data of Lamport, Caughey and Clark.)

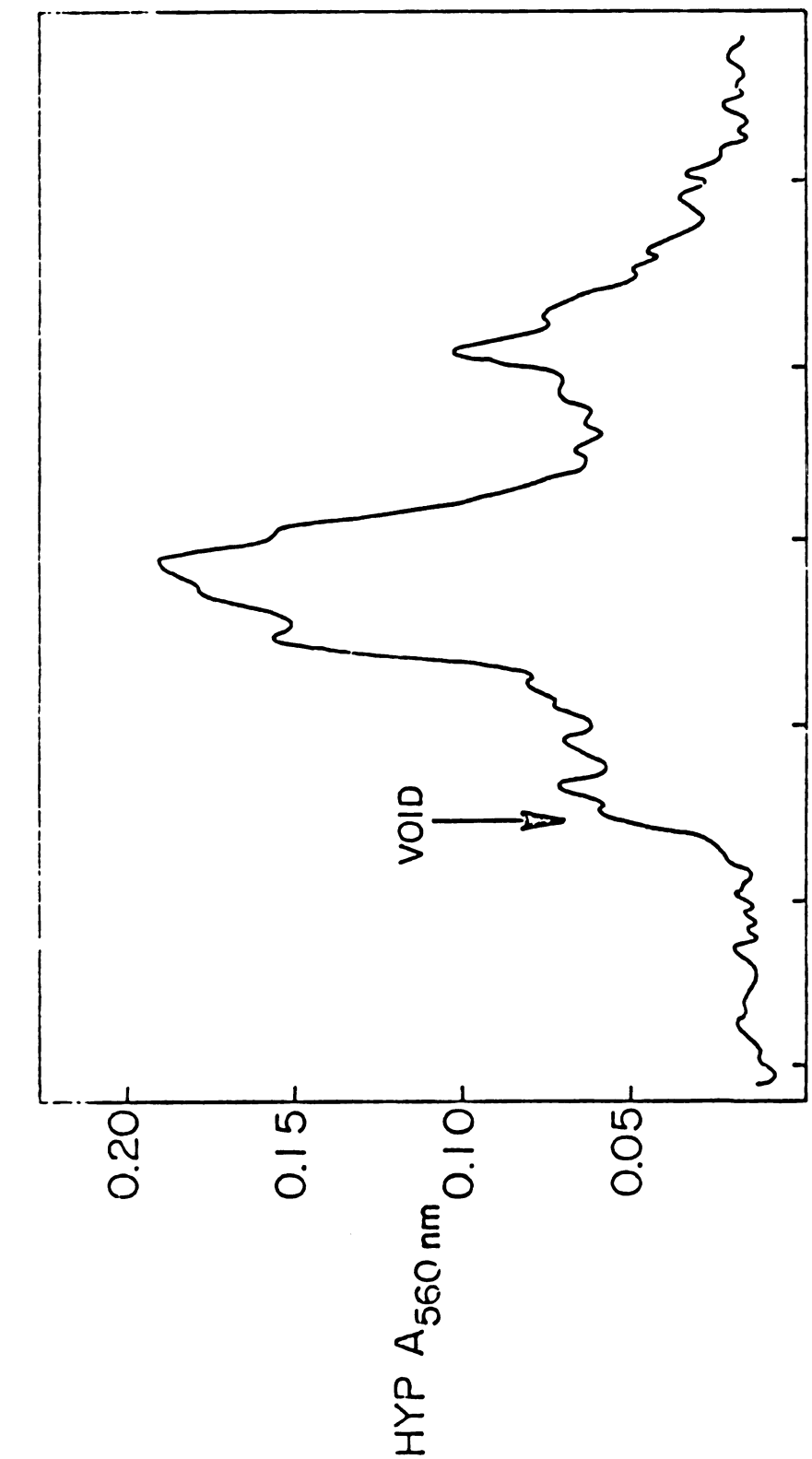

0.20
0.15
0.10
0.05
HYP $A_{560\,nm}$
VOID
1
10
20
30
40
50
FRACTION NUMBER (9.5 ml)

Figure 9

Table 7. HF Deglycosylation of a Soluble Hydroxyproline-Rich Glycoprotein Secreted by Sycamore-Maple Cultures

	Residues per Mole of Hyp	
	Before HF	After HF and Sephadex G-100 Chromatography
Rha	2	0.2
Fuc	1.2	0.1
Ara	16.8	1.4
Xyl	6.7	0.2
Gal	13.2	1.0
Glc	57.1	0.7
GalU	19.4	0
	Mole Percent of Total Amino Acids	
Hyp	6.7	14.2
Asp	4.9	11.3
Thr	8.2	9.0
Ser	9.6	12.3
Glu	9.8	7.5
Pro	n.d.	n.d.
Gly	10.4	7.1
Ala	9.3	12.3
Val	7.6	5.2
Cys	0	0
Met	1.1	0
Ile	4.9	3.3
Leu	7.8	5.2
Tyr	1.1	0.5
Phe	5.1	3.3
Lys	7.1	4.7
His	3.3	2.4
Arg	3.1	1.9

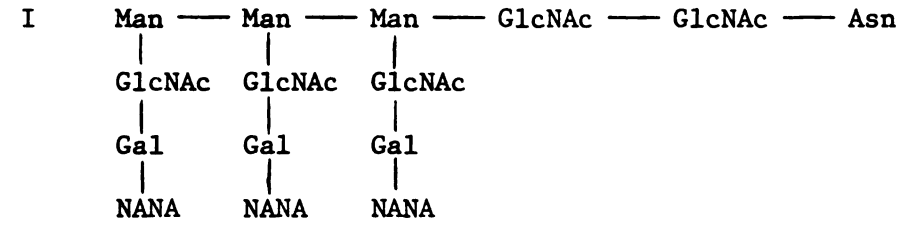
Source: Lamport, Caughey and Clark.

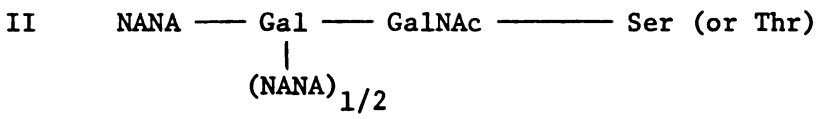
two-fold enrichment of Hyp compared with other amino acids.

c. Fetuin

Having verified that liquid HF cleaves O-glycosidic linkages of neutral and acidic sugars, I turned my attention to amino sugar N- and O-glycosidic linkages, which occur frequently as glycopeptide linkages, and are therefore especially important.

Spiro and co-workers (52) showed that the fetuin molecule contains six oligosaccharides, of which three are N-glycosidically linked (type I) and three O-glycosidically linked (type II).





Treatment of fetuin with 10 ml HF for 1 hour at 0 C with 1 ml anisole as a scavenger removed most of the attached neutral sugar residues (Table 8), but substantial amounts of the N-acetylated hexosamines remained.

The sugar composition of the treated fetuin could be explained if only the neutral sugar linkages were cleaved by the HF. From the structures proposed by Spiro (52), the asparagine linked oligosaccharides would be cleaved to a short disaccharide of two N-acetyl glucosamine

Table 8. Sugar Composition of Fetuin Before and After HF Solvolysis for 1 Hour at 0 C.

	Before HF Solvolysis		After HF Solvolysis		
	mg Sugar / 100 mg Fetuin Peptide	Sugar Residues[a] / Mole Fetuin	mg Sugar / 100 mg Fetuin Peptide	Sugar Residues[b] / Mole Fetuin	Percent Sugar Remaining
NANA	14.4	13	1.2	1.7	8.7
GlcNAc	7.4	15	3.0	6.0	41.0
GalNAc	1.3	3	1.3	2.8	94.0
Man	3.8	9	0	0	0
Gal	4.6	12	0.5	1.4	12

[a]As determined by Spiro and Bhoyroo (53).

[b]Calculated as: percent of the remaining sugar multiplied by its initial number of residues/mole fetuin.

residues and the serine and threonine linked ones to a single N-acetyl-galactosamine. The results shown in Table 8 are all consistent with this hypothesis apart from the retention of 12% of the galactose and 8.7% of the NANA. There are at least two possibilities to explain the retention of these sugars: (1) Some of the threonine or serine linked oligomers are unavailable to the HF for reaction, and (2) some of the sugar fluorides formed during the HF cleavage reattached themselves to the protein rather than to the anisole scavenger. These linkages to the protein could be either glycosidic to serine or threonine hydroxyl groups, glycosidic to the hydroxyl groups of the amino sugars still attached to the protein, or ester links to the carboxyl groups of aspartic or glutamic acid (Lenard found that methanol in HF causes the methyl esterification of aspartic acid (27)).

Harsher conditions (HF for 3 hours at 23 C) resulted in removel of all the N-acetylgalactosamine, leaving only 2-3 residues of N-acetyl-glucosamine per mole of fetuin (Table 9). I conclude that HF at 23 C cleaves GlcNAc-GlcNAc (cf. effect of HF on chitin) and GalNAc-Ser but <u>not</u> the GlcNAc-Asn linkage. Results of amino acid analyses of fetuin were similar both before and after treatment with HF at 0 C followed by dialysis (Table 10). Thus HF did not appreciably degrade or alkylate fetuin.

d. Pig Submaxillary Mucin

Pig submaxillary mucin (PSM) consists of about 60% carbohydrate and 40% protein. The carbohydrate is present as many short oligosaccharides O-glycosidically attached to at least 85% of the threonine and

Table 9. Sugar Composition of Fetuin Before and After HF Solvolysis for 3 Hours at 23 C.

	Before HF Solvolysis		After HF Solvolysis		
	my Sugar / 100 mg Fetuin Peptide	Sugar Residues / Mole Fetuin	mg Sugar / 100 mg Fetuin Peptide	Sugar Residues / Mole Fetuin	Percent Sugar Remaining
NANA	14.4	13	tr.	-	-
GlcNAc	7.4	15	1.2	2.4	16.0
GalNAc	1.3	3	tr.	-	-
Man	3.8	9	tr.	-	-
Gal	4.6	12	0.3	0.9	7.4

Calculations as for Table 8.

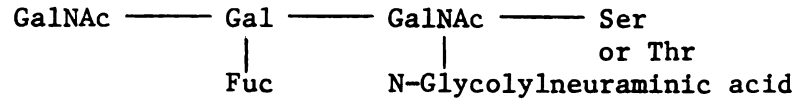
Table 10. Amino Acid Composition of Fetuin Before and After HF Solvolysis for 1 Hour at 0 C.

	Amino Acid Residues[a]	
	Before HF	After HF
Ala	60	61
Gly	44	44
Val[b]	78	68
Thr	37	36
Ser	44	47
Leu	50	44
Ile	23	22
Pro	65	61
Hyp	0	0
Met	0	0
Asp	61	60
Phe	21	21
Glu	69	69
Lys	31	31
Tyr	13	14
Arg	25	25
His	15	18
Cys	0	0

[a]Normalized to 69 Glu residues for comparison with the published analysis (54) and to offset the inflated valine value.

[b]Glucosamine elutes with valine on our amino acid analyzer and therefore gives a high value for valine in the untreated fetuin.

serine residues (55). Carlson (56) characterized a series of PSM oligosaccharides, the largest of the series being:



After treatment of PSM with HF (anisole as scavenger) for 1 hour at 0 C , followed by dialysis of the aqueous extract, only N-acetylgalactosamine remained as a major sugar component attached to the peptide (Table 11). From the work of Carlson (56) we know that the presence (or absence) of terminal N-acetylgalactosamine in the PSM oligosaccharides determines the presence (or absence) of blood group A type activity. Thus PSM pooled from a number of glands contains a mixture of blood group types and one can infer from the data of Payza et al. (55) that about half the PSM from pooled glands contains two GalNAc residues/oligosaccharide while half contains only one GalNAc residue/oligosaccharide; i.e., pooled PSM averages ca. 1.5 GalNAc residues/oligosaccharide. HF treatment (1 hour at 0 C) of pooled PSM actually resulted in the loss of ca. 33% N-acetylgalactosamine (Table 11) which is consistent with the data just mentioned and indicates that in PSM, as in fetuin, HF solvolysis at 0 C cleaves only neutral sugar linkages, leaving both GalNAc-Ser and GalNAc-Thr links intact. But more rigorous HF treatment of PSM for 3 hours at 23 C (anisole scavenger) almost completely stripped the protein of its sugars, only 6% of the original N-acetylgalactosamine remaining (Table 11). After only 2 hours at room temperature in HF, PSM retained 7-10% of its GalNAc. I conclude that

Table 11. Sugar Composition of Pig Submaxillary Mucin Before and After HF Solvolysis

	Before HF Solvolysis	After HF Solvolysis 1 hr. at 0 C	Percent Sugar Remaining After 1 hr. at 0 C	After HF Solvolysis 3 hrs. at 23 C	Percent Sugar Remaining After 3 hrs. at 23 C
	mg Sugar 100 mg PSM Polypeptide	mg Sugar 100 mg PSM Peptide		mg Sugar 100 mg PSM Peptide	
GalNAc	81	54	67	5	6
Gal	32	2.4	7	tr.	0
Fuc	27	0	0	0	0
NGNA	42	0	0	0	0

these more rigorous conditions of HF solvolysis cleave the GalNAc-Ser and GalNAc-Thr linkages in PSM, as in fetuin.

The amino acid composition of PSM did not change appreciably after HF treatment for 3 hours at 23 C followed by dialysis of the aqueous solution (Table 12).

4. Evidence for the Selectivity of HF

a. Gel Electrophoresis of BSA and Fetuin Before and After Treatment

Bovine serum albumin, an unglycosylated protein, was treated in HF, in the presence of anisole, at 0 C for 1 hour and subjected to SDS gel electrophoresis. No new bands appeared in the gels indicating no degradation of the protein (Figure 10).

When treated and untreated fetuin were subjected to electrophoresis in 5.6% gels in SDS and mercaptoethanol, as described by Fairbanks et al. (57), it was clear that the protein was considerably lower in molecular weight after the treatment but that the fetuin remained essentially as a single band on the gel (Figure 11). The band for treated fetuin was sharper than the untreated fetuin perhaps indicating the removal of heterogeneity.

b. Retention of N-Acetyl Groups by N-Acetylglucosamine After HF Treatment

As most hexosaminidases require the amino group of their substrate to be acetylated (58) I thought it important to prove that HF does not deacetylate amino sugars. Thus treatment of free N-acetylglucosamine with HF at 0 C for 1 hour followed by analysis as the TMS derivative

Table 12. Amino Acid Composition of PSM Before and After HF Solvolysis for 3 Hours at 23 C.

	Amino Acid Residues (mole percent)	
	Before HF	After HF
Ala	13	13
Gly	18	18
Val	7	7
Thr	11	11
Ser	18	18
Leu	3	3
Ile	3	3
Pro	6	6
Met	0	0
Asp	4	4
Phe	2	2
Glu	8	6
Lys	3	3
Tyr	1	1
Arg	3	3
His	1	1
Cys	0	0

Figure 10. SDS gel electrophoresis of bovine serum albumin before and after HF treatment.

Bovine serum albumin was treated in HF at 0 C for 1 hour in the presence of anisole. After evaporation of the HF the residue was taken up in 50% acetic acid, dialyzed and then electrophoresed as described by Fairbanks et al. (58).

Left: Untreated

Middle: Treated

Right: Untreated + Treated

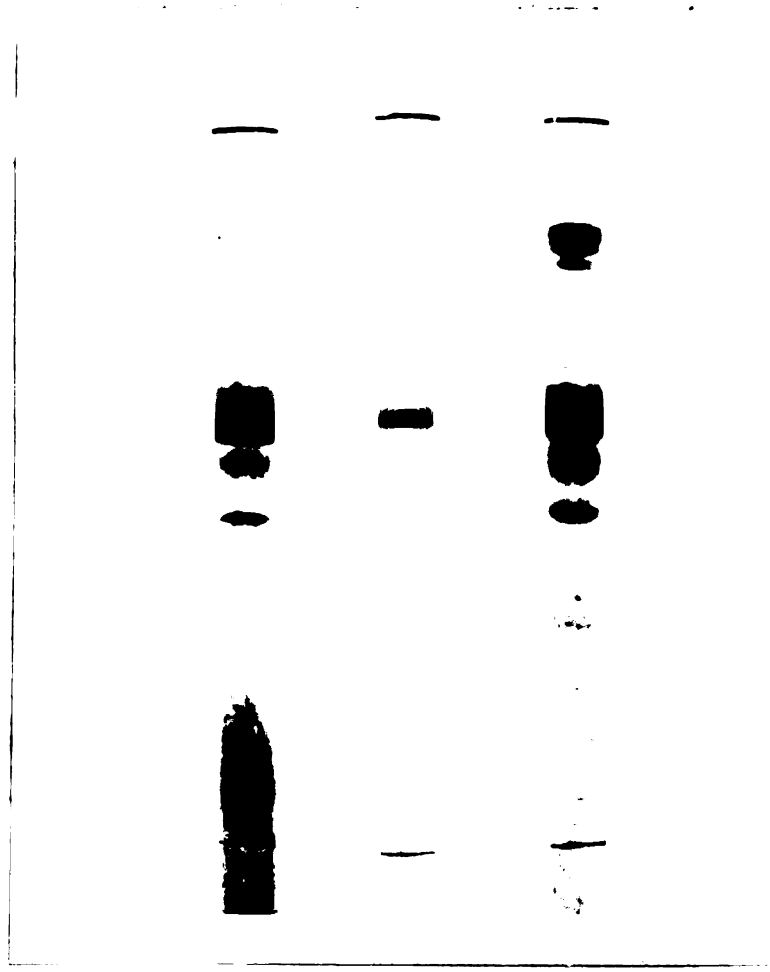


Figure 10

Figure 11. SDS gel electrophoresis of fetuin before and after HF treatment.

Fetuin was treated in HF at 0 C for 1 hour in the presence of anisole. After evaporation of the HF, the residue was dissolved in 50% acetic acid and then dialyzed against distilled water. The fetuin was then electrophoresed as described for bovine serum albumin.

Left: Untreated

Middle: Treated

Right: Untreated + Treated

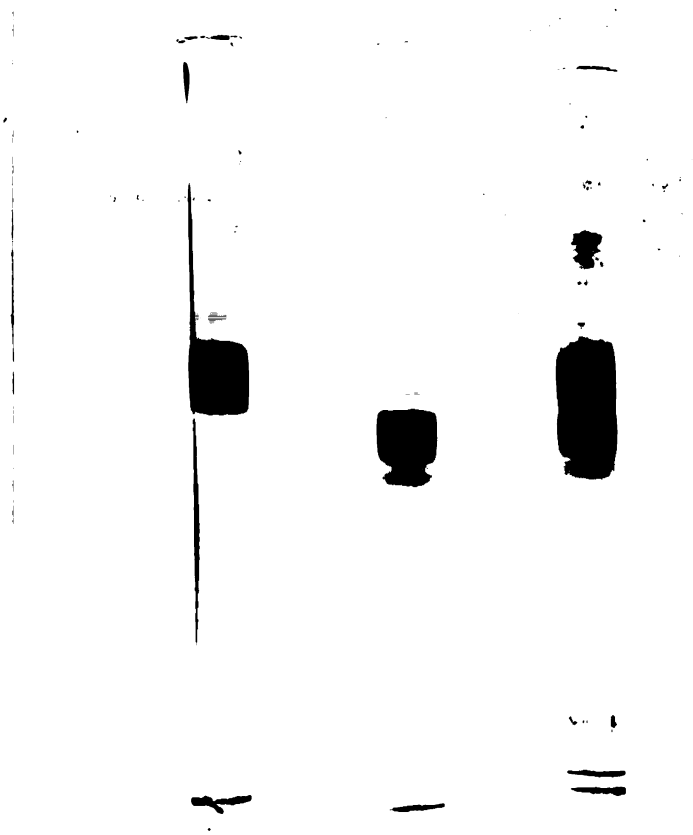


Figure 11

via combined gas chromatography and mass spectroscopy showed only TMS-N-acetylglucosamine (Figure 12).

D. Discussion

Anhydrous hydrogen fluoride is a reagent often used in the chemistry of synthetic peptides because of its selective cleavage properties, but it has not been previously reported as useful for the deglycosylation of glycoproteins. As summarized below, my experiments show that anhydrous HF cleaves the glycosidic linkages of neutral and acidic sugars within 1 hour at 0 C , and the O-glycosidic linkages of amino sugars within 3 hours at 23 C , but leaves intact peptide bonds and N-glycosidic linkages of amino sugars.

1. Glycosidic linkages of neutral and acidic sugars are cleaved in 1 hour at 0 C in HF.

a) The neutral and acidic sugars of plant cell walls were rendered completely water soluble after a 1 hour treatment in HF at 0 C. The sugars after this treatment were also soluble in methanol indicating a very low degree of polymerization.

b) When glycopeptides extracted from cell walls by sodium chlorite oxidation were treated in HF for 1 hour at 0 C the degree of glycosylation was reduced to an amount which could only be explained if most of the Hyp-O-arabinose and Ser-O-galactose linkages to the peptide had been broken.

c) HF treatment of fetuin and pig submaxillary mucin at 0 C for 1 hour removed the mannose and galactose from the proteins. Both of

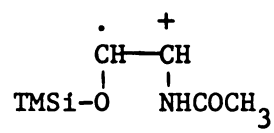
Figure 12. The mass spectra of authentic and HF-treated N-acetyl glucosamine as their trimethylsilyl derivatives.

The trimethylsilylated samples were injected into the LKB-9000 Glc/Mass spectrometer of Dr. C. C. Sweeley (Department of Biochemistry, MSU) and the mass spectra of the appropriate peaks taken. The difference in intensity of some of the ions of the two samples was probably due to slight variations in the conditions of the machine as the spectra were taken three months apart.

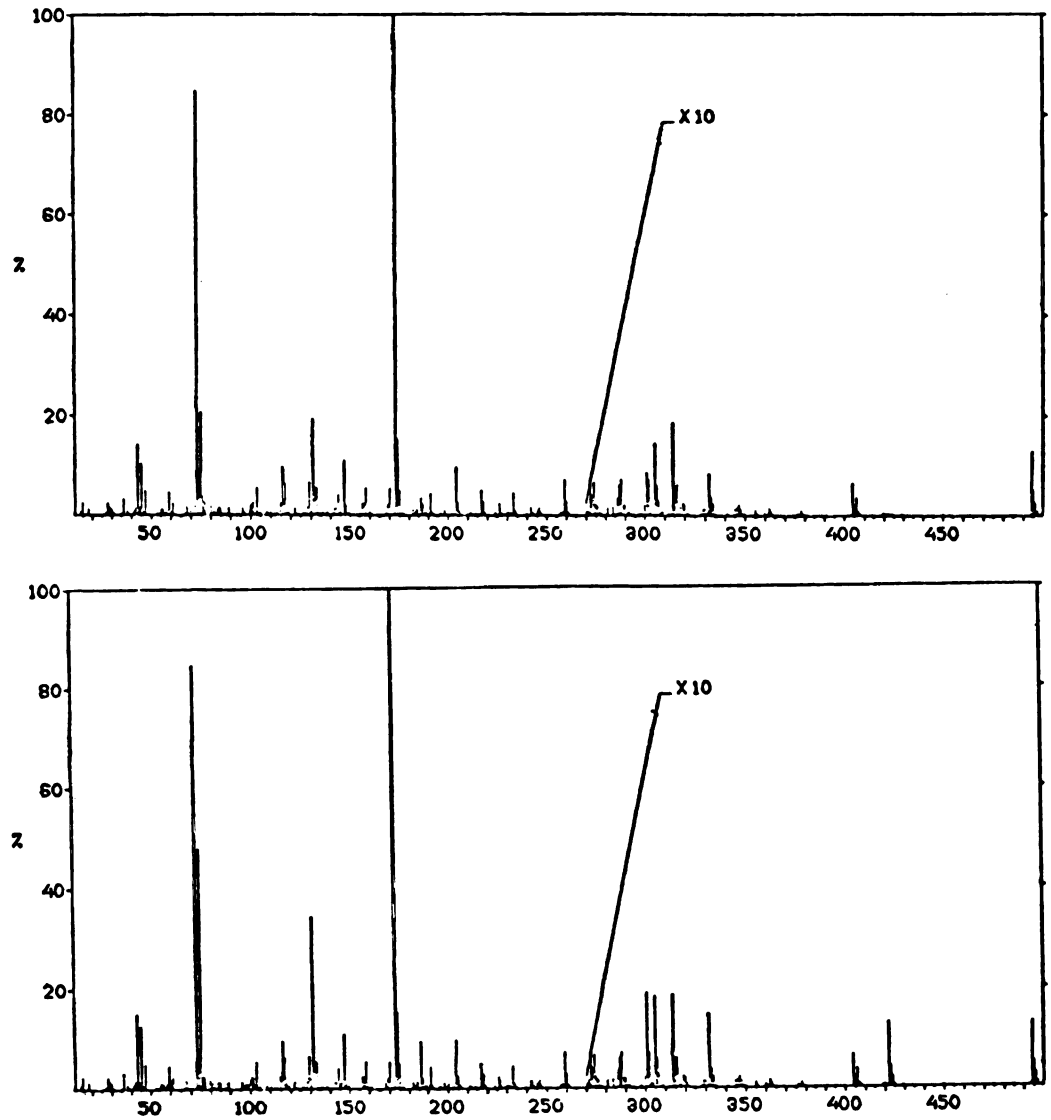
The fragmentation patterns of both samples clearly indicate an acetyl group on the amino group of the sugar and that the two samples are identical.

