HYDROXYPROLINE IN WHEAT ENDOSPERM AND WHEAT SEEDLING ROOTS

by

Annemarie Heyne

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas 66506

1976

Approved by:

Philip Nordin

Major Professor: Philip Nordin
Department of Biochemistry

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Lamport (1971) reported that hydroxyproline-arabinosides form a structural entity in the cell walls of numerous species throughout the plant kingdom. His investigations included two Bryophyta and two members of the Pteriodophyta (Lamport and Miller, 1971). In Spermatophyta hydroxyproline-arabinosides were present in Angiospermae (both monocotyledons and dicotyldeons) as well as in Gymnospermae. More work has been done on dicotyledons than on monocotyledons. It should be mentioned that the material used for investigation in general was support tissue rather than storage tissue. Due to the different functions, it is likely that the cell walls in the latter differ from those in the former with respect to their chemical composition.

When this work was started, no report had been published on the hydroxyproline content of the cell walls of any storage tissue in monocotyledons. Flour was chosen as an example of endosperm of gramineae because it is readily available and non-starchy polysaccharides had already been extracted from it. The chemical composition of flour is comparatively well known. Pentosans - classified into water-solubles and water-insolubles, the latter being sometimes referred to as hemicelluloses (Kulp, 1968)- have been extensively investigated.

Thus a search for hydroxyproline in water-soluble and water-insoluble pentosans was started. During the course of this work, Fincher and Stone (1974a) published a method for extraction of a hydroxyproline-rich glycopeptide from wheat flour. Their water extraction of ethanol-pretreated flour seemed to be suitable for examining flour for the hydroxyproline content of pentosans. A trichloroacetic acid (TCA) extraction method also was used to obtain hydroxyproline containing polymers. The TCA procedure allows one to roughly estimate the origin of the extract as cytoplasmic. It became the preferred method throughout this work.

LITERATURE REVIEW

I. THE PRIMARY CELL WALL

The primary wall is considered to be that part of the wall which the young growing cell deposits on both sides of the middle lamella. It is a thin, non-rigid lamella; however, it already possesses great mechanical strength. As long as the cell developes and increases its surface, the wall is recognized as primary.

The secondary wall is formed after growth has ceased. The wall then is thickened by apposition of mainly cellulosic material onto the primary wall (Hall et al., 1974).

The plant systems under investigation in this thesis are wheat seedling roots and wheat endosperm, whose cell walls show a structure characteristic for primary walls (Mares and Stone, 1973a). Thus, mainly the primary walls are reviewed.

Of what materials does a primary wall consist? Approximately 90% of the structural material of the wall is polysaccharide, the remaining 10% is protein (Albersheim, 1975; Northcote, 1969). Cellulose is embedded in a matrix of pectic substances, hemicellulose and protein. Northcote (1969) defined pectins as the fraction of cell wall matrix polysaccharides that is soluble in water or in solutions of chelating agents for

calcium ions, and hemicelluloses as the fraction that is soluble in alkali solution. Pectins are rich in galacturonic acid, and usually also contain L-arabinose and D-galactose as the main monosaccharide residues, though other sugars including rhamnose, fucose, glucose and xylose may be present. The hemicelluloses are very