Both mass spectra, especially that of the HF-treated N-acetyl glucosamine, are very similar to the spectrum published for trimethylsilylated N-acetyl galactosamine (59).

The base peak of the mass spectra is m/e 173, which corresponds to



This structure only forms from N-acetylated hexosamines in the pyranose form. There is no molecular ion but a relatively high abundance of M^+-15 ($^{\cdot}CH_3$)(m/e 494), a peak at m/e 404 corresponding to M^+-Me$\cdot$-TMSiOH, and one at m/e 314, possibly M^+-Me$\cdot$-2TMSiOH. Thus HF had no effect on the structure of N-acetyl glucosamine.


100
80
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X10
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400
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100
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%
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20
X10
50
100
150
200
250
300
350
400
450

Figure 12

these sugars were glycosidically linked to amino sugars which remained on the proteins. Therefore, the glycosidic linkages must have been broken. In the case of pig submaxillary mucin the NGNA was also removed from the protein showing that the glycosidic linkage of NGNA to GalNAc had also been cleaved.

2. O-glycosidic linkages of amino sugars are cleaved in 3 hours at room temperature in HF.

a) The chitin of crab shells remained polymeric after 1 hour in HF at 0 C yet became predominantly monomeric after 3 hours in HF at room temperature.

b) The GalNAc-O-Ser and GalNAc-O-Thr linkages of fetuin and pig submaxillary mucin were cleaved after 3 hours at room temperature in HF but not after 1 hour at 0 C.

3. The N-glycosidic linkage between asparagine and GlcNAc is not cleaved after 3 hours in HF at room temperature.

a) The GlcNAc that remains as part of high molecular weight material after a 3 hour treatment of crab shells in HF at room temperature remains associated with protein.

b) After a 3 hour treatment of fetuin in HF at room temperature there remain 3 moles of GlcNAc per mole of protein, the same as the number of asparaginyl linked oligosaccharides.

Aqueous hydrofluoric acid also has selective cleavage properties, although rather different ones from those of anhydrous HF. For example, 60% aqueous HF at 0 C specifically cleaves the phosphate esters of teichoic acids from Bacillus subtilis, yielding mainly glucosyl glycerol

(60). Table 13 shows that, depending on the reaction conditions, HF shows a surprisingly wide range of specificities for cleaving various linkages in biologically important molecules. Anhydrous HF, in particular, appears to be a highly versatile reagent. We have just begun to explore its usefulness in glycoprotein and carbohydrate chemistry. I list the following applications as worthy of further study:

1. Complete sugar analysis of polysaccharides.

This should be especially useful for those polysaccharides which are difficult to hydrolyze by conventional methods; such as, uronic acid-containing polymers, cellulose, and mixed polymers.

2. Identification of glycopeptide linkages.

a) O-glycosidic linkages such as GalNAc-Ser

For example, after HF treatment of pig submaxillary mucin at 0 C only N-acetylgalactosamine remained peptide bound. The amino acid involved in the glycopeptide linkage could have been identified by β-elimination in the presence of sulfite (53).

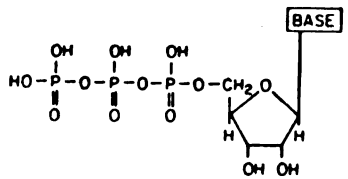
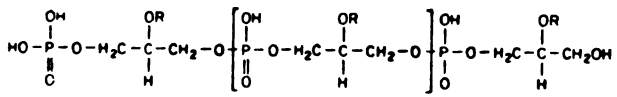
b) N-glycosidic linkages: GlcNAc-Asn.

Because treatment of glycoproteins with HF for 3 hours at 23 C cleaves all except N-glycosidic linkages, HF can be used to confirm the presence of the GlcNAc-Asn linkage.

3. Structural determination of oligosaccharides containing amino sugars.

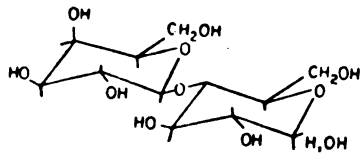
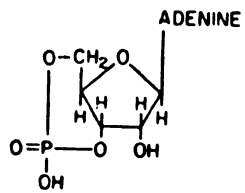
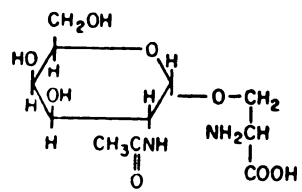
My data show that at 0 C HF cleaves glycosidic linkages of neutral sugars but leaves intact the glycosidic linkages of amino sugars. Thus HF treatment of glycoproteins (in the absence of scavenger) should

Table 13. HF Cleavage of Various Linkages[a] Listed in Order of Increasingly Severe Conditions

Type of Linkage	Example	Conditions: 60% Aqueous HF
Pyrophosphates 	5-Iodo-UTP ⟶ 5-Iodo-UMP (61)	-50 C, 1 hour
Phosphate esters 	5-Iodo-UMP ⟶ 5-Iodouridine (61)	0 C, 1 hour
	Glucosylpolyglycerolphosphate (a teichoic acid) ⟶ glycosyl glycerol (60)	0 C, 3-5 hours
	Phosphatidyl diglucosyl diglyceride ⟶ diglyceride + diglycosyl diglyceride (62)	0 C, 24 hours
	Acyl Carrier Protein -O-P-O pantetheine (16)	2 C, 24 hours

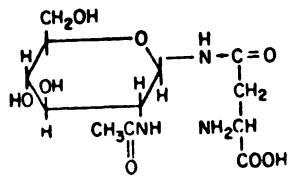
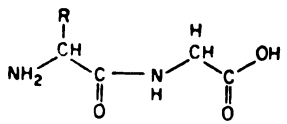
continued

Table 13--Continued

Type of Linkage	Example	Conditions: Anhydrous HF
Neutral sugar glycosides	Arabinogalactan, cellulose (22)	0 C, 1 hour
	Man-O-GlcNAc	
	Hyp-O-ara	
	Ser-O-gal	
Acidic sugar glycosides	Cell wall pectin (rhamnogalacturonan)	0 C, 1 hour
N-glycosides to purine bases	3'5-cyclic AMP ⟶ ribose + adenine	Room temperature short time unspecified
		
Amino sugar O-glycosides	Chitin..(GlcNAc-GlcNAc....)	Room temperature 3 hours
	Serine or Threonine-O-N-acetylglucosamine	

continued

Table 13--Continued

Type of Linkage	Example	Conditions
Amino sugar N-glycosides 	Asparaginyl N-acetylglucosamine	Stable for at least 3 hours at 23 C
Peptide 	Methionyl glycine (29)	Cleaved 36 hours at 23 C

[a]Conditions for cleavage of many of the protecting groups used in chemical peptide synthesis are listed in reference 15.

yield straight chain or branched oligosaccharide fragments with neutral sugars at the reducing terminus and amino sugars at the non-reducing terminus. In conjunction with deaminative cleavage at amino sugars (64) oligosaccharide fragments could be obtained to allow complete sequencing of the short sugar chains.

4. Facile generation of substrates for glycosyl transferases.

Although the N-asparagine linked N-acetylglucosamine cannot be cleaved from glycoproteins under the conditions I have tested, natural substrates for the glycosyl transferases of O-glycosidically linked oligosaccharides can be generated easily.

5. The role of sugars in glycoproteins.

The biological activity of some glycoproteins (65) is absolutely dependent on the sugar components which often seem to act as a recognition code for "topographical location" (66) of the glycoprotein. In other glycoproteins the role of sugar components is less than clear, perhaps because it is more subtle. For example, after HF treatment for 1 hour at 0 C , ovomucoid largely retained its activity as a trypsin inhibitor. This is consistent with the observation (18,20,41) that some nonglycosylated proteins such as lysozyme and RNase retain their biological activity after HF treatment at 0 C , although not after treatment at 30 C.

6. Solubilization of proteoglycan networks.

This was my original interest, because I expected anhydrous HF to render soluble the protein of plant cell walls. However, while HF

solubilized the bulk of the cell wall (ca. 90%), the residue which contained the protein was quite insoluble in various protein solvents such as SDS and urea, indicating the presence of as yet unidentified cross links between the protein molecules of the wall. In the absence of protein-protein cross links, I predict that anhydrous HF will solubilize the cell wall proteins of yeasts, bacteria, etc.

7. As a simple method for purifying the protein component of some glycoproteins.

Microheterogeneity caused by varying degrees of glycosylation contributes to the difficulties inherent in the purification of highly glycosylated glycoproteins, making it especially difficult to establish the presence of one unique peptide species. HF deglycosylation removes the microheterogeneity and reduces the molecular weight of the (glyco) protein. Thus fractionating by size before and after deglycosylation may yield a virtually pure apoprotein (cf. section 3 b.).

8. Assistance in amino acid sequencing of glycoproteins.

The oligosaccharide units of highly glycosylated glycoproteins present two obstacles to sequence determination: first, steric hindrance to proteolytic attack, and second, the absence of an identifiable product after Edman degradation of a glycosylated amino acid residue.

Except for the GlcNAc-Asn linkage, anhydrous HF removes these obstacles. It should now be possible to sequence previously intractable proteins such as the soluble hydroxyproline-rich glycoprotein (15).

Complications may arise because of the influence of the HF on the peptide portion of the protein. HF is a very strong hydrogen bonding

agent and so, may denature the protein, causing it to be less soluble in aqueous solvents, or heterogenous in size. Aimoto and Shimonishi (67) found that after 1 hour at 0 C in HF, lysozyme split into three peaks on a Sephadex G-50 column; the latest eluting being native lysozyme. After 1 hour at 25 C the peak corresponding to native lysozyme had disappeared and the majority of the protein was in the second peak. They suggested that the earliest peak was a dimer. However, when fetuin treated with HF at 0 C for 1 hour was subjected to electrophoresis on a SDS gel after reduction with mercaptoethanol, I found no evidence of dimerization or degradation.

Because of the extreme toxicity of HF and the expense, approximately $3,000 of a Kel F line, I have considered the use of other deprotecting agents. I have obtained very good results with a short cell wall glycopeptide and intact cell walls using HBr gas in anhydrous trifluoroacetic acid as described in (18) but the conditions of the reaction (3 hours at room temperature) with HBr are not conducive to the retention of biological activity.

Other promising reagents are boron tris-trifluoroacetate (68) or trifluoromethane-sulfonic acid (69) in anhydrous trifluoroacetic acid.

I hope in future experiments to investigate the use of these other specific cleavage reagents.

From the data presented here and that of previous workers with HF, I conclude that the use of HF will greatly assist in the determination of the structure of many glycoproteins and polysaccharides.

PART II

SOLUBILIZATION AND CHARACTERIZATION OF CELL WALL PROTEIN GLYCOPEPTIDES BY $NaClO_2$ OXIDATION

A. Introduction

As mentioned in Part I, I was unable to solubilize cell wall protein by HBr in TFA, or HF solvolysis. One of the reasons for this could have been cross linking of the protein via phenolic compounds (70). All my attempts to avoid this possible phenolic cross linking failed. The addition of ascorbic acid or sodium dithionite to keep the phenols which had been oxidized in the hydroguinone form, or the addition of soluble polyvinylpyrrolydone to complex the phenols, made no difference in the subsequent ability of HBr to solubilize cell wall protein. Therefore, I attempted to destroy the phenols selectively after the wall preparation but prior to the HBr TFA solvolysis. The most selective way to destroy phenols chemically is to oxidize them with sodium chlorite. This oxidation bleaches cell walls to a brilliant white and in one experiment allowed their complete dissolution with a combination of $NaClO_2$ oxidation followed by HBr in TFA. When an amino acid analysis of the oxidized walls was performed I realized that a large proportion of the cell wall had been solubilized by the $NaClO_2$ treatment alone, especially the cell wall protein. From the known non-reaction of sodium chlorite with polysaccharides this raised the possibility that $NaClO_2$ oxidation could be used to release glycopeptides

from cell walls under conditions which would not remove or degrade the oligo- or polysaccharides attached to the protein. What follows is a description of my attempts to characterize the glycopeptides solubilized from cell walls by $NaClO_2$ oxidation.

1. The Use of $NaClO_2$ in the Carbohydrate Chemistry of Wood

To allow the extraction of hemicelluloses from wood in good yield one must first degrade a large part of the lignin. The lignin must be removed in a way which does not change the hemicelluloses by derivatization or degradation.

There is no method which removes lignin from wood without some degradation of the polysaccharides. Two methods are commonly used to prepare wood chips or powder for hemicellulose extraction: (1) chlorination of the lignin or (2) oxidation with sodium chlorite or chlorous acid (71). The milder more selective of these two methods is the sodium chlorite oxidation.

Chlorine dioxide oxidation was known as early as 1921 (72) to selectively remove lignin from wood. Schmidt and co-workers (73) also studied the effect of chlorous acid (ClO_2 dissolved in water) on various biological compounds and found that polysaccharides were unreactive, as were most amino acids except the sulfur and aromatic amino acids excluding phenylalanine.

Jayme (74) and Wise et al. (75) have modified the delignification procedure of Schmidt et al. (76) by using sodium chlorite in hot weak acetic acid solution (instead of ClO_2 gas), thus allowing delignification in from 3 to 6 hours as opposed to 14 hours and making the

procedure less cumbersome. Using chlorite oxidation, Wise et al. (75) could account quantitatively for wood even after fractionation into the cellulose and hemicellulose fractions indicating that little degradation and no loss of polysaccharides had resulted from the delignification process.

After 6 hours of chlorite oxidation Timell et al. (77) found the degree of polymerization of cellulose to have fallen to about 1/3 that of the native cellulose but purified cotton cellulose was much more readily degraded by chlorite (78) (as if the hemicelluloses and lignin protect the cellulose from oxidation). The degree of polymerization of hemicelluloses dropped to about 60% of that in untreated wood during the same treatment.

In more recent experiments, Goring and co-workers have investigated the course of delignification of spruce by sodium chlorite oxidation as compared to commercial pulping processes. Ahlgren and Goring (79) found that no polysaccharide was released from the wood until at least 60% delignification was achieved using chlorite but that from the beginning of bisulfite (80), acid sulfite (81) or Kraft delignification (81), there is substantial loss of hemicelluloses. The sugar losses are reflected by the size of the lignin fragments which are solubilized during the process (82) and by the pore size of the wood (determined by solvent exclusion (83)). With chlorite treatment the average molecular weight of solubilized nondialyzable lignin is around 9,000 until at least 60% delignification (84) whereas the molecular weight of Kraft and sulfite solubilized lignin increases from 9,000 at the start of the

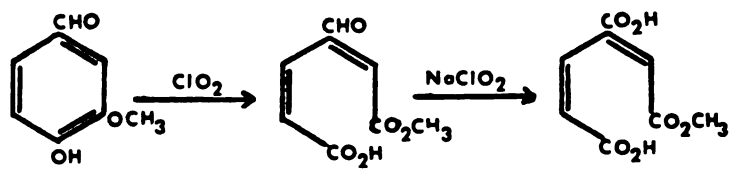
reaction to 25,000 and 75,000 respectively (82). The pore size of chlorite treated wood remains around 30 Å but in Kraft and sulfite treated wood grows to 40 Å and 55 Å respectively.

The mechanism by which sodium chlorite oxidation delignifies wood is not fully understood. During oxidation with sodium chlorite there is generation of chlorine dioxide gas (ClO_2) which becomes hydrated to form chlorous acid (85). It appears to be this generation of chlorous acid which is important in the delignification process as Lindgren and Nilsson (85) found that chlorite oxidation was very specific for the oxidation of aldehydes to acids if a chlorine scavanger was added to the reaction mixture. When the scavenger was effective in removing all the chlorine generated from the chlorite no chlorine dioxide gas was formed. High yields of vanillic acid were obtained from vanillin in the presence of chlorite and the scavenger, sulphamic acid. If the sulphamic acid were not present, as in experiments by Husband et al. (86), the products were difficult to purify and characterize, but there was one identifiable product in a 17% yield, chlorovanillin.

Barton (87) attempted to identify the products of slash pine lignin after chlorite oxidation but could only determine the structure of some of the ether soluble components: fumaric acid, oxalic acid and monochloroacetic acid (perhaps from chlorination of the acetic acid in the reaction mixture). The lignin fragments had varied solubility characteristics. From the absence of a peak around 280 nm in the UV spectrum of the two major ether "insoluble" fractions, Barton suggested

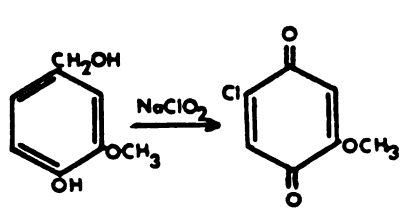
that drastic changes must have taken place in the phenolic nucleus of lignin subunits. Both fractions contained a large proportion of chlorine and oxygen.

Sarkanen et al. (86) used vanillin (I) as a model compound for lignin and found that chlorine dioxide oxidation of this compound resulted in a substantial amount of ring cleavage. The only product identified (a 30% yield) was β-formylmuconic acid monomethyl ester (II). If sodium chlorite was used in place of chlorine dioxide the aldehyde was also oxidized to an acid yielding β-carboxymuconic acid monomethyl ester (III).

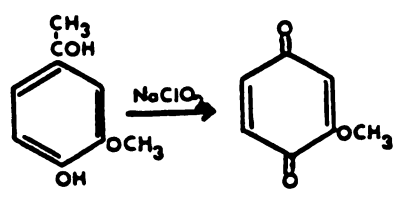


I II III

Using vanillyl IV, α-methyl vanillyl V, and syringyl alcohols, Dence et al. (89) found that chlorine dioxide or chlorite oxidation in addition to the oxidative ring cleavage described by Sarkanen et al. (88) could cause chlorination of the ring and oxidation of phenolic hydroxyl groups to quinones with a concommitant dealkylation at the position para to the hydroxyl.

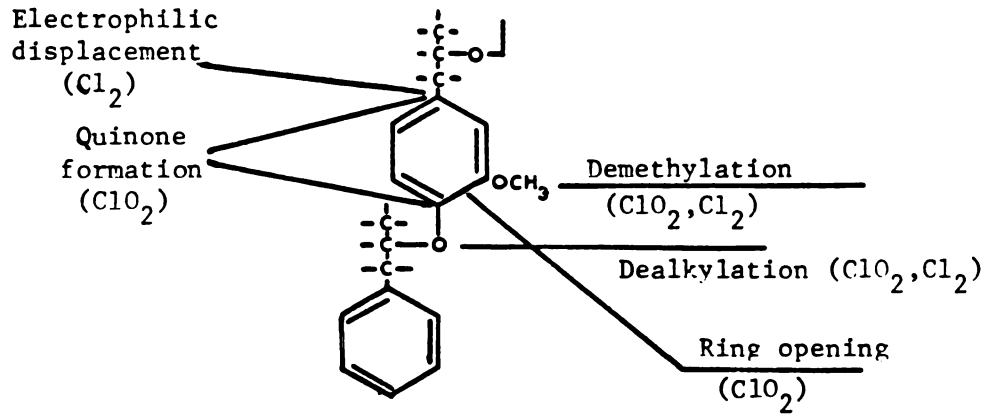


IV



V

From these reactions a mechanism for the delignification of wood was proposed.



2. Other Uses of $NaClO_2$

The ability of chlorite to oxidize aldehydes has been used very successfully but infrequently. Launer and Tomimatsu (90) found that chlorite oxidation could be used to quantitate glucose in the range from 4 to 300 μM allowing detection of 0.6 μgm of glucose by titration of the remaining oxidant. As only the free reducing carbon of a sugar is oxidized, this method could be used to determine the degree of polymerization of polysaccharides. This method is, however, complicated by the need to correct for the spontaneous decomposition of oxidant during the reaction.

Hofreiter et al. (91) have used sodium chlorite to oxidize periodate treated starch to a polyacid. The dialdehydes formed by the periodate oxidation could be quantitatively oxidized to diacids with little or no degradation of the chain as judged by the high viscosity of solutions of the resulting product.

Lindgren and Nilsson (85) have raised the possibility of a much wider use of chlorite oxidation of aldehydes by demonstrating, as mentioned above, that if a scavenger of chlorine is added to the reaction mixture no chlorine dioxide gas is formed. In the absence of ClO_2, chlorite is completely selective for the oxidation of aldehyde groups to acids in compounds as sensitive to oxidation as hydroxylated benzaldehydes.

B. Materials and Methods

1. General Materials and Methods

Most materials were generally commercially available. $NaClO_2$ was purchased from ROC/RIC, 507-509 Main St., Belleville, N. J. 07109.

Cell wall preparation, amino acid analysis, sugar analysis, and determination of Hyp in column effluents were as described in Part I.

Sugars were determined by phenol sulfuric acid assay (92). Uronic acids were determined by the hydroxydiphenyl assay of Blumenkrantz and Asboe-Hansen (93).

The following columns were used, equilibrated in 0.1 N acetic acid:

1. Bio-Gel A .5m, 45 x 1.8 cm
2. Bio-Gel A 1.5m, 43 x 1.8 cm
3. Ultragel AcA 22, 100 x .9 cm
4. Sephadex G-50, 75 x 1.8 cm
5. Sephadex G-50, 105 x .9 cm
6. Bio-Gel P-100, 115 x .9 cm

DEAE cellulose chromatography was performed on an 11 x 1.8 cm column equilibrated with 0.05 M NaAcetate buffer, eluted with a gradient generated by mixing in a 2 chamber gradient maker 75 ml of the same buffer with 75 ml of 0.05 M NaAcetate buffer made 0.5 N in NaCl.

2. Extraction of Pectins with Ammonium Oxalate

A large fraction of the pectin of the walls was removed by boiling for 2 hours in 0.5% ammonium oxalate and then thorough washing with water on a fritted glass funnel followed by drying with acetone washes and desiccation.

3. $NaClO_2$ Treatment of Cell Walls

Cell walls were treated with $NaClO_2$ in the minimum volume possible for ease of handling. Usually 2 to 3 grams of depectinized cell walls were treated in 250 ml of water, 2.5 ml acetic acid, plus 2.5 grams $NaClO_2$ in a 400 ml beaker stirred constantly on a hot plate in a fume hood. The walls were allowed to reach the reaction temperature and become fully hydrated before addition of the $NaClO_2$ initiating the reaction. At the end of the desired time the reaction was terminated by the addition of solid ascorbic acid (care being taken not to allow the reaction mixture to froth out of the beaker) until the yellow color due to dissolved ClO_2 gas had disappeared. To be sure that the $NaClO_2$ had all been reduced, the reaction mixture must have remained white for at least 1 minute. The mixture was filtered on a coarse fritted glass filter funnel and washed until there was no significant reaction of the filtrate in the phenol sulphuric acid assay for sugars. The filtrates

were combined and then dialyzed overnight in large dialysis tubing. It is important to remove the low molecular weight compounds from the cell wall extract as the mixture quite rapidly becomes colored and could contaminate the cell wall polymers. The dialyzed material was then lyophilized.

4. Alkaline β-Elimination

β-Elimination of the serine-O-glycosides was performed by a slight modification of the conditions described by Lamport (94). Between 10 and 20 mg of glycopeptide was suspended in 1 ml of dimethyl sulfoxide mixed with 0.2 ml ethanol and 0.6 ml H_2O and 68 mg $NaBH_4$ in a 5 ml microvial with a vane magnetic stirrer. 200 µl of 2N NaOH were added to make the mixture 0.2 N base. Much of the material then precipitated. The vial was stirred in a 37 C room for 6 hours. The reaction mixture was neutralized with glacial acetic acid, great care being necessary to keep the mixture from bubbling out of the microvial. The resulting suspension was degassed, centrifuged, and the supernatant chromatographed on a Sephadex G-50 column in 0.1 M acetic acid. The precipitate was solubilized in 0.1 M acetic acid and chromatographed separately.

5. CsCl Gradient Centrifugation

CsCl gradients were centrifuged in a Beckman L2 65B ultracentrifuge at 40,000 rpm in a SW65K rotor for 3 days at 4 C to allow equilibrium to be established. One ml of sample plus 2 ml of a CsCl solution of density 1.74 g/ml gave rise to a gradient of density 1.4 to 1.65 g/ml, which spread the chlorite solubilized material over the

bottom half of the tube. Light mineral oil was used to fill the tubes and balance them. The gradients were removed from the tubes either by puncturing the bottom of the tube and collecting 3 drop fractions or by drawing the gradient from the top of the tube with an auto Densi-Flow II (Searle Buchler Instruments, Fort Lee, N. J.). If during removal of the gradient there is contamination of the later fractions by material which failed to leave the tube in the earlier fractions, these two methods of collection should show opposite artifactual effects. The density of every eighth fraction was determined by measuring its refractive index with a Bausch and Lomb Abbe-3L Refractometer.

6. Methylation Analysis

Methylation analyses were performed by a slight modification of the Hakamori method (95). The dimethylsulfinyl sodium was generated as described by Sanford and Conrad (96), but the concentration of dimethylsulfinyl carbanion used (100 µl 2N/ml DMSO) was intermediate between the conditions of Sanford and Conrad (96) and those of Lindberg (97). There seems to be no generally accepted optimum concentration or ionization time for the reaction with the carbanion. In the hopes of obtaining complete methylation I left the polysaccharide in the ionization solution overnight with constant agitation, as did Lindberg and Rosell (98). One hundred to 200 µl of methyl iodide were added in 25 µl aliquots over 1 hour at the end of the ionization period. The mixture was then left an additional 1 hour before diluting 1:10 with a 1:1 chloroform/methanol solution followed by dialysis against distilled water. The dialysate was dried on a rotary evaporator, taken up in chloroform/methanol and

transferred to a Pyrex tube. This was then evaporated and hydrolyzed for 75 minutes at 121 C with 0.5 ml of 2 N TFA containing inositol as an internal reference. The soluble contents were transferred to another tube and dried at 50 C with a stream of nitrogen. The sample was reduced with 250 µl of a 20 mg/ml solution of sodium borohydride in 3 N ammonium hydroxide for 1 hour and the remaining reductant destroyed by adding a few drops of acetic acid. The borate was then removed by repeated evaporation of methanol from the tube. The reduced methylated sugars were acetylated by heating in acetic anhydride at 121 C for 1 hour.

Methylated, acetylated derivatives were tentatively identified by chromatography on 3 different columns:

1. A 6 ft. column of 0.2% poly (ethylene glycol adipate), 0.2% poly (ethylene glycol succinate), and 0.4% XF-1150 programmed at 1 C per minute from 110 to 180 C with an initial hold of 6 minutes (99).
2. A 50 ft. column of 3% OV-225 (SCOT) programmed at 1 C per minute from 165 to 225 C with an initial hold of 4 minutes (99).
3. A 6 ft. column of 0.3% OV-275 and 0.4% XF-1150 programmed at 1 C per minute from 120 to 185 C with a 4 minute hold (100).

Standards used for the identification of the sugar derivatives were: sycamore cell walls (99), xylan (101), arabinogalactan of larch (101), gum arabic (102), cellobiose, and laminarin.

The identities of the methylated sugars were confirmed by combined gas chromatography/mass spectrometry using the facilities of Dr. C. C. Sweeley (Department of Biochemistry, MSU).

C. Results

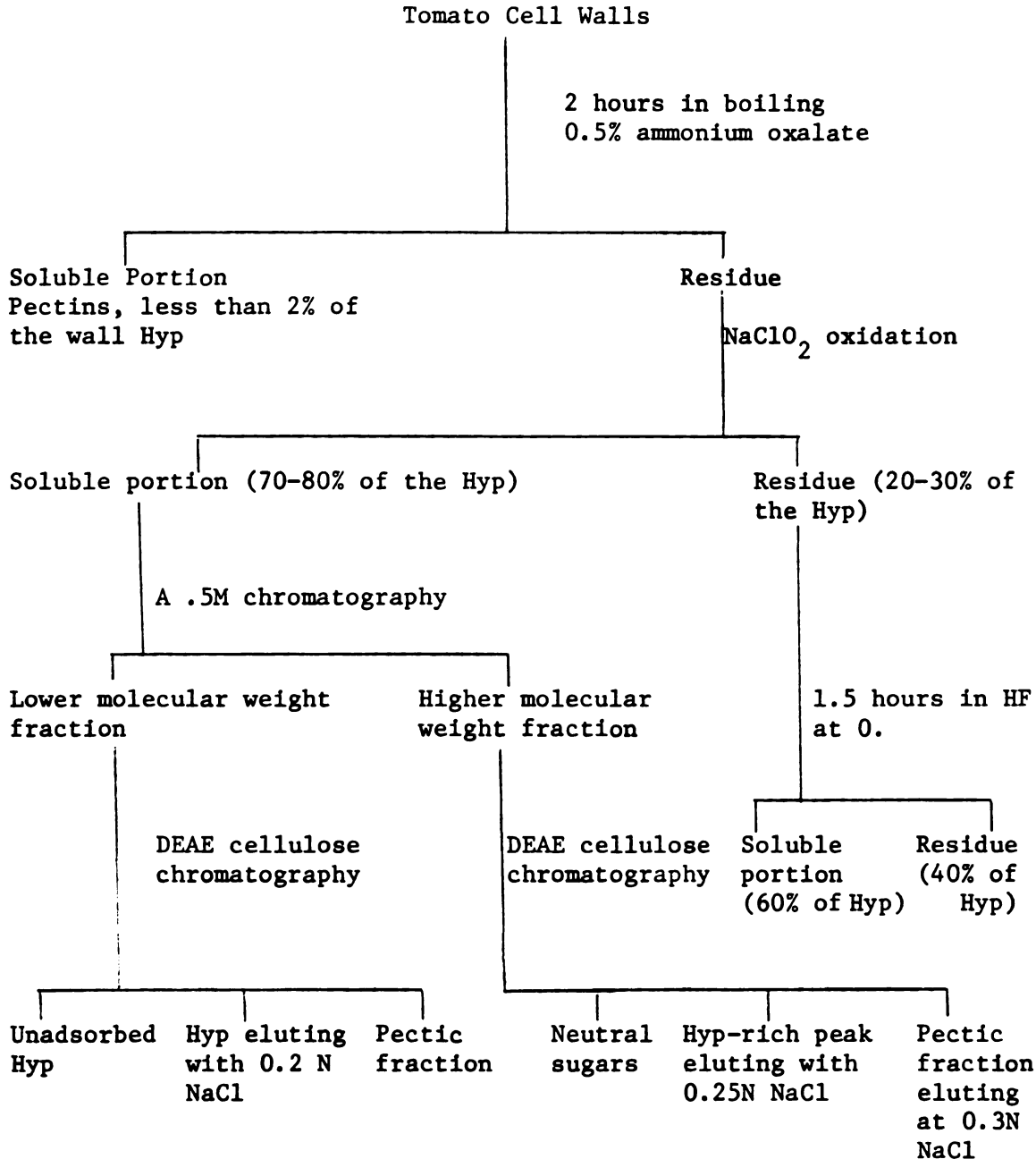
A general outline of my fractionation procedures is provided in Figure 13 to assist the reader in following the experiments of Part II.

1. Solubilization of Hyp-Rich Material from Tomato Cell Walls by $NaClO_2$ Oxidation

In the initial experiments with $NaClO_2$, I was hoping to remove phenols from cell walls to allow total dissolution of walls via a treatment with HBr in TFA, as described in Part I. The amino acid composition and Hyp content of the cell walls after the $NaClO_2$ treatment showed that either the sodium chlorite was destroying the Hyp or solubilizing a large portion of it. When I analyzed the reaction medium and washings from the cell walls I found the missing amino acids, neutral sugars and uronic acids. If the walls were treated under the same conditions (75 C for 30 minutes in 1% acetic acid) but without the $NaClO_2$ some of the uronic acids were solubilized but little or none of the Hyp. Thus, in subsequent experiments, the walls were depectinized by boiling for 2 hours in a 0.5% ammonium oxalate solution and then thoroughly washed to avoid contamination of the glycopeptides released by the $NaClO_2$ with uronic acids solubilized by the hot acetic acid alone.

Boiling in phosphate buffer (pH 7.2) was only half as effective as oxalate in depectinizing the walls as judged by loss of weight from the cell walls. The oxalate solubilized about 40% by weight of the walls, phosphate 20%. As the original experiments were performed as described for the preparation of holocellulose (103) the conditions of $NaClO_2$ oxidation were 70 C for 30 minutes. This treatment solubilizes between

Figure 13. Guide to fractionation procedures.



50 and 75% of the wall Hyp. In later experiments other conditions were tested but with very similar results. The amino acid, and sugar analyses of the cell walls before and after the treatments are given in Tables 14 and 15. These data show that the depectinization treatment removes only proteins which do not contain Hyp but that the $NaClO_2$ treatment solubilizes Hyp containing peptides somewhat selectively.

From the sugar analysis of the untreated and depectinized walls (Table 15) it appears that boiling ammonium oxalate releases similar polymers from tomato cell walls as does the polygalacturonase used by Talmadge et al. (99) in their investigation of sycamore-maple cell walls. The major sugars removed are galacturonic acid and rhamnose, but some arabinose, galactose, and glucose are also solubilized. Thus the cellulose, xyloglucan, and cell wall protein are the polymers which are not extracted to any appreciable extent by ammonium oxalate. The only major change in the sugar composition going from depectinized walls to $NaClO_2$ oxidized walls is the drop in arabinose content, indicating that the chlorite is selectively removing Hyp-ara containing glycopeptides from the walls.

2. Analysis of the Residual Cell Wall

About 60% of the Hyp that remained in the tomato cell walls after $NaClO_2$ oxidation could be rendered water-soluble by a 1.5 hour treatment in HF at 0 C in the absence of anisole scavenger. Sixty percent of the Hyp in the HF solubilized peptides eluted in the void volume of a Sephadex G-50 column (Figure 14). These peptides are very rich in Hyp (40 mole percent) but the ones retarded on the column are much poorer

Table 14. Amino Acid Analysis of Cell Walls After Various Sequential Treatments

	No Treatment		Depectinization		Sodium Chlorite Oxidation	
	Mole percent	Per 30 Hyp	Mole percent	Per 30 Hyp	Mole percent	Per 30 Hyp
Ala	4.0	4.1	3.2	2.8	8.4	42.9
Gly	4.4	4.4	3.7	3.3	11.0	56.3
Val	6.0	6.1	5.7	5.0	6.6	33.6
Thr	3.7	3.8	3.9	3.4	5.7	28.9
Ser	10.4	10.5	10.3	9.0	8.6	44.1
Leu	4.3	4.3	3.7	3.3	9.2	46.7
Ile	2.6	2.7	3.5	3.0	4.4	22.3
Pro	4.6	4.7	4.8	4.2	3.9	19.7
Hyp	29.7	30.0	34.4	30.0	5.9	30.0
Met	.5	.5	.2	.2	.0	.0
Asp	5.3	5.3	4.6	4.8	11.5	58.6
Phe	2.2	2.2	1.7	1.5	4.1	20.7
Glu	4.6	4.7	3.7	3.3	10.5	53.5
Lys	9.9	10.0	9.9	8.7	5.0	25.7
Tyr	3.3	3.3	3.1	2.7	.2	.9
Ada	.0	.0	.0	.0	.0	.0
Arg	2.2	2.2	1.5	1.3	3.3	16.9
His	2.1	2.1	1.8	1.6	1.8	9.1
Cys	.0	.0	.0	.0	.0	.0
Weight percent Hyp		1.34	40% weight loss →	2.28	17% weight loss →	0.45
Weight percent Amino Acids		4.46		6.4		7.0

Amino acid analyses were determined by amino acid analyzer after 18 hour hydrolysis of an aliquot of walls in constant boiling HCl. The analyses were normalized to 30 Hyp for comparison to those of Lamport (13).

Table 15. Sugar Composition of Tomato Cell Walls After Various Sequential Treatments

	No Treatment	Depectinization	Sodium Chlorite Oxidation
		Mole Percent of Each Sugar Present	
Ara	10.2	10.9	3.6
Rha	3.3	1.3	2.6
Fuc	.0	.1	.0
Xyl	7.9	12.7	11.5
GalU	23.8	5.0	10.9
Man	4.0	4.5	5.0
Gal	8.5	8.0	6.6
Glc	40.7	56.8	59.6
Weight percent of the wall recovered as sugars:			
	81%	60%	84%

Sugar compositions were determined by total dissolution of the wall polysaccharides in HF containing 10% methanol followed by methanolysis and trimethylsilylation as described in Part I, Section C, 2b. Different wall preparations were used for each analysis.

Figure 14. Sephadex G-50 chromatography of the soluble portion of the HF treated residue of $NaClO_2$ treated tomato cell walls.

155 mg of $NaClO_2$ extracted cell walls were treated in approximately 20 ml of HF (no methanol or anisole scavenger) for 1.5 hours at 0 C. After evaporation of the HF the residue was suspended in 15 ml distilled water and the insoluble portion removed by centrifugation. The insoluble portion was washed with water and then lyophilized. Only 12.5 mg (8% of the starting material) remained insoluble. After lyophilization the soluble portion was chromatographed on Sephadex G-50. The effluent from the column was assayed for Hyp.

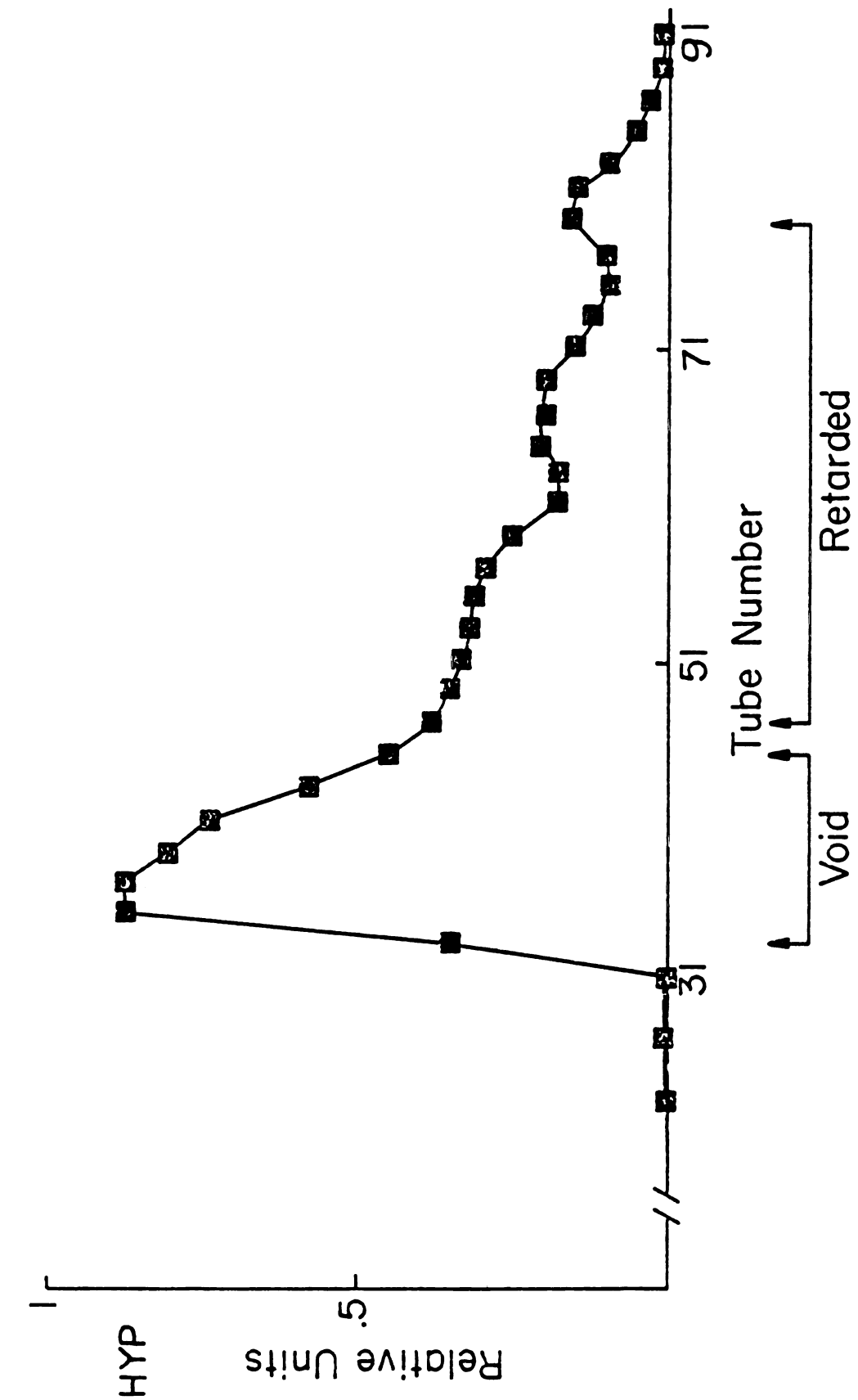


Figure 14

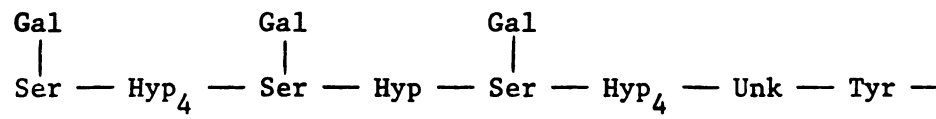
in Hyp, as is the residue wall (Table 16). The insoluble fraction of the walls after HF treatment, as with the HF treated native walls, is only 40% protein again indicating the presence of some as yet unidentified material.

3. Changes in the Peptide Portion of Proteins During $NaClO_2$ Oxidation

In a preliminary investigation of the effects of $NaClO_2$ on proteins, I treated a well-characterized cell wall glycopeptide and chicken egg white lysozyme, one of the best characterized proteins available.

a. A Model Cell Wall Glycopeptide

When a tryptic peptide from tomato cell walls isolated by Katona in this laboratory, as described by Lamport (11), with the sequence



was treated with $NaClO_2$ at 75 C for 30 minutes, the tyrosine and unknown amino acid were destroyed with the appearance of about 0.6 equivalent of aspartic acid (c.f. effects of $NaClO_2$ on lignin model compounds (87)). No destruction of serine or hydroxyproline was apparent. Fifty percent of the lysine was lost with about a 25% conversion to a new amino acid. This amino acid was identified as α-amino adipic acid as it co-chromatographed with authentic α-amino adipic acid on both the amino acid analyzer and on gas chromatography as its heptafluorobutyryl ester.

Table 16. Amino Acid Analysis of Fractions Obtained by HF Treatment from the Cell Wall Residue after $NaClO_2$ Oxidation

	Void of G-50		Retarded on G-50		Insoluble Residue	
	Mole percent	Per 30 Hyp	Mole percent	Per 30 Hyp	Mole percent	Per 30 Hyp
Ala	.8	.5	5.1	12.8	9.4	47.4
Gly	1.2	.8	11.3	28.2	9.1	46.1
Val	3.4	2.4	5.5	13.6	6.8	34.3
Thr	3.2	2.3	4.3	10.9	5.1	25.6
Ser	13.8	9.7	10.4	25.9	7.9	40.0
Leu	2.8	2.0	6.1	15.2	9.3	46.8
Ile	1.3	.9	12.2	30.5	5.0	25.3
Pro	3.8	2.7	3.6	9.0	4.9	24.8
Hyp	42.6	30.0	12.0	30.0	5.6	30.0
Met	.0	.0	.9	2.3	.0	.0
Asp	5.3	3.7	7.1	17.8	8.9	44.8
Phe	1.5	1.1	2.4	5.9	4.8	24.5
Glu	4.5	3.2	6.9	17.2	9.7	49.0
Lys	8.1	5.7	4.6	11.4	3.9	19.8
Tyr	.5	.4	.9	2.3	.8	4.3
Ada	4.0	2.8	3.2	7.9	3.4	17.4
Arg	3.1	2.1	1.6	3.9	3.6	18.3
His	.0	.0	.0	.0	1.2	6.2
Cys	.0	.0	.0	.0	.0	.0

Amino Acid analyses determined by Glc.

b. Lysozyme

To determine whether $NaClO_2$ oxidation degrades protein, I treated chicken egg white lysozyme with $NaClO_2$ at 75 C for 30 minutes and then chromatographed it on a Sephadex G-50 column. During the reaction the protein first precipitated and the solution turned yellow from the ClO_2 gas. The protein redissolved in about 20 minutes. After the addition of a few milligrams of ascorbic acid the solution went colorless and almost clear. The elution profile of the treated and untreated lysozyme is shown in Figure 15. This shows that the protein was degraded somewhat but not into small pieces. More experiments would be needed to determine if $NaClO_2$ oxidation breaks specific peptide bonds. But as $NaClO_2$ does seem to break some peptide bonds the mechanism by which the $NaClO_2$ is releasing the protein from the cell walls may be by peptide bond breakage, breakage of phenolic cross links or both. It seems unlikely that the peptide of cell walls is breaking at all the tyrosine residues since there are many tyrosine residues in the tryptic peptides characterized by Lamport (11). This would cause some of the peptides released by $NaClO_2$ to be very short, but, as will be described later, they appear to be large.

c. Comparison of the Effects of $NaClO_2$ and N-Bromosuccinimide

Another chemical reagent specific for aromatic compounds, N-bromosuccinimide (104), also released cell wall glycopeptides.

A treatment of sycamore maple cell walls with 50 mM N-bromosuccinimide in 50% acetic acid for 20 hours at room temperature solubilized 50% of the Hyp (compared with 50-75% release in $NaClO_2$ as mentioned

Figure 15. Sephadex G-50 elution profile of $NaClO_2$ treated lysozyme.

A small aliquot of lysozyme was treated with $NaClO_2$ in 1% acetic acid at 75 C for 30 minutes. The reaction was terminated by the addition of ascorbic acid until the yellow color of ClO_2 was no longer present. The product was chromatographed on a Sephadex G-50 column. Protein was determined by the method of Lowry *et al*. (46). The elution profile of untreated lysozyme is superimposed to show the effect of $NaClO_2$.

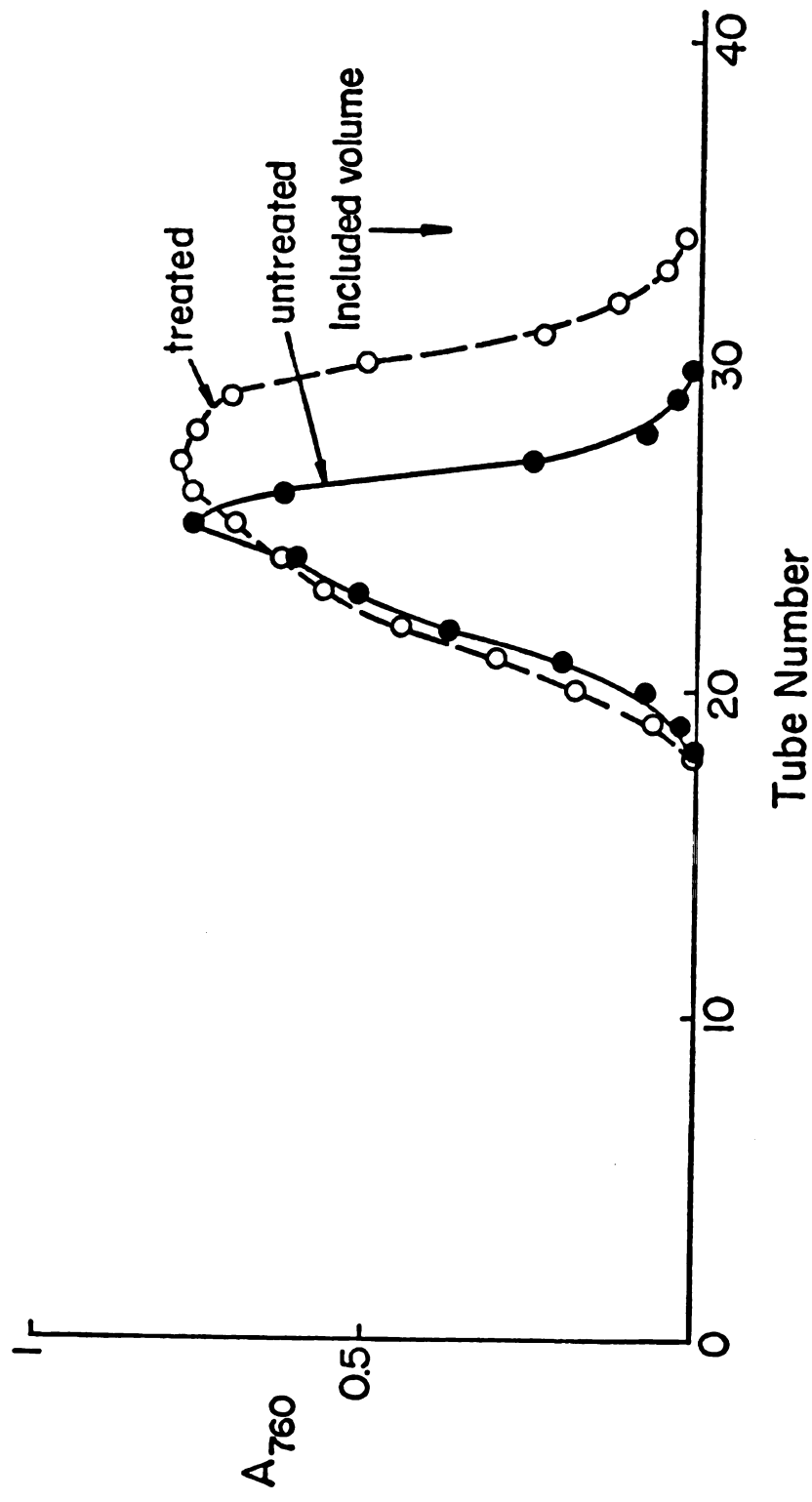

treated
untreated
Included volume
A_{760}
1
0.5
0
10
20
30
40
Tube Number

Figure 15

earlier). This reagent may be releasing the peptides solely by breakage of tyrosyl and histidyl bonds but may also be breaking the phenolic crosslinks that I have postulated. A study of these peptides may be undertaken fruitfully in the future.

4. Fractionation of the $NaClO_2$ Released Peptides by Size

a. Chromatography on Bio-Gel A .5 m and A 1.5 m

The material solubilized by $NaClO_2$ oxidation from tomato cell walls can be divided into two major fractions by chromatography on a Bio-Gel A .5 M or A 1.5 m column as shown in Figure 16. These columns exclude spherical molecules of molecular weight 500,000 and 1,500,000 respectively. The majority of the uronic acids elute near the void volume on either of these columns, but in some experiments, especially the ones in which the $NaClO_2$ treatment was short, uronic acids appeared throughout the column effluent. The ratio of void to retarded Hyp peaks varied from one experiment to another, perhaps due to the variation in cell wall cross linking via phenols. Some preparations of cell walls were whiter than others. There was also variation in the loss of lysine during the $NaClO_2$ treatment. This also appeared to be connected to the color of the walls. The browner the walls the less the loss of lysine, as though the phenols competitively protected the amino group from oxidation.

The material in the void volume is rich in Hyp, arabinose, and galacturonic acid (see Table 17); whereas, the retarded material is less enriched in Hyp and almost devoid of galacturonic acid.

Figure 16. Chromatography of total $NaClO_2$ extracted glycopeptides on Bio-Gel A .5 m.

Three grams of washed tomato cell walls were extracted with boiling ammonium oxalate solution, washed with water, and dried with acetone. The depectinized walls were then treated with $NaClO_2$ at 75 C for 45 minutes. The extract was reduced with ascorbic acid, dialyzed, and lyophylized. About one-third of the extract was applied to the column as in concentrated solution the glycopeptides were very viscous. Each fraction was analyzed for the presence of Hyp.

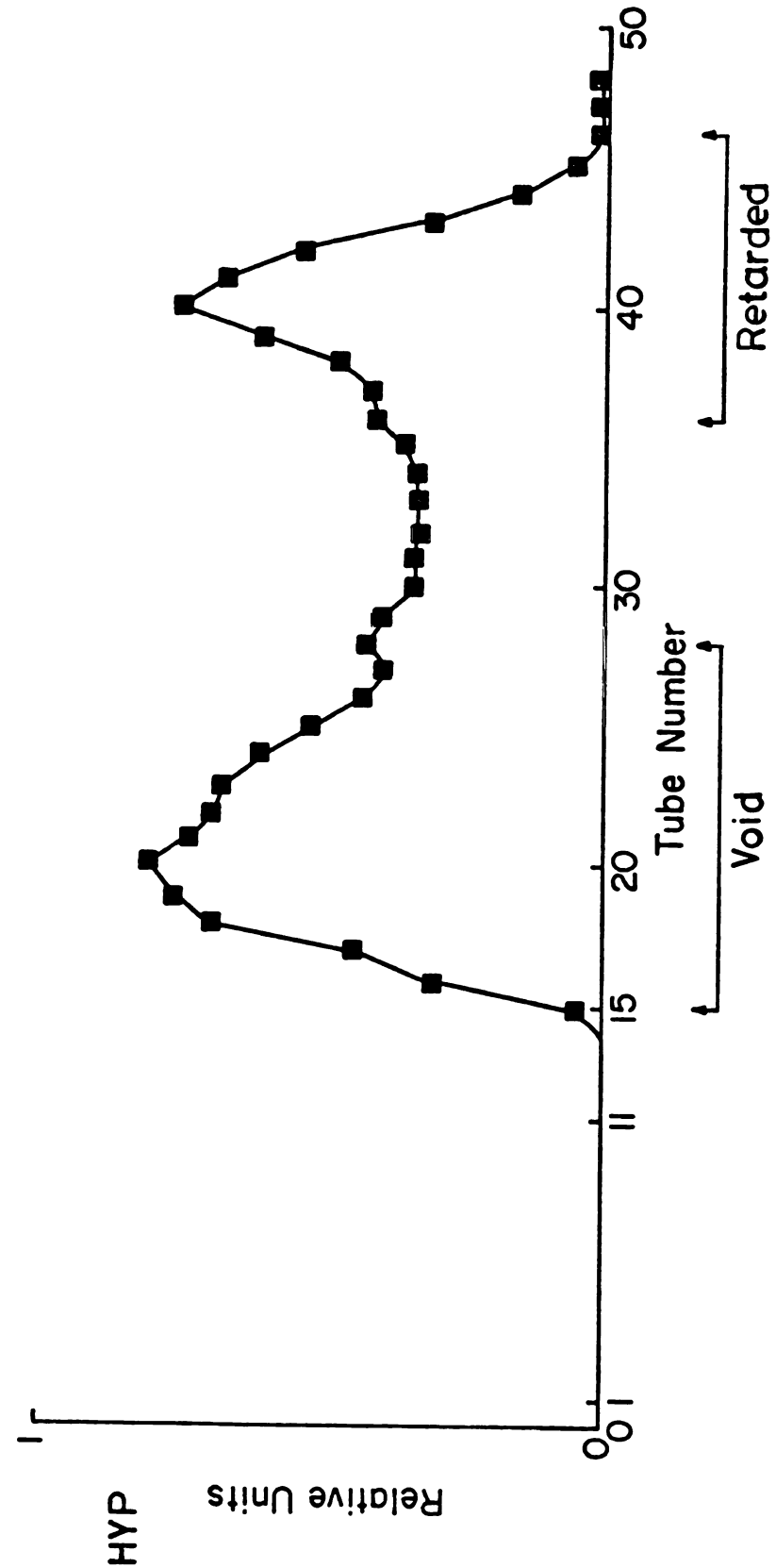

DAH
Relative Units
1
0
0
1
11
15
20
30
40
50
Tube Number
Void
Retarded

Figure 16

Table 17. Amino Acid and Sugar Analysis of the $NaClO_2$ Extract Eluting in the Void Volume of an A 1.5 m Column

	Mole Percent	Per 30 Hyp
Ala	trace	trace
Gly	trace	trace
Val	5.2	2.7
Thr	1.3	.7
Ser	16.2	8.5
Leu	1.4	.8
Ile	.8	.4
Pro	1.7	.9
Hyp	56.9	30.0
Met	n.d.	n.d.
Asp	4.4	2.3
Phe	trace	trace
Glu	1.0	.6
Lys	5.7	3.0
Tyr	1.7	.9
Ada	2.8	1.5
Arg	.8	.4
His	n.d.	n.d.
Cys	n.d.	n.d.
Ara	48.9	
Rha	8.1	
Fuc	.0	
Xyl	1.4	
GalU	28.0	
Man	trace	
Gal	9.5	
Glc	4.0	

Note: Amino acid analysis by G.L.C. Sugar analysis by G.L.C. as trimethylsilylated methyl glycosides.

Thus, there appear to be two size classes of Hyp containing peptides released by the chlorite oxidation, the high molecular weight peptides being associated with uronic acids, the low ones to a lesser extent. If the association of the uronic acid with the protein were proved covalent, then the place of the protein in the initial model of the primary cell wall of Keegstra et al. (2) would be verified (Figure 1). If it is only associated because of its similar chromatographic properties, a revised model would be necessary.

$NaClO_2$ also solubilizes a large portion, 50% or more, of the Hyp-containing protein of tobacco and sycamore-maple cell walls. The Hyp in sycamore-maple extract behaves chromatographically the same as Hyp solubilized by $NaClO_2$ from tomato but that of tobacco does not appear to be of as high a molecular weight.

b. Chromatography of the High Molecular Weight Peptides after HF Treatment

If the $NaClO_2$ solubilized glycopeptides eluting in the void volume of an A .5 m column are deglycosylated by a 1 hour treatment in HF at 0 C the majority of the peptides elute in the void volume of a Sephadex G-50 column (Figure 17). Thus, it appears that $NaClO_2$ has not severely degraded the peptide backbone of the protein.

5. Co-purification of Hyp-Containing Peptides with Uronic Acids

One way to test whether or not the protein and polysaccharides are covalently attached is to chromatograph the complex under conditions which would retard the protein but not the carbohydrate. As the sugars include galacturonic acid, retardation of the sugars along with the

Figure 17. Sephadex G-50 elution profile of the higher molecular weight glycopeptides off an A .5 m column after HF deglycosylation.

The $NaClO_2$ solubilized peptides eluting in the void volume of an A .5 m column (Figure 16) were deglycosylated by treatment in HF at 0 C for 1 hour. After evaporation of the HF the peptides were dissolved in 0.1 N acetic acid and chromatographed on a Sephadex G-50 column. The column effluent was assayed for Hyp.

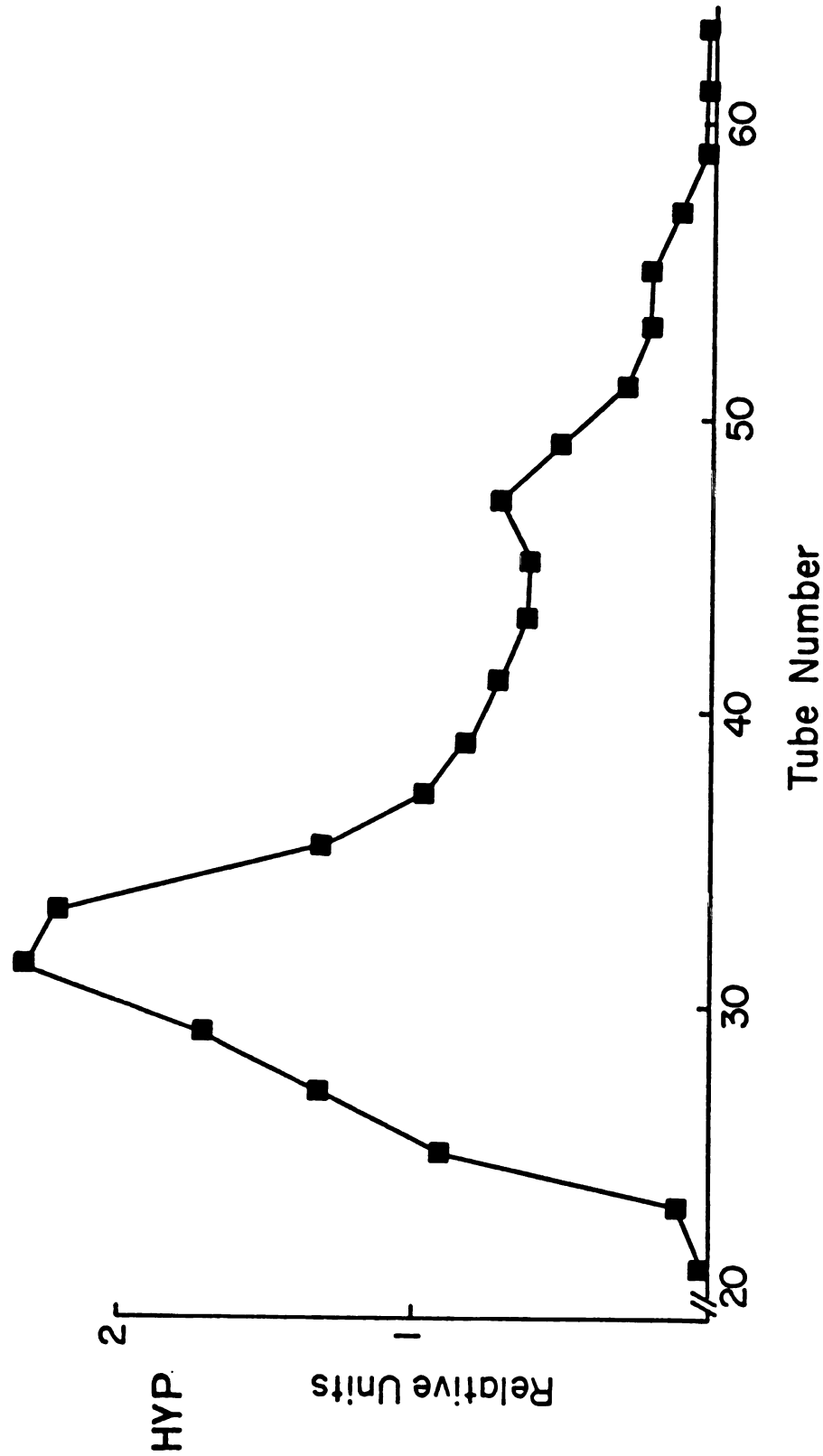


Figure 17

protein on a cation exchange column would be a strong indication of covalent attachment of the two.

Chromatography of the void fraction from an A .5 m column on a CM cellex column equilibrated in 50 mM pH 4.2 sodium acetate buffer and on a cellex P column at pH 2 showed, however, that both the protein and polysaccharide were negatively charged or that protein was prevented from binding to the column because of attached uronic acids.

a. Co-chromatography on DEAE Cellulose (Anion Exchange)

On DEAE cellulose chromatography at pH 5.2 most of the Hyp is retained, as are most of the neutral sugars and all of the galacturonic acid. The elution profile varies from experiment to experiment but there is consistently the major peak of Hyp eluting with about 0.25 M NaCl. Often there is a peak of uronic acids following this (Figure 18), but in some experiments this was not present (Figure 19). If the Hyp-rich peak is rechromatographed on a shallower gradient, .1666-.5 M NaCl, there is still coincidence of uronic acid, neutral sugars and Hyp (Figure 20).

The amino acid composition of the Hyp-rich peaks from DEAE cellulose columns of various experiments are given in Table 18. This fraction is extremely rich in Hyp (over 50 mole percent) and rich in serine (15 to 20 mole percent). This composition is very reminiscent of the tryptic peptides described by Lamport (11). The tryptic peptides of Lamport had a maximum length of 15 amino acids but the material in the peak of Hyp from the DEAE column is excluded from a A .5 m column, which only excludes molecules which act as though they are larger than

Figure 18. Elution profile of A .5 m void on DEAE cellulose.

Fifty milligrams of the material eluting in the void volume of an A .5 m column (Figure 16) was chromatographed on DEAE cellulose using a linear gradient of sodium chloride as eluant. The fractions were assayed for sugars, uronic acid and Hyp.

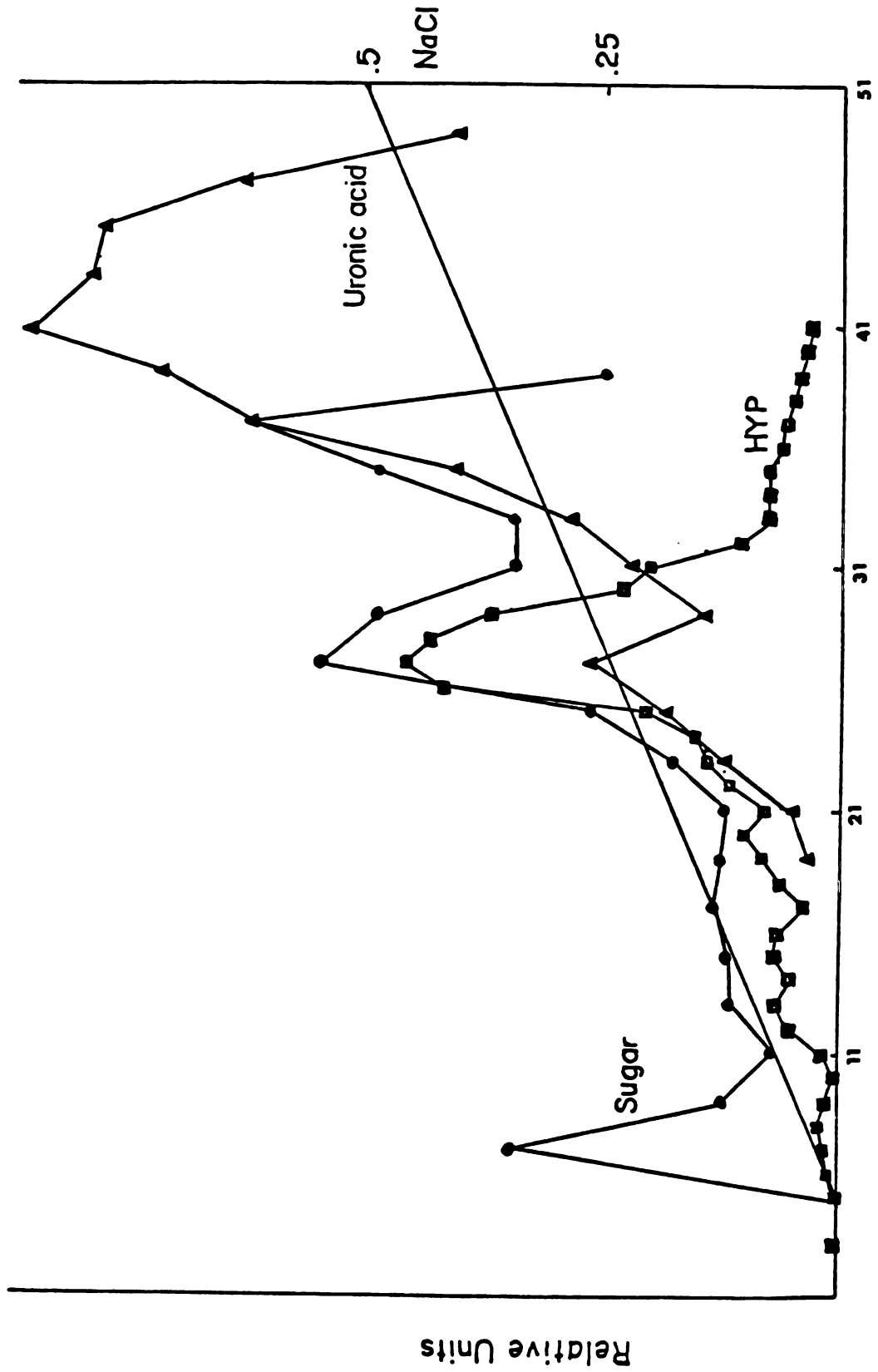

Relative Units
Tube Number
Sugar
HYP
Uronic acid
NaCl
.5
.25
1
11
21
31
41
51

Figure 18

Figure 19. Elution profile of A .5 m void on DEAE cellulose.

Conditions as for Figure 18.

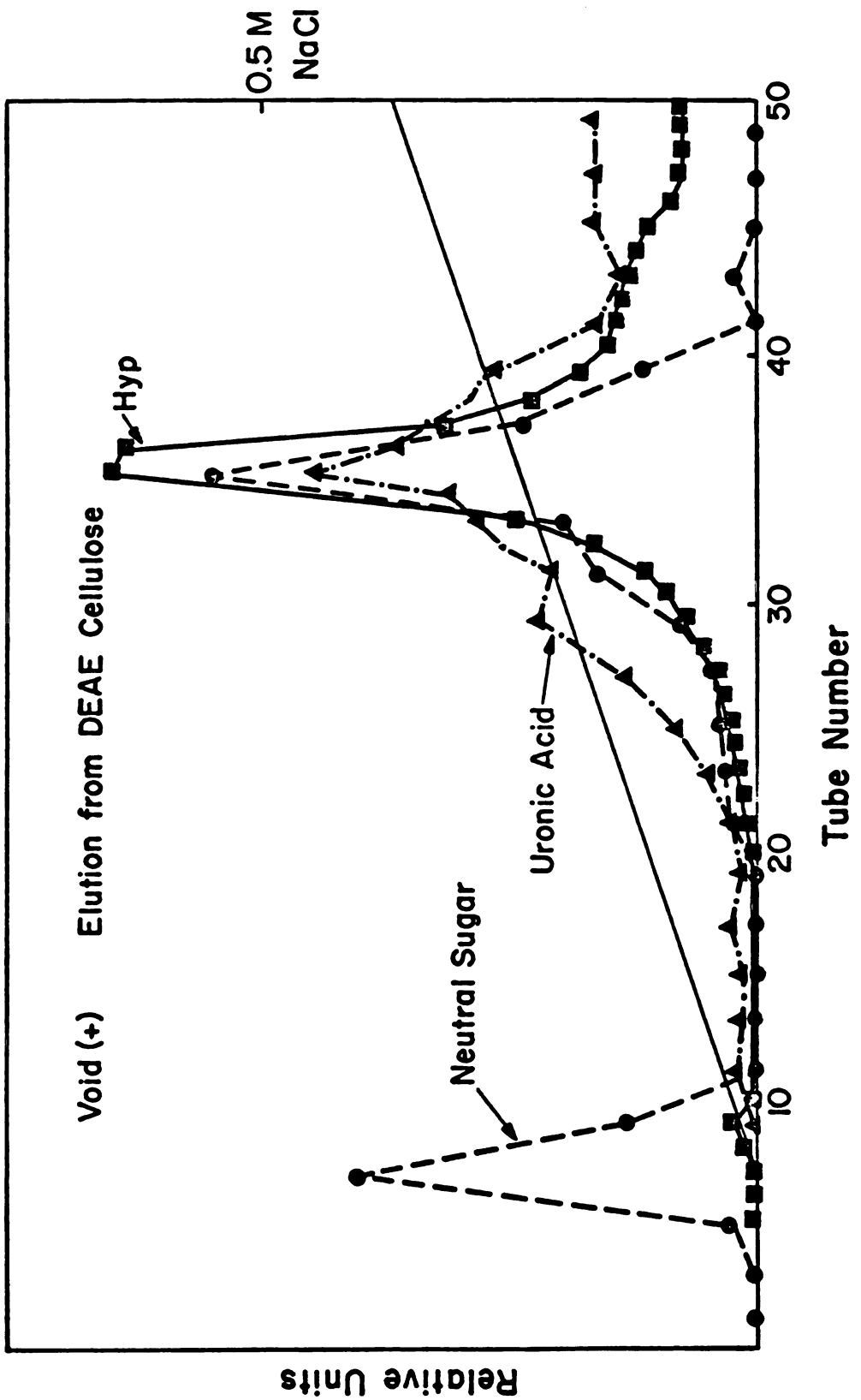

Relative Units
Void (+)
Elution from DEAE Cellulose
0.5 M NaCl
Hyp
Uronic Acid
Neutral Sugar
10
20
30
40
50
Tube Number

Figure 19

Figure 20. Rechromatography of the Hyp-rich peak on DEAE cellulose using a shallower gradient.

The Hyp-rich fractions of the eluate from the DEAE cellulose column described in Figure 19 were pooled, dialyzed, lyophylized, and then rechromatographed on DEAE cellulose using a gradient from 0.166 to 0.5 M sodium chloride rather than 0.0 M to 0.5 M.

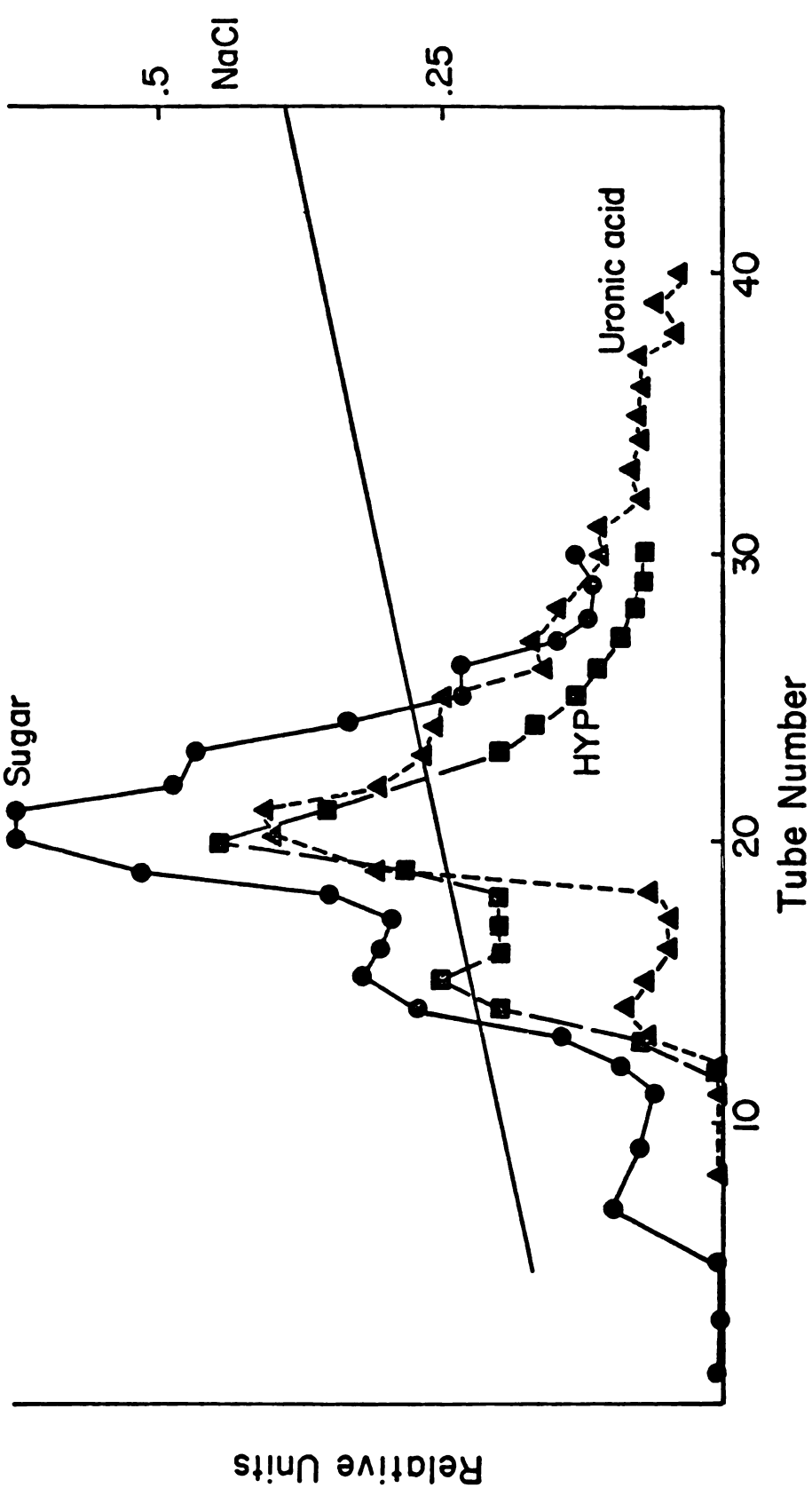

.5
NaCl
.25
Sugar
HYP
Uronic acid
10
20
30
40
Tube Number
Relative Units

Figure 20

Table 18. Amino Acid Analyses of the Pooled Fractions Rich in Hyp on DEAE Cellulose

	Sample 1		Sample 2		Sample 3	
	Mole percent	Per 30 Hyp	Mole percent	Per 30 Hyp	Mole percent	Per 30 Hyp
Ala	.0	.0	6.0	3.3	1.8	1.1
Gly	.0	.0	2.3	1.2	1.8	1.1
Val	3.5	1.9	3.7	2.1	4.9	2.9
Thr	1.2	.7	.9	.5	2.6	1.5
Ser	19.1	10.4	15.6	8.6	15.0	8.8
Leu	.8	.5	.5	.2	.8	.4
Ilu	.3	.2	.6	.3	.7	.4
Pro	3.1	1.7	2.3	1.3	3.4	2.0
Hyp	54.9	30.0	54.2	30.0	50.9	30.0
Met	.0	.0	.0	.0	.0	.0
Asp	4.1	2.2	3.0	1.7	5.1	3.0
Phe	.6	.3	.2	.1	.0	.0
Glu	2.7	1.5	1.6	.9	1.8	1.1
Lys	4.0	2.1	5.5	3.0	9.0[a]	5.3
Tyr	2.7	1.5	.5	.3	.5	.3
Ada	1.9	1.0	1.6	.9	.0	.0
Arg	.1	.0	.3	.1	.4	.2
His	.9	.5	.0	.0	1.3	.9
Cys	.0	.0	.0	.0	.0	.0

[a]This sample was prepared from a particularly brown batch of cell walls. It is probable that the lysine was protected from oxidation to α-amino adipic acid by the presence of a higher than normal amount of phenols.

500,000 daltons molecular weight. Thus, the large peptides solubilized by chlorite must consist of long stretches of amino acids similar in sequence to that in the tryptic peptides, or of short cross linked peptides. This cross linking, if present, is not through glycosidic linkages as the deglycosylated peptides elute in the void volume of a Sephadex G-50 column (section 4-b).

Sugar analyses of the Hyp containing peak obtained from the DEAE column were much more variable than the amino acid analyses. As can be seen from the profiles (Figures 18, 19 and 20), the ratio of neutral sugars and uronic acids to Hyp varies across the peak. The higher the salt concentration required for elution from the DEAE cellulose column, the higher is the uronic acid and neutral sugar content, as would be expected if the uronic acids are the major charged groups binding the protein on the column. The sugar composition of the pooled peak tubes do show, however, that there is more arabinose present than could be accounted for by the Hyp arabinosides described by Lamport and Miller (8). In tomato one would expect an average of 3.06 arabinose/Hyp, but I found between 4 and 6 arabinose/Hyp. Hydrazinolysis of this fraction destroys 80% of the serine residues (14) indicating that 80% of the serines are glycosylated. Thus, if the serines were glycosylated by a single galactose, one would expect only 0.8 galactose residues/serine. I found between 2 and 8 galactose residues/serine. In addition to the arabinose and galactose, there was galacturonic acid, rhamnose, and glucose. The association of these sugars with the Hyp containing peak on the DEAE cellulose column lend support to the proposition by

Keegstra et al. (2) that an arabinogalactan links the cell wall protein to the rhamnogalacturonan. However, since rhamnogalacturonan free of protein would also be adsorbed to the DEAE cellulose column, more evidence is needed before a covalent attachment of the cell wall protein to the cell wall pectin can be proved.

b. Chromatography on Ultrogel AcA 22

Chromatography of the Hyp-rich peak eluted from a DEAE cellulose column on an AcA 22 column also points to the likelihood of a covalent association between uronic acids and protein, but the behavior of the material on the column was anomalous. It was very much retarded on the column although from other experiments with sizing columns (Biogel A 1.5 m) it should have been only slightly retarded or excluded by the AcA 22 column which has an exclusion limit of 1,000,000 for globular proteins. There was, however, good coincidence of the uronic acids and Hyp (Figure 21).

c. Precipitation with Alkaline DMSO

While performing β-elimination experiments (see next section) I noticed that a precipitate formed when the DMSO was made alkaline but before the addition of the sodium borohydride. By analysis of the supernatant and precipitate I found that Hyp-containing peptides not associated with pectin were soluble in alkaline 50% DMSO; whereas, those containing much uronic acid precipitated. When the precipitated glycopeptides were run on a DEAE cellulose column they remained adsorbed in the presence of a higher salt concentration than the soluble ones, which may indicate a higher content of covalently attached uronic acid.

Figure 21. Chromatography of the Hyp-rich peak obtained from a DEAE cellulose column on Ultrogel AcA 22.

The fractions rich in Hyp in the eluate from a DEAE cellulose column were pooled and then chromatographed on an ACA 22 column. The fractions were assayed for sugars, uronic acids, and Hyp.

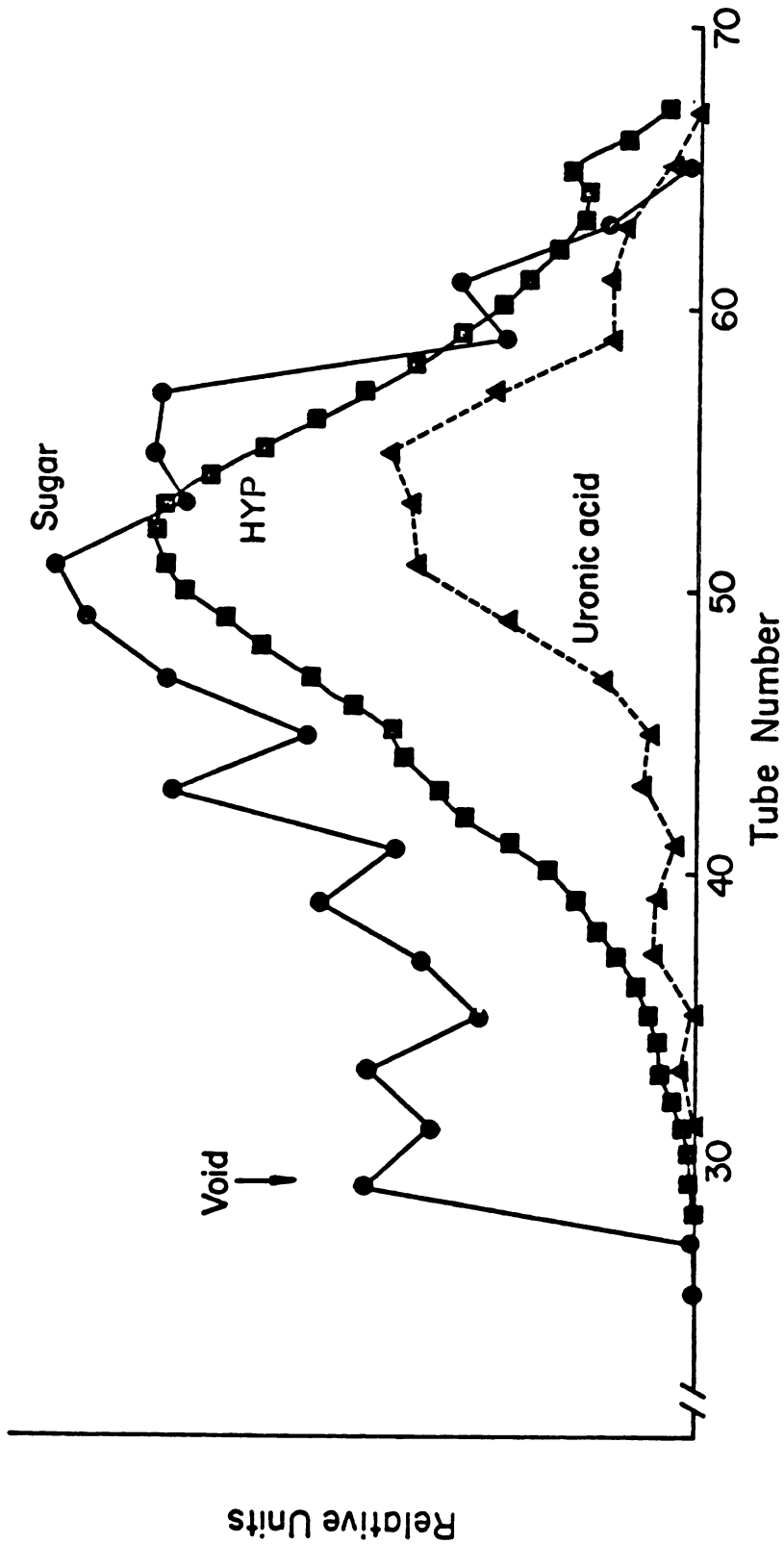

Relative Units
Void
Sugar
HYP
Uronic acid
30
40
50
60
70
Tube Number

Figure 21

d. Isoelectric Focusing

In an experiment performed by Lamport and Caughey the peptides solubilized by chlorite and which elute in the void volume of a G-100 column (rather than an A .5 m column) were put on an isoelectric focusing column with a pH range of 3 to 10. Again, there was overlap of the uronic acids and Hyp (Figure 22).

e. CsCl Density Gradient Centrifugation

The experiments described above are consistent with the possibility that a polysaccharide linking pectin to the protein is present in the sodium chlorite solubilized glycopeptides, yet do not prove it. I hoped that by CsCl density centrifugation I would obtain a clear separation of the Hyp from the pectin, thereby proving there is no covalent linkage between protein and pectin or a peak of pectin co-sedimenting with protein at a density removed from that of free pectin, proving a covalent linkage or at least an extremely strong interaction.

Silpananta, Dunstone and Ogston (105) have used equilibrium CsCl gradient centrifugation to fractionate a hyaluronic acid preparation from ox synovial fluid into 3 major fractions: a protein-rich light fraction, a purified hyaluronic acid protein-poor fraction and a dense chondroitin sulfate-protein complex. Franek and Dunstone (106) have also used CsCl gradient centrifugation to fractionate proteinpolysaccharides from bovine nasal cartilage and to purify a glycoprotein from their preparations by floating it away from the proteinpolysaccharide. Creeth and Denborough (107) and Dunstone (108) have used CsCl gradients to fractionate ovarian cyst glycoproteins. Dunstone (108) found that

Figure 22. Isoelectric focusing of $NaClO_2$ solubilized tomato wall peptides.

Tomato walls were treated with $NaClO_2$ for 45 minutes at 75 C , and the soluble fraction (after dialysis) was passed through a Sephadex G-100 column. The bulk of the Hyp which was excluded by the column was chromatographed on DEAE cellulose, and that which was retarded by the DEAE cellulose was put into an LKB 8101 isoelectric focusing apparatus. The material was focused in a pH gradient of 3 to 10. The fractions collected from the gradient were assayed for Hyp and uronic acids.

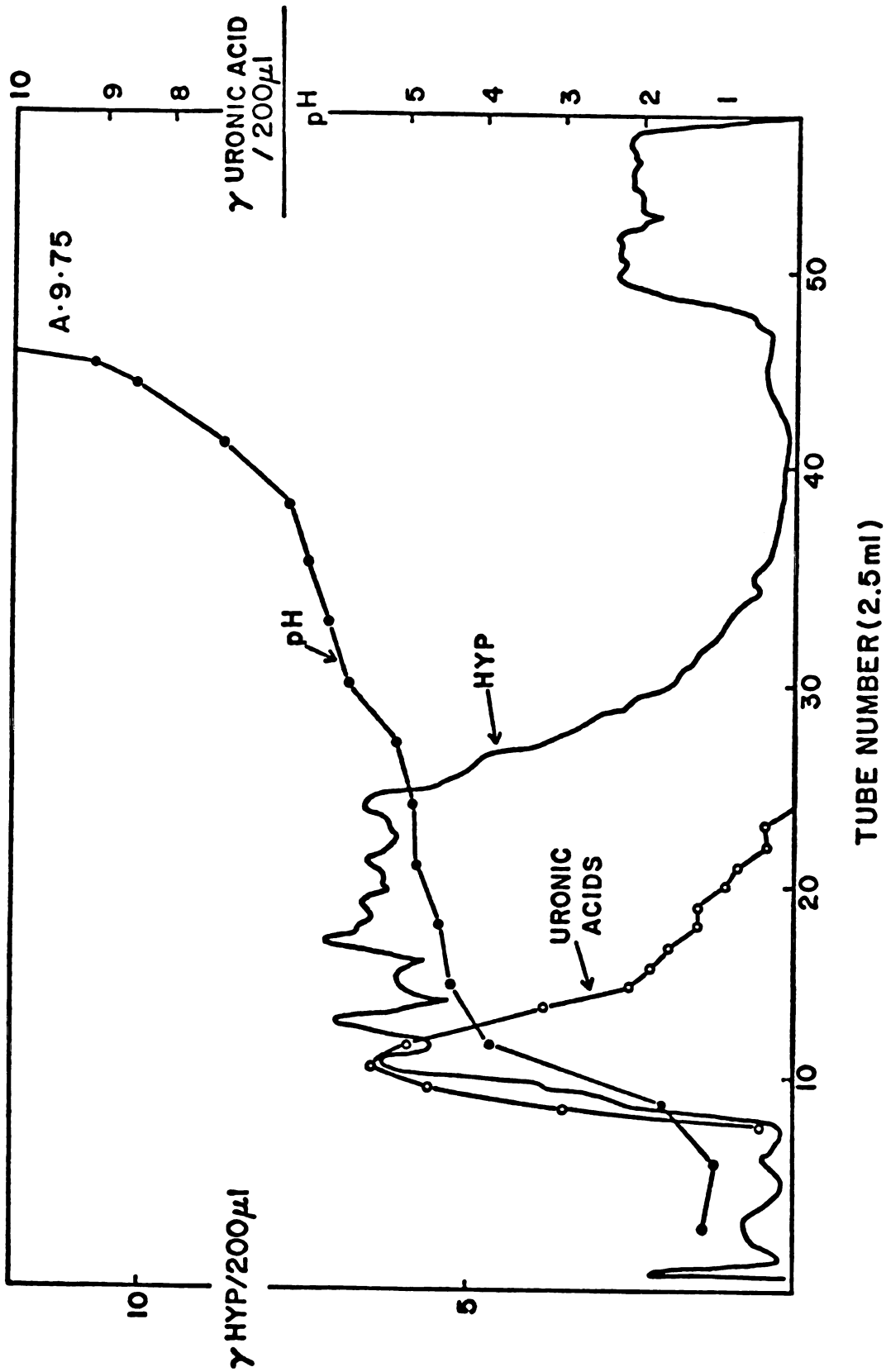

A·9·75
γ HYP/200µl
γ URONIC ACID / 200µl
pH
HYP
URONIC ACIDS
TUBE NUMBER(2.5ml)

Figure 22

denser glycopeptide fractions contained a higher ratio of carbohydrate to protein yet all the fractions contained blood group activity, indicating a heterogenous population of glycoproteins with blood group activity. Creeth and Denborough (107), using a less purified cyst fraction, were able to purify the blood group substances from contaminating serum proteins (about 90% of the material) in a single CsCl density gradient step. The serum proteins floated to the top of the gradient and the proteins with blood group activity were spread over the gradient, those being more highly glycosylated sedimenting to higher densities.

The basis of these separations is the large difference in the buoyant density of protein, approximately 1.3 g/ml (109), and that of polysaccharides (greater than 1.6 g/ml) (110). The density of polysaccharides seems to vary much more than that of proteins. Erikson and Szybalski (110) found that glucose-containing polymers have densities around 1.6 g/ml, but the densities of sulfated glycuronoglycans are higher 1.65-1.75 g/ml (111). Unfortunately, as far as I know, there is no table of buoyant densities of plant polysaccharides in the literature. The buoyant density of polysaccharides is very dependent on the degree of hydration of the polymer. Mashburn et al. (111) found that upon changing from CsCl to $CsSO_4$ or adding 3 M guanidine hydrochloride the buoyant density of polysaccharides is drastically reduced.

When the peak of Hyp-containing material, which had been eluted from a DEAE cellulose column and which had previously been excluded by a Biogel A .5 m column, was centrifuged to equilibrium in a cesium

chloride gradient, density 1.45 to 1.64 g/ml, the distribution of Hyp in the gradient showed that the polypeptides were heterogeneous with respect to density, indicating varying degrees of glycosylation (Figure 23). As expected, the greater the density, the larger the proportion of uronic acids. At the bottom of the tube (high density) the Hyp-proline concentration is very low; whereas, the uronic acid concentration is high. Since the buoyant density of polygalacturonic acid has not been reported, I thought it necessary to determine the density of protein-free pectin to be sure that the association of pectin with protein in the gradient was indicative of a covalent linkage between the two. Material containing uronic acids which was released from the tomato cell walls by boiling in ammonium oxalate, sedimented to a density of between 1.6 to 1.63 g/ml but some of the uronic acids acted as though they had a density less than free pectin and so must be covalently attached to something less dense, e.g., protein (Figure 24). This lighter fraction of pectin does contain a small amount of Hyp.

If the fractions rich in uronic acids eluting in the trailing edge of the Hyp-rich peak from the DEAE cellulose column (see section 5-a) are centrifuged to equilibrium in a CsCl gradient, the major peak, as one would expect, is at a high density, 1.62 g/ml, but a shoulder appears at a density of around 1.54 g/ml which is Hyp-rich and has an amino acid composition similar to that of the major Hyp peak from the DEAE cellulose column (Figure 25).

The sugar compositions of the four pooled fractions indicated in Figures 23 and 25, are given in Table 19. It is peculiar that both the

Figure 23. CsCl density gradient centrifugation of the major fraction of Hyp on a DEAE cellulose column.

The fractions rich in Hyp from a DEAE cellulose column (Figure 18) were pooled, dialyzed, and lyophylized. A portion of the material, approximately 5 mg in 1 ml, was put on top of 2 ml CsCl solution with a density of 1.74. This was centrifuged at 40,000 RPM for 3 days at 4 C. The gradient was removed from the centrifuge tube with an auto Densi Flow II, which takes the solution from the top of the gradient thus avoiding any biasing of the ligher fractions towards higher densities. The fractions were analyzed for uronic acid and Hyp, and pooled as indicated.

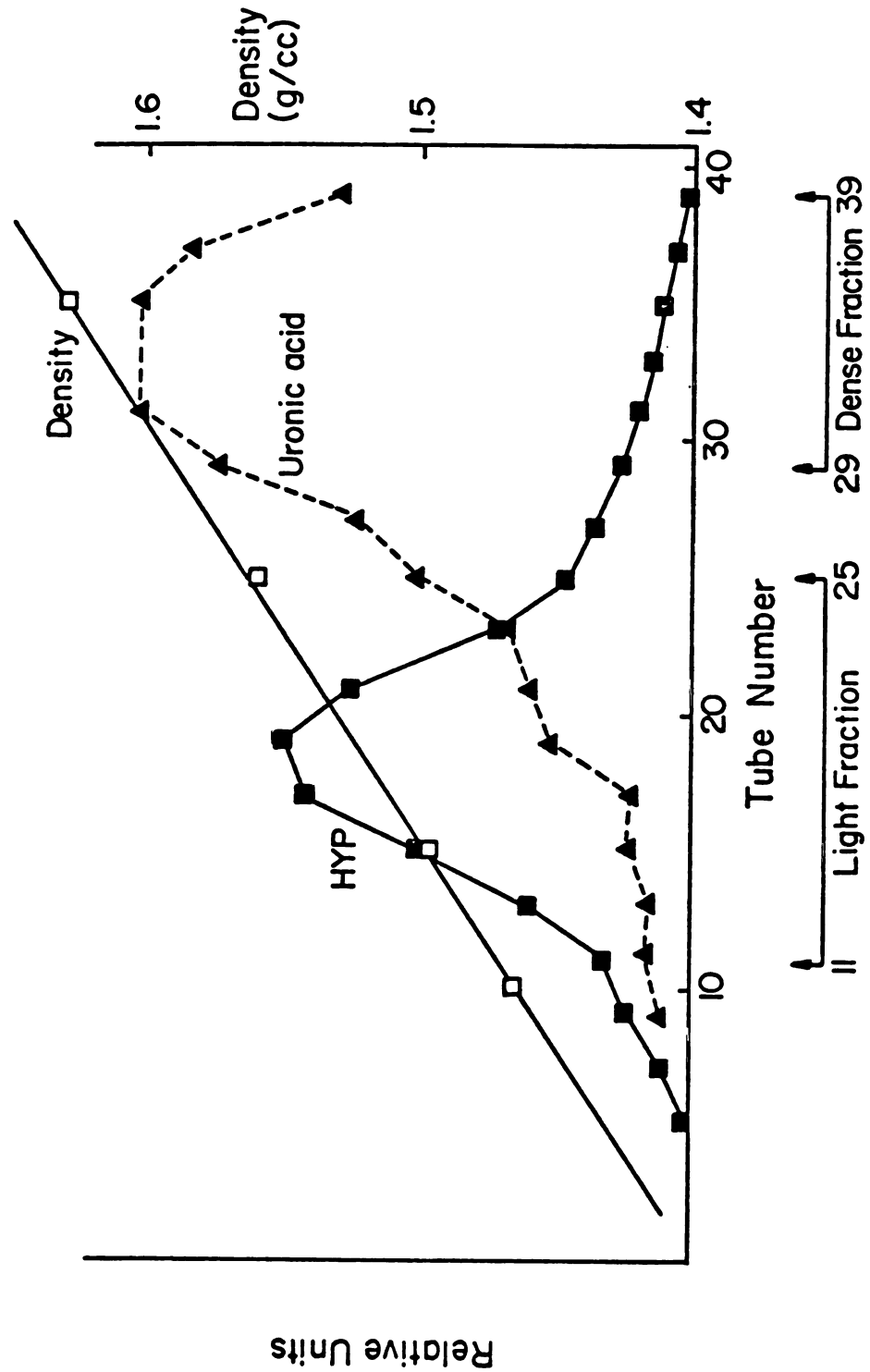

Density (g/cc)
1.6
1.5
1.4
Density
Uronic acid
HYP
Relative Units
10
20
30
40
Tube Number
11 Light Fraction 25
29 Dense Fraction 39

Figure 23

Figure 24. CsCl gradient centrifugation of bulk pectin extracted from tomato cell walls by boiling ammonium oxalate.

The ammonium oxalate soluble cell wall polymers prepared as described in Materials and Methods were precipitated by making the solution 70% in ethanol. The precipitate was washed with 70% ethanol. Approximately 5 mg of this material was centrifuged for 3 days at 40,000 rpm at 4 C. The gradient was removed from the centrifuge tube by puncturing the bottom of the tube and collecting 3 drop fractions. The fractions indicated were pooled and assayed for Hyp.

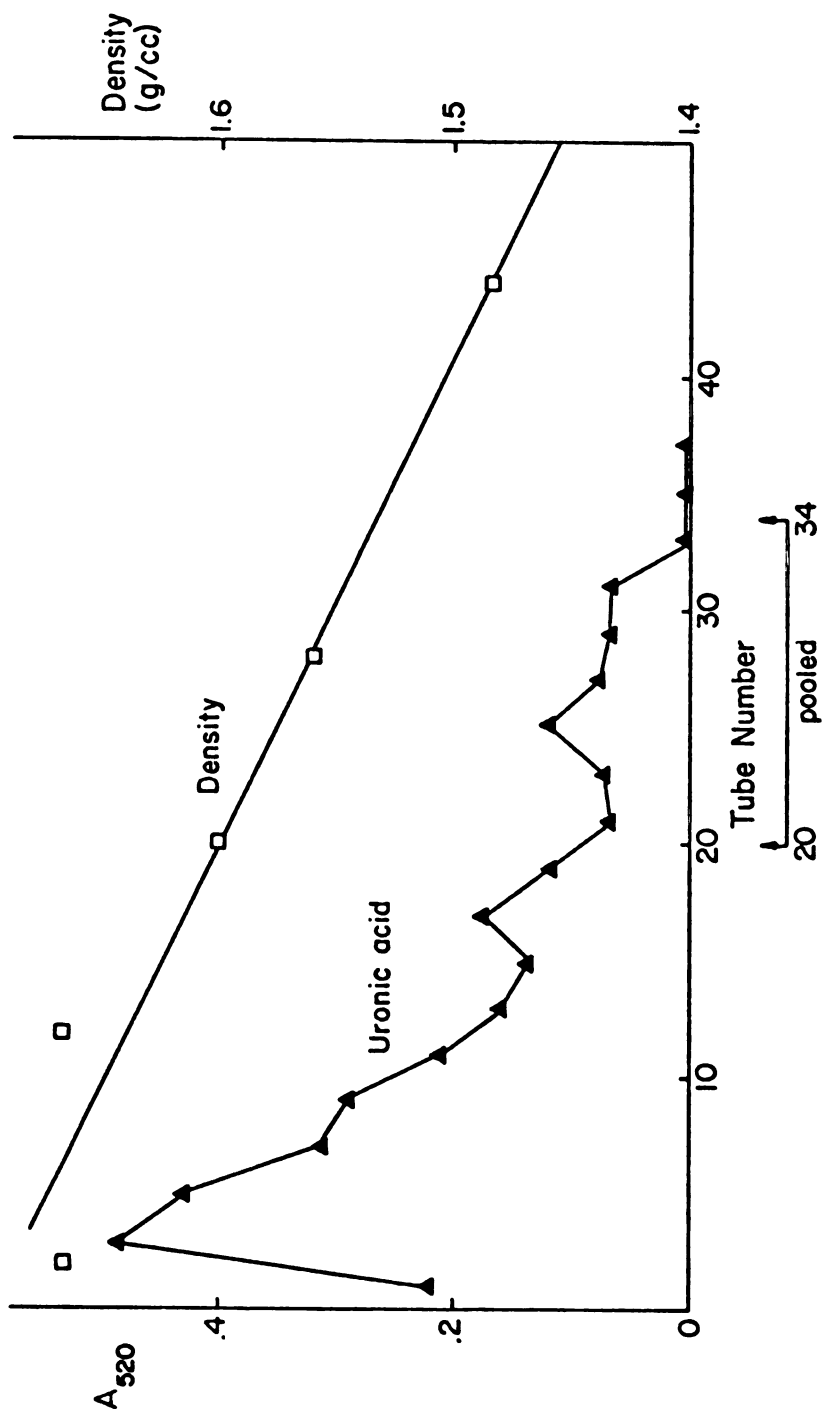

Density (g/cc)
1.6
1.5
1.4
Density
Uronic acid
A_{520}
.4
.2
0
10
20
30
40
Tube Number
20
pooled
34

Figure 24

Figure 25. CsCl density gradient centrifugation of the trailing edge of the Hyp-rich peak on DEAE cellulose.

The fractions containing Hyp which were eluted at a higher salt concentration than the major Hyp peak on DEAE cellulose (Figure 18), were pooled, dialyzed, lyophylized, and then centrifuged in a CsCl gradient as described for Figures 23 and 24. The gradient was collected using an auto Densi Flow II. The fractions indicated were pooled for further analysis (c.f. Table 19).

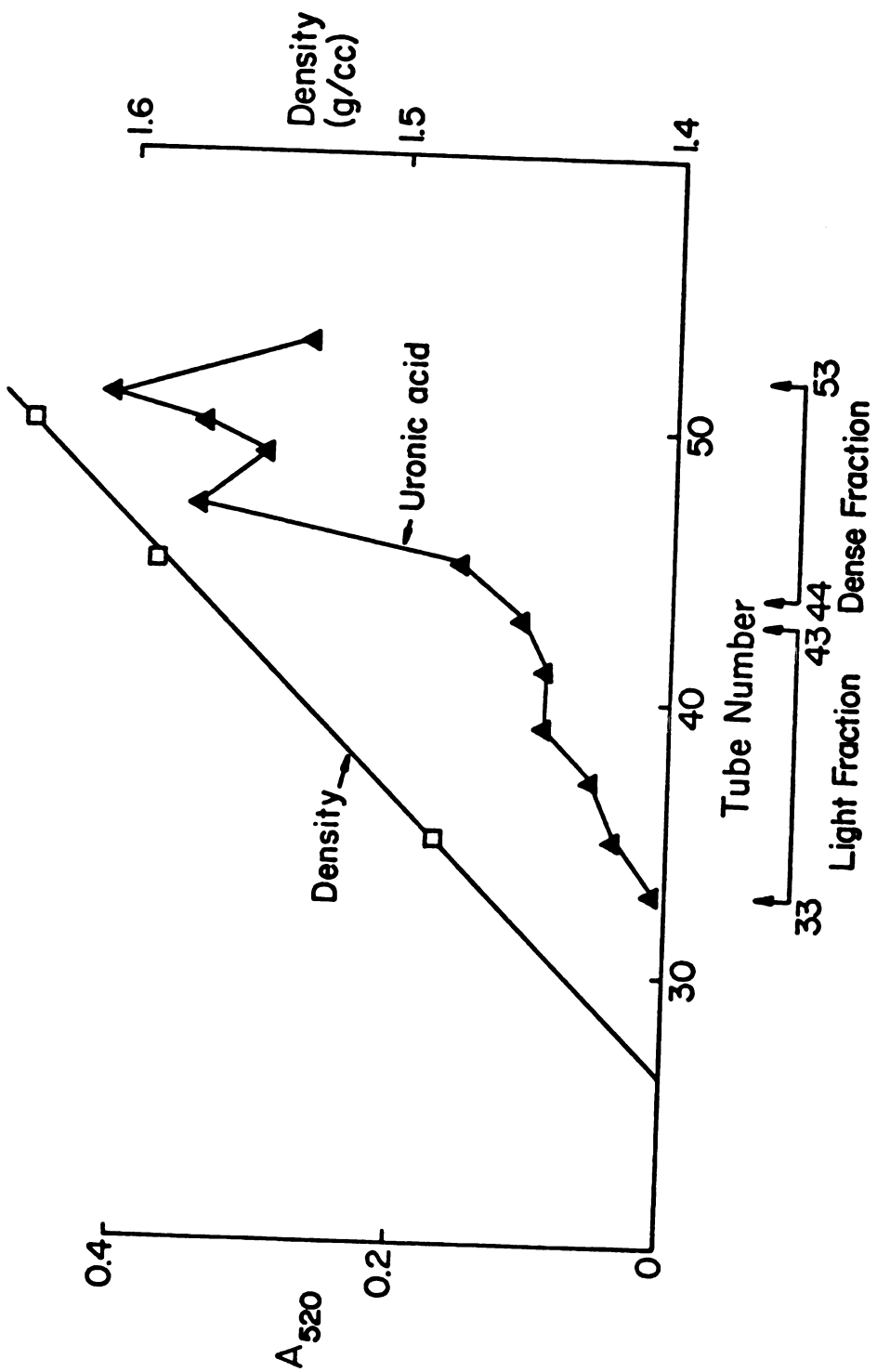

Density (g/cc)
1.6
1.5
1.4
Uronic acid
Density
A_{520}
0.4
0.2
0
30
40
50
Tube Number
33
43 44
53
Light Fraction
Dense Fraction

Figure 25

Table 19. Sugar Composition of Various Fractions from Cesium Chloride Gradients

	Light Hyp (Figure 23)	Dense Hyp (Figure 23)	Light pectin (Figure 25)	Dense pectin (Figure 25)
	Mole percent	Mole percent	Mole percent	Mole percent
Ara	77.3	15.9	42.1	7.5
Rha	.4	2.3	12.3	10.8
Fuc	.0	.2	.0	.0
Xyl	.0	2.6	.0	3.2
GalU	8.7	45.0	21.1	46.4
Man	1.2	3.8	.0	7.4
Gal	9.8	24.7	19.5	14.8
Glc	2.6	5.6	5.0	10.0
	3.7 ara/Hyp	7.3 ara/Hyp	3.7 ara/Hyp	n.d.

light and heavy fractions of the Hyp-rich peak on the DEAE cellulose column have a very high galacturonic acid to rhamnose ratio, almost 20:1; whereas, the pectin fractions eluted from DEAE-cellulose with a higher salt concentration have a much lower ratio, 2-4:1. The fact that the majority of the Hyp is associated with a rhamnose-poor pectic fraction is consistent with the hypothesis that the pectins of the wall are linked to extensin via the reducing end of the polyrhamnogalacturonan. It is also interesting that there is glucose present in all the fractions, most predominantly in the dense pectic fraction. This is not at all what one would expect from the model put forward by Keegstra et al. (2). One would only expect a small amount of glucose associated with the xylose present as xyloglucan. This extra glucose may indicate the presence of a glucan which would have been removed by the amylase treatment that Talmadge et al. (99) used in the preparation of their walls. It is very unlikely that the glucose is in starch as it has adsorbed to the DEAE cellulose column.

6. β-Elimination of Polysaccharides from Chlorite Solubilized Glycopeptides

Another way of testing covalent attachment of polysaccharides to the cell wall protein would be to subject the glycopeptides solubilized by $NaClO_2$ to a treatment which cleaves the serine-O-glycoside linkage but does not cleave peptide bonds or other glycosidic linkages and then show that the polysaccharide and protein can then be separated by methods which would not separate them before the treatment. Serine-O-glycosides are susceptible to β-elimination under alkaline conditions.

Unfortunately the conditions necessary to cleave this linkage also causes some peptide bond cleavage (112). The degradation of the peptide is probably specific to certain amino acid residues (112,113) most likely serine and threonine, as peptides (which can be fractionated by ion exchange chromatography into seemingly discrete peptides) have been obtained from tomato cell walls (94) by a β-elimination procedure.

The susceptibility of peptide bonds to the molarity of sodium hydroxide used in the elimination conditions of Lamport (94) (0.2 N NaOH at 45 C for 5 hours) has not been investigated thoroughly but results of workers using similar conditions indicate that racemization of amino acids and peptide bond hydrolysis at serine residues will occur. Pickering and Li (113) using 0.1 N NaOH found that after 15 minutes in a boiling water bath, 15 to 20% of the Tyr-Ser bond of adenocorticotropin had been cleaved and many of the amino acids had been racemized. Warner (114) found that 1 in 8 peptide bonds of ovalbumin were cleaved by 0.28 N NaOH at 68 C for 3 hours. Downs et al. (112) using conditions almost identical to those of Lamport (94) noted that for at least 1 hour of reaction in the alkali bovine submaxillary mucin remained completely nondialyzable yet after 24 hours almost 20% of the amino acids of the protein were dialyzable.

When the Hyp-rich peak from the DEAE cellulose column (see Figure 18) was subjected to the β-elimination conditions of Lamport (94) with the modification as described in the Methods, the material was partially soluble in the elimination mixture and partially insoluble. When the soluble portion was chromatographed on a Sephadex G-50 column the hydroxyproline was retarded and eluted as two fused peaks (Figure 26).

Figure 26. Elution profile on Sephadex G-50 of the soluble portion of the β-eliminated Hyp peak from a DEAE cellulose column.

Ten to fifteen mg of the Hyp peak material from a DEAE cellulose column were dissolved in 1 ml of DMSO in a 3 ml microvial to which was added 0.2 ml of ethanol, 0.6 ml of water and 0.2 ml of 2 N NaOH. Upon the addition of the NaOH a large precipitate formed. The suspension was made 1 M in $NaBH_4$ and then left stirring for 6 hours in a 37 C room. After the 6 hours the reaction mixture was neutralized with glacial acetic acid and degassed under vacuum. The precipitate was removed by centrifugation and the supernatant chromatographed on Sephadex G-50. The fractions were assayed for Hyp.

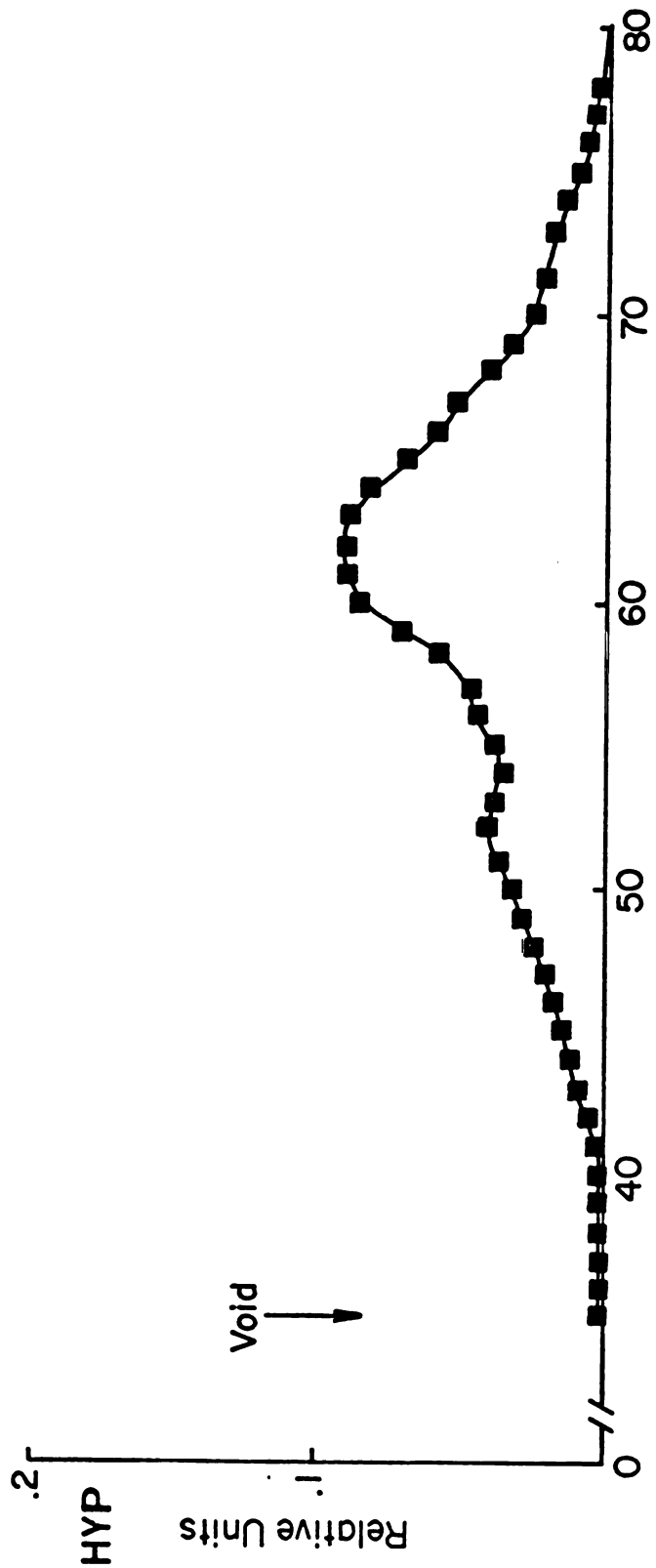


Figure 26

The peptide must, therefore, have been degraded by the alkaline treatment, as before alkali treatment the totally deglycosylated peptides were excluded by a G-50 column (Figure 17). The sugar and Hyp composition of the larger of the two peaks (Table 20) shows that much of the galactose, rhamnose, and arabinose that are associated with the peptides no longer co-chromatographs with the Hyp.

In the void volume of the G-50 column there is a very high ratio of arabinose to Hyp, 21 ara/Hyp indicating the presence of polysaccharides not linked to peptides.

The material insoluble in DMSO β-elimination reaction mixture eluted in the void volume of a Biogel A .5 m column and had a composition much enriched in sugars as compared with the starting material but still contained some Hyp, probably due to incomplete β-elimination. It is unlikely that the reaction would go to completion as the highly glycosylated Hyp-containing peptides are insoluble in the β-elimination reaction mixture.

Since the alkaline conditions used to bring about β-elimination of the glycopeptides lead to some peptide bond breakage the results of the experiment are not conclusive. They are, however, consistent with the β-elimination of a polysaccharide from the glycopeptides.

7. Partial Characterization of the Lower Molecular Weight Glycopeptides

The ratio of Hyp retarded by the A .5 m column to that excluded by the column varies from about 1:1 to 2:1. Thus, these smaller peptides are a significant portion of the chlorite extract. They do not

Table 20. Sugar Composition of Hyp-containing Fractions Before and After β-Elimination

	Before Elimination	Total Soluble in Reaction Mixture	Peak 1 on Sephadex G-50 (Figure 26)
Ara/Hyp	6-8	5.1	4.9
Gal/Ser	2-3	0.8	0.7
Glc/Ser	1	trace	0.3
Rha/Ser	0.6	trace	-

appear to arise from the larger peptides as the ratio of large to small was smaller when the chlorite treatment was carried out for only 8 minutes. The larger peptides after this short treatment did not peak as sharply in the void of an A 1.5 m column as the 30 or 45 minute chlorite extracts. The majority of the Hyp retarded on the A 1.5 m column eluted in the void volume of a Sephadex G-50 column (Figure 27). The amino acid composition of the peptides eluted in the void volume of the G-50 column was different from that of larger peptides discussed previously (Table 21), the smaller peptides being richer in lysine and valine and a little poorer in serine.

When the peptides eluted in the void volume of a Sephadex G-50 column were chromatographed in the same way as those in the void volume of the A .5 m column on a DEAE cellulose column the peptides split into two fractions, one which was not adsorbed to the column and a broad adsorbed peak (Figure 28). Again, there is co-chromatography of uronic acids and Hyp although there is a lower proportion of uronic acid to Hyp than for the larger peptides. Little of either of the two Hyp-rich fractions was retarded on a Biogel-P100 column.

8. Methylation of Glycopeptides

If a unique oligo- or polysaccharide is responsible for linking extensin to the hemicelluloses or pectins of the cell wall, methylation analysis of $NaClO_2$-released glycopeptides should reveal its presence, if not its complete identity. As mentioned previously, the data put forward by Keegstra et al. (2) to support their proposal that a 3,6-linked arabinogalactan links extensin to the pectin of the wall was

Figure 27. The elution profile of the $NaClO_2$ solubilized peptides retained by a Bio-Gel A .5 m column on Sephadex G-50.

The fractions from the second Hyp peak on A .5 m (see Figure 16) were pooled and lyophilized and then taken up in 2 ml of 0.1 N acetic acid and chromatographed on Sephadex G-50. There was no problem with viscosity in the lower molecular weight fraction. A peak of brown unidentified material (chlorite oxidation products) eluted in the included volume. The fractions were assayed for Hyp.

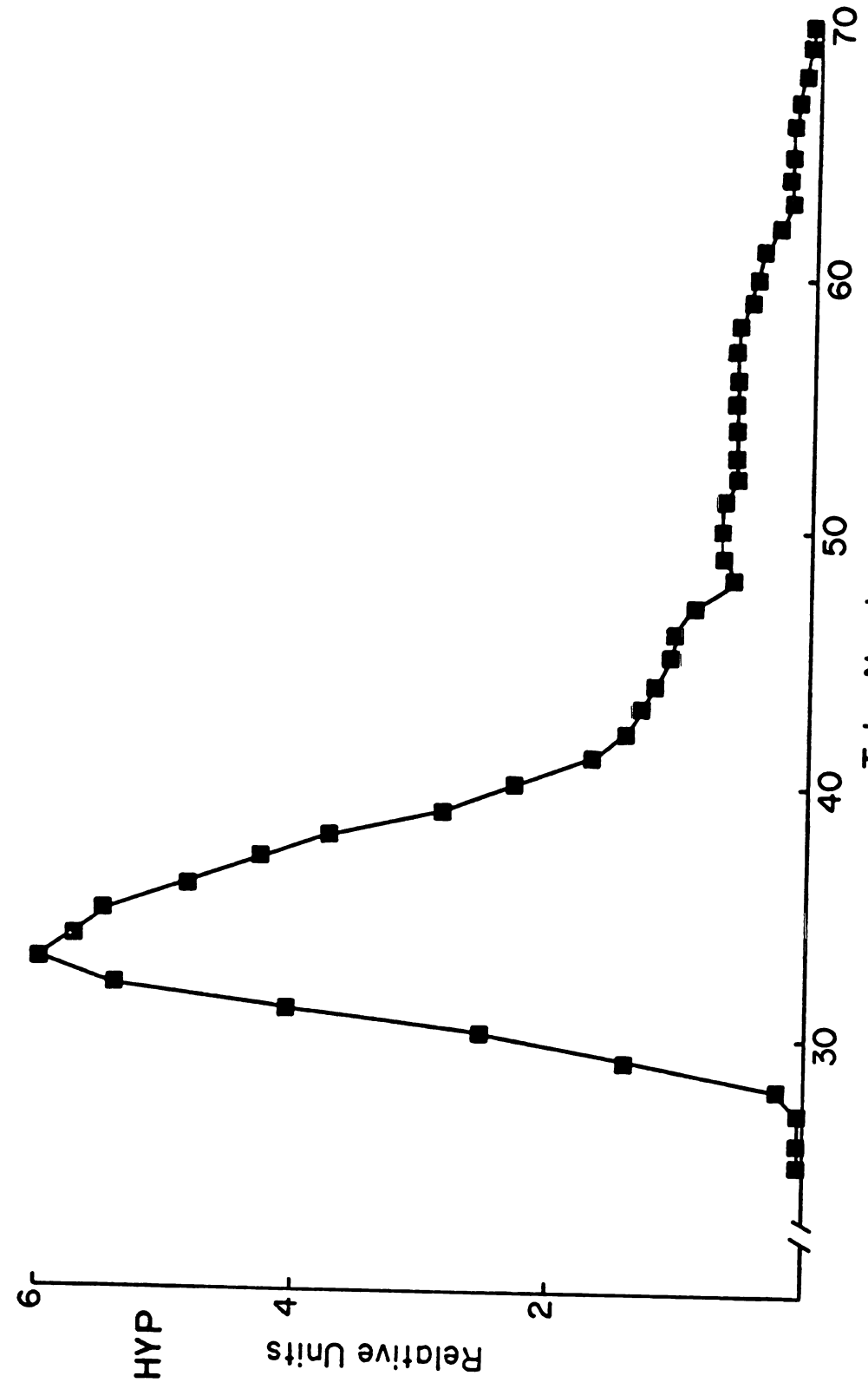


Figure 27

Table 21. Comparison of Amino Acid Analyses of the Various Sized Fractions Obtained from Cell Walls by Sodium Chlorite Extraction

A.A.	Excluded by A .5 m Mole Percent	Excluded by A .5 m Per 30 Hyp	Excluded by G-50 Mole Percent	Excluded by G-50 Per 30 Hyp	Retained by G-50 Mole Percent	Retained by G-50 Per 30 Hyp
Ala	.0	.0	.0	.0	3.8	6.0
Gly	.0	.0	.0	.0	6.3	10.0
Val	5.2	2.7	6.6	3.7	4.8	7.6
Thr	1.3	.7	2.5	1.4	4.8	7.8
Ser	16.2	8.5	12.4	6.8	12.5	20.0
Leu	1.4	.8	.8	.5	4.4	7.0
Ile	.8	.4	.6	.3	2.8	4.4
Pro	1.7	.9	3.9	2.2	5.0	8.0
Hyp	56.9	30.0	54.4	30.0	18.8	30.0
Met	.0	.0	.0	.0	.0	.0
Asp	4.4	2.3	2.9	1.6	8.2	13.2
Phe	.0	.0	.8	.4	1.9	3.0
Glu	1.0	.6	.7	.4	7.3	11.7
Lys	5.7	3.0	9.0	5.0	6.6	10.6
Tyr	1.7	.9	1.2	.7	.8	1.3
Ada	2.8	1.5	4.1	2.3	4.1	6.6
Arg	.8	.4	.0	.0	1.7	2.8
His	.0	.0	.0	.0	.0	.0
Cys	.0	.0	.0	.0	.0	.0

Amino acid analyses were performed by gas liquid chromatography as described in the Methods.

Figure 28. Chromatograph of the smaller glycopeptides solubilized by $NaClO_2$ on DEAE cellulose.

The peptides retarded by A .5 m but excluded by Sephadex G-50 were applied to a DEAE cellulose column and eluted with a linear gradient of NaCl, 0-0.5 M. The eluate was assayed for Hyp, sugars and uronic acids.

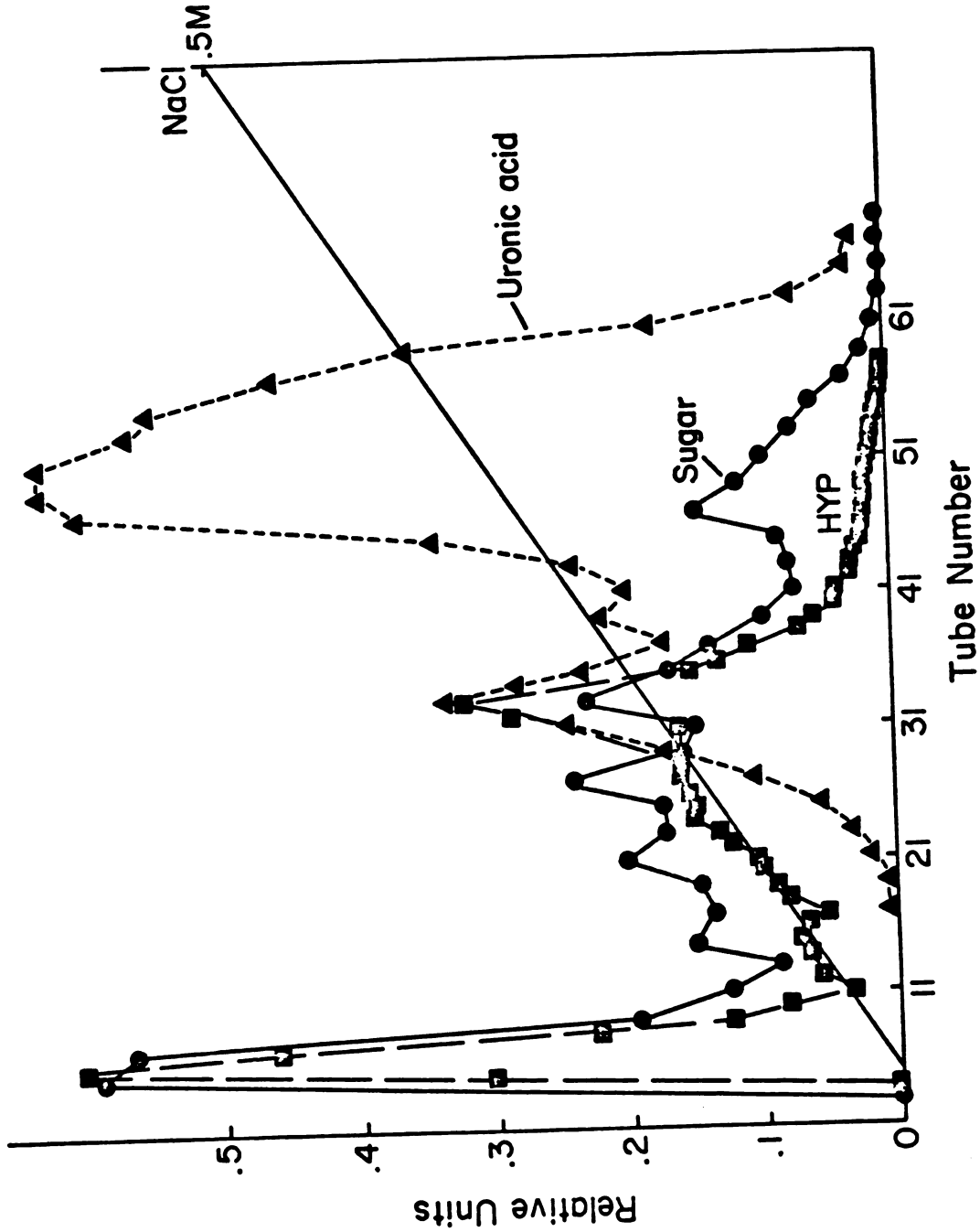

NaCl .5M
Uronic acid
Sugar
HYP
Relative Units
0
.1
.2
.3
.4
.5
11
21
31
41
51
61
Tube Number

Figure 28

very tenuous. The protein-containing fraction from cell walls which they were using to investigate this linkage contained very little protein and only a low percentage of 3,6-linked galactose. Most of the polysaccharides were pectinaceous. $NaClO_2$ released peptides are a relatively high percentage protein with little or no pectin, thus they should contain higher amounts of this proposed arabinogalactan.

a. Large $NaClO_2$-released Glycopeptides

The higher molecular weight glycopeptides which had been purified by DEAE cellulose chromatography and CsCl density centrifugation were methylated and analyzed on 3 different GLC columns. On all three columns there was a peak which co-chromatographed with 3,6-linked galactose.

The major Hyp-containing fraction from a CsCl gradient, "light" Hyp (see Figure 23), contained predominantly T, 2- and 3-linked arabinose, indicative of the Hyp-arabinosides (Figure 29). The next most abundant sugar is terminal galactose. Although it is very difficult to quantitate methylated sugars as there is not yet a good internal standard to correct for all the losses incurred during the methylation procedure, in this case one can estimate how much Hyp must be represented by the methylated sugars if one assumes that the 2-linked arabinose is only associated with Hyp-arabinosides. Knowing the ratio of Hyp to ser, the linkages of the Hyp-arabinosides: T-ara, 1-3 ara, 1-2 ara, 1-2 ara, 1-4 Hyp for $Hyp\text{-}Ara_4$ (115), and the fractions of $Hyp\text{-}Ara_{4\text{ to }0}$(8), one can estimate that there is more than enough T-galactose to account for one T-gal for all the seryl hydroxyl groups present. This concurs with the result that hydrazinolysis of the glycopeptides (14)

Figure 29. Methylation analysis of the less dense Hyp fraction from a CsCl density gradient.

The fractions designated light Hyp in Figure 23 were pooled, dialyzed, and methylated as described in the Methods. After acetylation 2-3 µl were injected on a 6 foot 0.3% OV 275, 0.4% XF 1150 column.

The peaks are identified in all subsequent methylation analyses by the same numbers:

1) T-Ara, 2) T-Xyl, 3) T-Fuc, 4) 2-Ara, 5) 2-Rha, 6) 3-Ara, 7) 5-Ara, 8) T-Gal, 9) 2 or 4-Xyl, 10) unidentified, 11) 2,4-Rha, 12) 3-Gal, 13) unidentified, 14) unidentified, 15) 4-Gal, 16) 4-Glc, 17) 2,3,5-Ara, 18) 6-Gal, 19) unidentified, 20) unidentified, 21) 4,6-Gal, 22) 4,6-Glc, 23) 3,6-Gal, 24) free Gal, 25) free Glc, 26) Inositol.

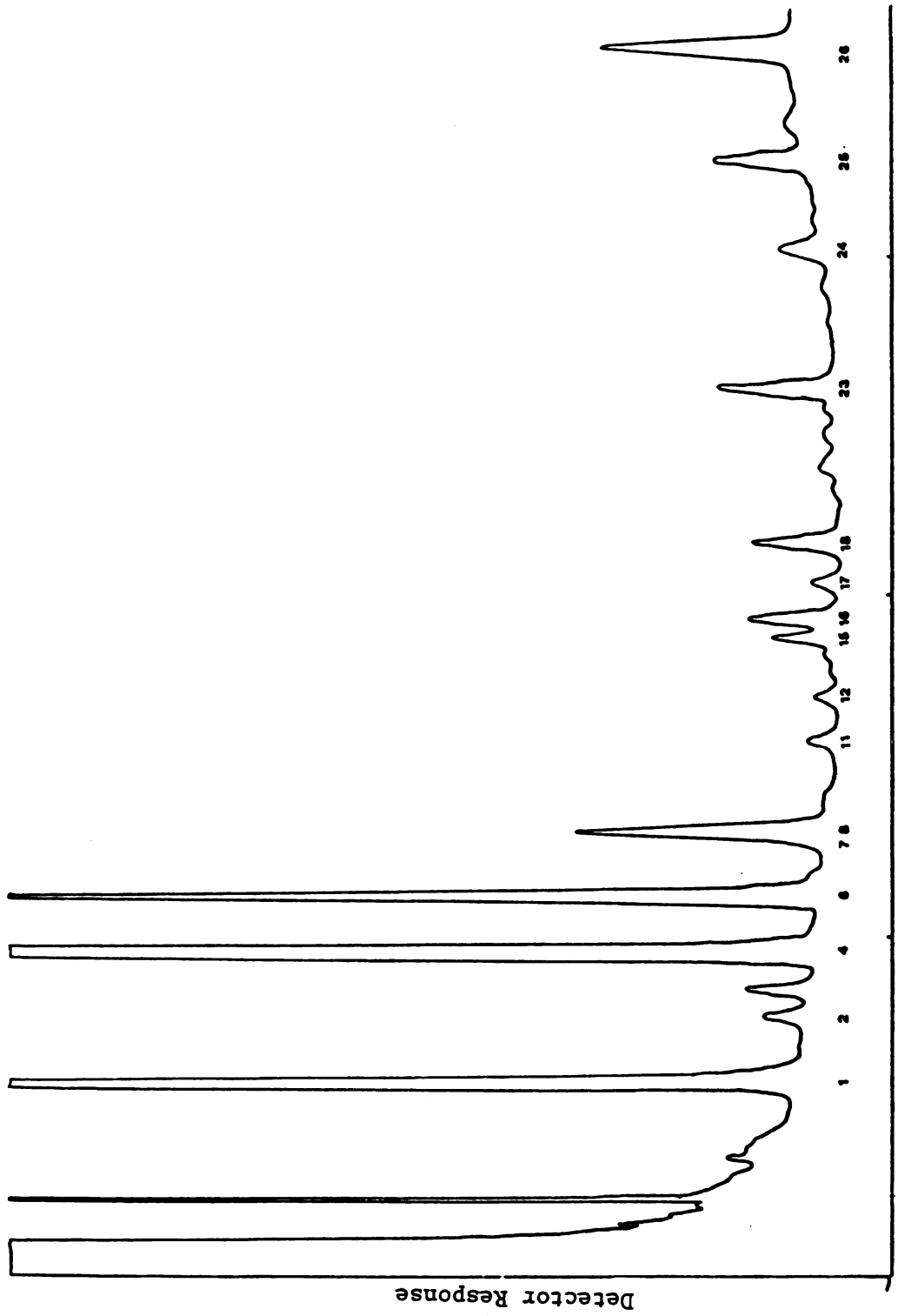

Detector Response
0
20
40
60
Time (minutes)
1
2
4
6
7 8
11
12
15 16
17
18
23
24
25
26

Figure 29

causes 80% destruction of the serines, indicating 80% glycosylation of the serines.

I had expected that the galactosyl-serine linkage would be broken in a β-elimination reaction caused by the strongly alkaline conditions of the methylation procedure. Lindberg (97) reported that O-acetyl groups are cleaved during the methylation reaction. But Baenziger and Kornfeld (116) found no evidence of β-elimination when working with O-glycosidically linked oligosaccharides of IgA, and Lindberg (97) finds no evidence of the destruction of methyl esterified uronic acids which are known to be susceptible to β-elimination induced by alkali.

Present in only slightly lesser quantities than T-galactose are 3,6-; 3-; and 6-linked galactose (Figure 29), the sugars characteristic of the oligosaccharide Keegstra et al. hypothesized links extensin to the pectins of cell walls. There are in addition small amounts of the sugars characteristic of pectins: 4-linked galactose, 5-linked arabinose and 2,4-linked rhamnose, but only a trace if any of 2-linked rhamnose. There is also the unexplained presence of 4-linked glucose.

The denser fractions (see Figure 23) off the CsCl gradient of the Hyp-rich peak on DEAE cellulose are much richer in sugars and poorer in Hyp. These fractions also contain 3,6-; 3-; and 6-linked galactose, but lesser amounts of 2- and 3-linked arabinose, consistent with the lower percentage Hyp (Figure 30). There is a much increased presence of 4-linked galactose, 5-linked arabinose, 2,4-linked rhamnose, and the appearance of a small amount of 2-linked rhamnose, indicating a greater proportion of pectin. Again, there is the unexplained presence of

Figure 30. Methylation analysis of the denser Hyp fractions from a CsCl gradient.

The fractions designated dense Hyp in Figure 23 were pooled, dialyzed, and methylated as described in the Methods.

For peak identities see Figure 29.

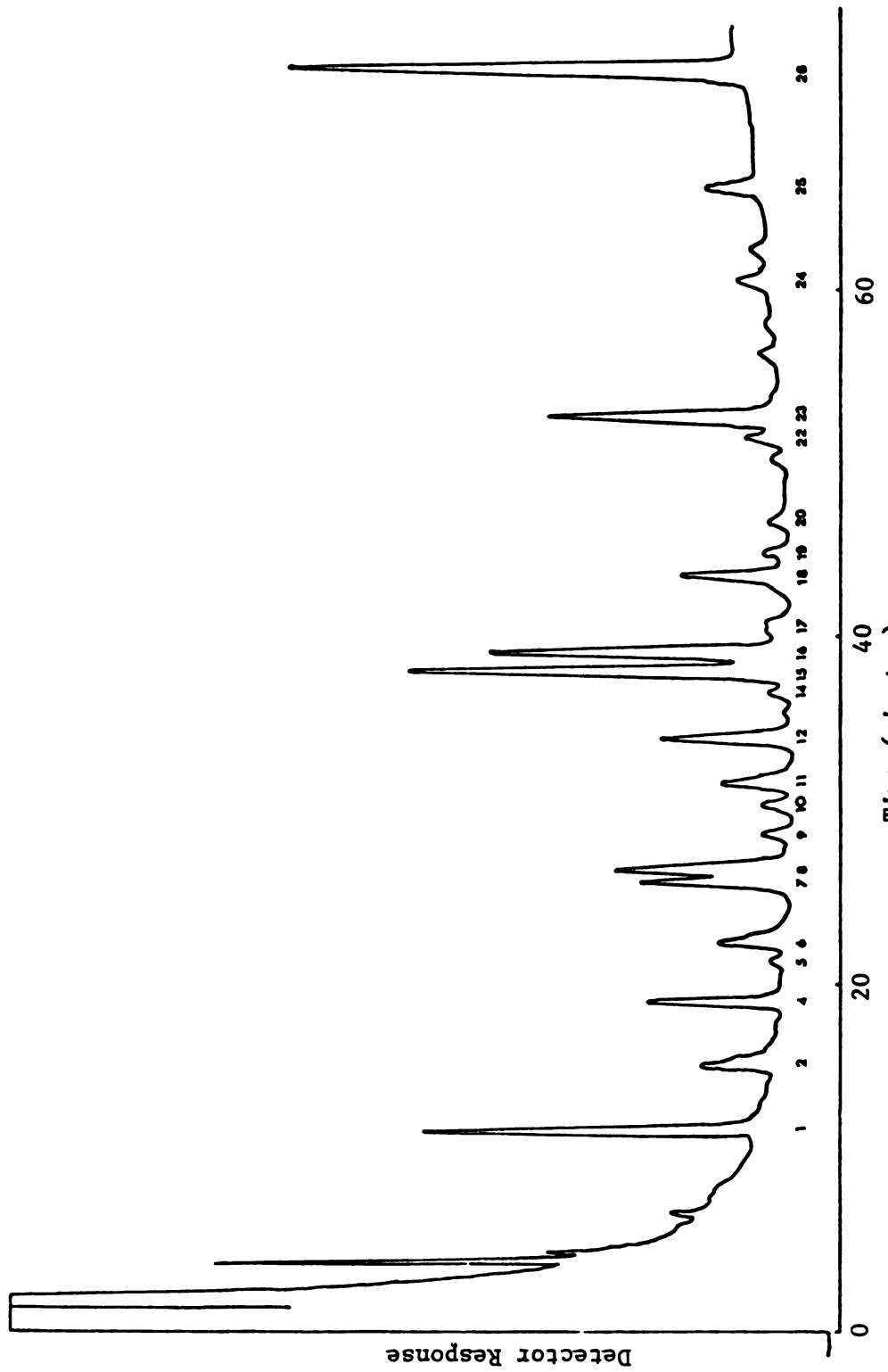

Detector Response
0
20
40
60
Time (minutes)
1
2
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
22
23
24
25
26

Figure 30

4-linked glucose, suggesting that either tomato walls are a little different from those of sycamore-maple, or that the amylase treatment used by Talmadge et al. (99) was removing something other than just starch from the walls.

The dense fraction off the CsCl gradient of the pectinaceous fraction on DEAE cellulose (see Figure 25) contains little 3,6; 6-; and 3-linked galactose but a large amount of 2-linked and 2,4-linked rhamnose, T- and 4-linked galactose, and again 4-linked glucose (Figure 31). The GLC profile for this fraction resembles that of methylated pectin released from the cell walls by boiling ammonium oxalate (Figure 31).

b, Smaller Molecular Weight Peptides

The smaller molecular weight peptides (in the void of Sephadex G-50, retained on A .5 m), which were adsorbed on DEAE cellulose and purified from any contamination by free polysaccharides by CsCl centrifugation, were methylated and found to contain almost exclusively the T-, 2-, and 3-linked arabinose expected for Hyp-arabinosides and T-galactose adequate to account for the hydrazinolysis data indicating 80% glycosylation of the serine residues of the peptides. Only traces of the other linkages present in the large molecular weight peptides were present (Figure 32).

Figure 31. A comparison of the pectinaceous fraction of a DEAE cellulose column effluent with the washed ammonium oxalate extract from tomato cell walls.

Top trace: The dense fractions pooled as indicated in Figure 25.

Bottom trace: Washed ammonium oxalate extract.

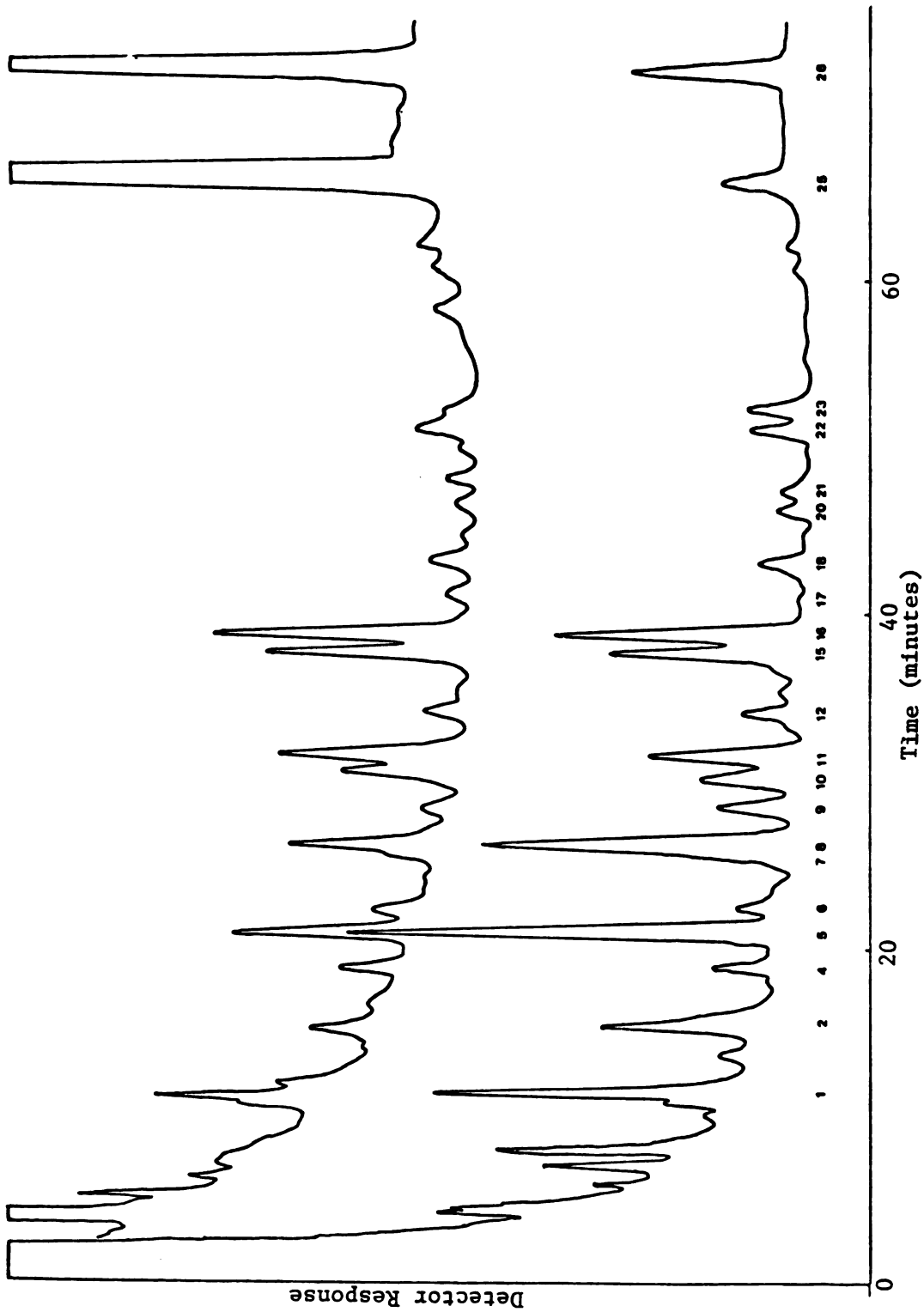

Detector Response
0
20
40
60
Time (minutes)
1
2
4
5
6
7
8
9
10
11
12
15
16
17
18
20
21
22
23
25
26

Figure 31

Figure 32. Methylation analysis of the smaller molecular weight peptides. For peak identities see Figure 29.

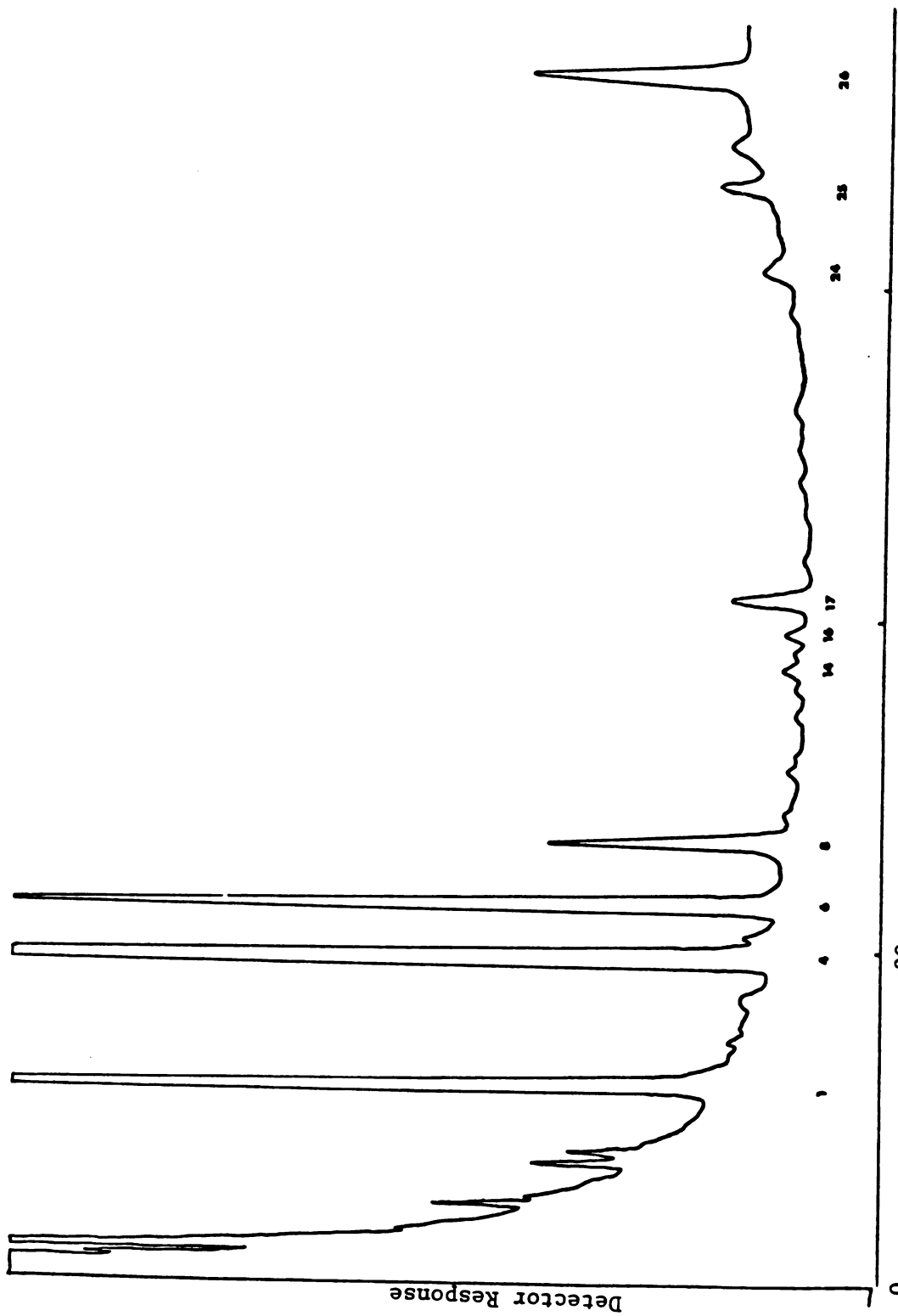


Figure 32

D. Discussion

The experiments in Section II-C indicate that $NaClO_2$ oxidation solubilizes large fragments of extensin from cell walls. These fragments are for the most part extremely rich in Hyp, over 50 mole percent, and contain the amino acids found in the short tryptic peptides described and sequenced earlier by Lamport (11). The peptide portion of the glycopeptides solubilized by $NaClO_2$ cannot have been degraded to a great extent, since the size of lysozyme treated with $NaClO_2$ under the same conditions is not drastically reduced. However, lysine is changed to α-amino adipic acid, and tyrosine and the unidentified amino acid are destroyed or changed to aspartic acid. From the literature (see Section II-A) one would not expect $NaClO_2$ oxidation to change the polysaccharides attached to the peptides and so the oligosaccharides of the glycopeptides released should represent those present in untreated walls. Unfortunately, the conditions used for the $NaClO_2$ oxidation are such that pectins whether or not linked to protein would be extracted from the walls along with the glycopeptides. Thus, strong evidence for the covalent linkage of any oligosaccharide to the peptides must be provided before any conclusions can be made about the linkages of extensin to the wall polysaccharides.

In addition to the problem of co-extraction of free polysaccharides and glycopeptides, there is the problem of random breakage of the polygalacturonic acid chains. Thus, if a peptide is linked directly or indirectly to the pectic polymers the size of its oligosaccharide side chains will be very variable.

Some evidence that the peptides are linked to a polymer containing galacturonic acid was given in Section II-C-5. The CsCl density gradient centrifugation experiments provide the strongest evidence for the attachment of pectins to extensin:

1. When the major peak fractions of hydroxyproline from a DEAE cellulose column (Figure 18) are centrifuged in CsCl, there is galacturonic acid associated with the resulting major Hyp peak (see Figure 23) at a density much lower than 1.6 g/ml.

2. There is Hyp which behaves as though it has a greater density than this major peak, indicating more highly glycosylated peptides (Figure 23).

3. The more tightly adsorbed pectinaceous material on a DEAE cellulose column contains a lesser amount of Hyp which floats away from the majority of the pectin on CsCl centrifugation but this Hyp also is associated with galacturonic acid (Figure 25).

Methylation analysis of the Hyp peaks (Figure 29) from the CsCl gradients show that they contain the sugar linkages of the 3,6-linked arabinogalactan proposed to link extensin to the pectins. These sugar linkages are present in only trace amounts in the pectic fractions not associated with Hyp (Figure 31). The Hyp-containing peptides also contain T-, 2-, and 3-linked arabinose and enough T-galactose to account for a single galactose residue on every serine residue (Figure 29).

These results strengthen the possibility, suggested by W. D. Bauer (personal communication) that extensin may allow cell wall growth by a transglycosylation mechanism. Growth would be mediated by an

enzyme which can either be glycosylated at its active site by a single galactose or a 3,6-linked galactan. Such an enzyme would be similar to dextran synthetase which uses sucrose as its substrate to make dextran and fructose, thereby dispensing with the need for a sugar phosphate intermediate. The interchange of galactose for the 3,6-linked galactan would make or break cell wall cross links thus allowing extension.

There are still quite a few questions to be answered before we can truly describe the structure of extensin in cell walls. It has become apparent from the work described here that some type of linkage in addition to glycosidic links are holding the protein in the wall. What are these linkages? The amino acid composition of the residue from cell walls after they have been treated with liquid HF shows that although there is very little sugar detectable there is only 40% of the residue which can be accounted for by amino acids. The rest is unidentified. When sycamore-maple or tomato cell walls were methylated and extracted with chloroform:methanol the residue again contained most of the cell wall protein along with some sugars, but surprisingly (99) there was relatively little glucose present. Most of the sugars were arabinose (T, 2 and 3-linked as in the Hyp-arabinoside). This residue appeared microscopically to be cell wall shaped (Figures 33 and 34), but from the sugar linkage analysis contained very little cellulose, again indicating that the protein holds together in the shape of a cell wall in the absence of polysaccharides. This suggests to me that there is an unidentified cross link between the protein molecules of the wall.

Figure 33. The appearance and methylated sugar composition of sycamore cell walls.

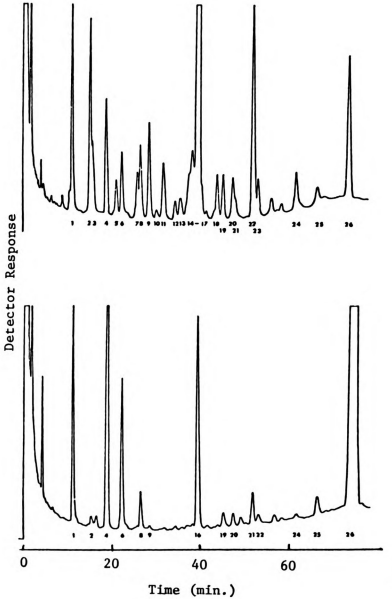
Top left: The chloroform/methanol soluble methylated sugars of sycamore cell walls.

Top right: The microscopic appearance of untreated sycamore cell walls.

Bottom left: The insoluble residue of methylated sycamore cell walls.

Bottom right: The microscopic appearance of the insoluble residue of methylated sycamore cell walls.

Peak identities as in Figure 29.



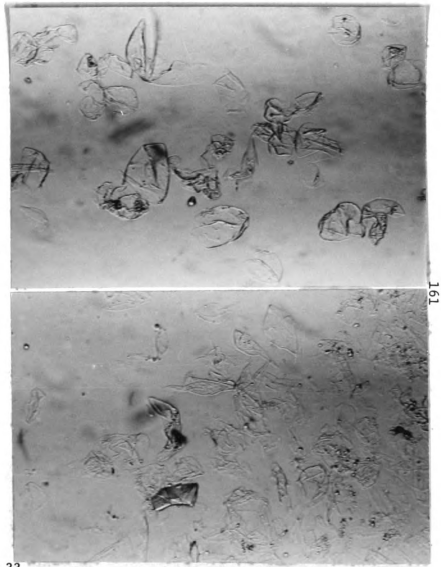


Figure 33

Figure 34. The appearance and methylated sugar composition of tomato cell walls.

Top left: The chloroform/methanol soluble methylated sugars of tomato cell walls.

Top right: The microscopic appearance of untreated tomato cell walls.

Center left: Methylation analysis of $NaClO_2$ extracted tomato cell walls.

Bottom left: The insoluble residue of methylated tomato cell walls.

Bottom right: The microscopic appearance of the insoluble residue of methylated tomato cell walls.

See Figure 29 for peak identities.

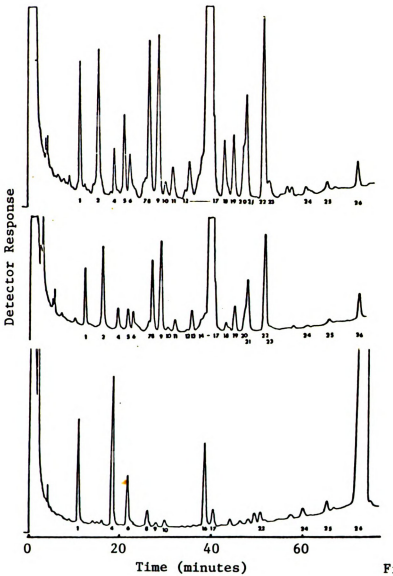


Figure 34

The resistance of these cross links to both HF and the strongly basic conditions of methylation analysis excludes the possibility that the cross links are either glycosidic or ester linkages. If the cross links were direct condensations between amino acids of adjacent peptides as in collagen (117) there is still the presence of the unidenfified material in the HF residue of cell walls. My failure to account for all of the cell walls as sugar and amino acids, points to a firmly attached additional component.

Are these cross links artifacts of the procedures used to isolate or treat the walls or are they naturally occurring? This question remains unanswered. Plants produce an abundance of secondary products including phenolic compounds which are compartmentalized in the intact cell but upon cell breakage are released and can interact with proteins. In the case of suspension cultures, the cells are surrounded by the same medium for anywhere up to 1 month. Therefore, any excreted products or released products from dead cells are in contact with the cell wall for a long period of time.

The reactions of proteins in HF, especially in the presence of large amounts of sugars, has not been well-characterized but could by some unknown mechanism cross link the protein. This would not explain the retention of cell wall shape after removal of most of the wall polysaccharides by methylation. A different unexplained mechanism would have to be involved for the generation of cross links under alkaline conditions. Thus, it is unlikely that the cross links are artifacts of the cell wall treatments (HF or methylation). The possibility that the

cross links are generated during the cell wall isolation cannot be excluded, although, as mentioned earlier, all my precautions to prevent interaction of soluble cell contents with the walls during their isolation failed to prevent the cross linking.

This leaves the possibility that the cross links are formed because the cells, being in suspension culture, were surrounded by their own by-products or that the cross links are normally present in the walls of the cells that these cultured cells represent in the whole plant.

Whitmore (118) has investigated the occurrence of lignin in wheat coleoptiles and its effect on cell elongation. Coleoptiles are one of the most popular plant parts for the study of the plant hormone auxin since they are very responsive to the hormone yet are one of the simplest tissues. Whitmore fed radioactive phenylalanine (a known lignin precursor) to coleoptiles and then partially characterized the labelled cell wall products by their extractability in various solvents. About one-fourth of the radioactivity was found in lignin-like molecules or precursors to lignin. These lignin-like molecules were only extractable in alkali, not in hot water, indicating that my ammonium oxalate extractions would not remove them (c.f. the low percentage of sugar in depectinized cell walls--Table 15). Whitmore found a residue which he called lignin that was insoluble in all his solvents including 6 N HCl at 100 C., 72% H_2SO_4, and 24% KOH. Almost all of the counts in the intact walls could be removed by a prolonged (4 hours) sodium chlorite treatment alone. Thus, the presence of lignin and compounds of a phenolic nature in the walls of growing tissue has been reported.

Côté et al. (119) have used very concentrated aqueous (up to 80%) HF, as Fredenhagen and Cadenbach suggested (22) to remove the sugars from wood to allow electron microscopic observation of the deposition of lignin. Their electron micrographs show that the cell walls, even though devoid of sugars, retain their original shape.

Plants and plant tissue cultures are renowned for their production of phenols. There have been many reports of phenol production by suspension cultures (120,121,122). The presence of phenols in walls of suspension cultured cells has not been reported specifically, but Nimz et al. (123) have detected hardwood-like lignin in washed, dried soybean suspension cultures by ^{13}NMR spectroscopy.

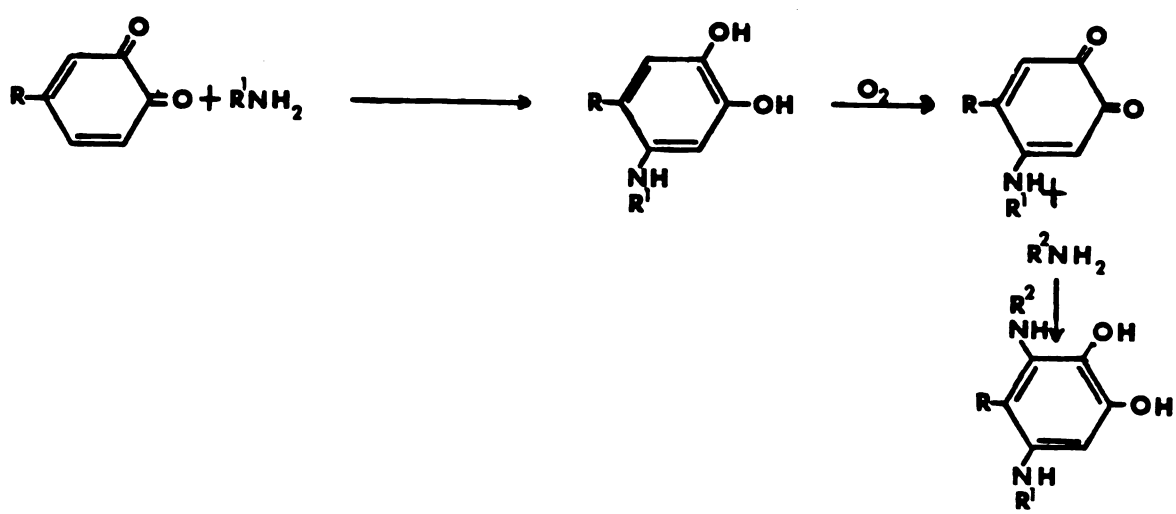
Another indication that there could be phenolic compounds in cell walls comes from the work of Burke et al. (124). Their estimate of the protein content of cell walls of rye grass cultures by the Lowry assay was three and a half times that of Smith and Stone (125) who determined total nitrogen. The sugar analysis of both groups agreed well, thus the protein estimates should be comparable. It is likely that the Lowry assay was measuring phenolic compounds extracted from the walls by the alkali of Lowry reagent A (46). In the same report (124), Burke et al. state that they could account for between 18 and 107% of the extracellular polymers precipitated by 70% ethanol from the culture medium of a range of plant suspension cultures as polysaccharides and protein. For all but two cultures they accounted for less than 60% of the polymers. What is the identity of this unidentified polymeric material? Again, they assayed for protein using the Lowry assay and found high protein

contents as they did in cell walls. Yet, in the only case in which protein was determined by total nitrogen, sycamore-maple, the protein content of the analyzed extracellular polysaccharides was less than 1%. In all the cultures tested by the Lowry assay, the protein content of the extracellular material accounted for 20-40% by weight.

Since the extracellular polymers of suspension cultured cells are in most cases very similar to those in the cell walls (124,126), it is likely that the unidentified polymers are also present in cell walls.

Phenols are readily oxidized to quinones which Morrison and others have found (127) readily form 1,4 addition products with amino groups and thiols, and an uncharacterized addition product with tyrosine or other phenols.

In plants there are enzymes which oxidize phenols to orthoquinones which like para-quinones are very reactive with amino groups (128).



Thus it is logical to propose that in the wall, extensin is not only linked to cellulose microfibrils indirectly via a 3,6-linked

galactan, but is also linked to itself by phenolic cross links. Therefore, to extract intact extensin it will be necessary to break both types of cross links without breaking the protein. At present this cannot be done as the cross links are not identified. The presence of phenolic cross links would explain why $NaClO_2$ oxidation releases protein from walls, and why no method which does not break peptide bonds has been devised to release extensin from wall. Perhaps the only way to obtain intact extensin will be to extract it from cells before it is put into the wall.

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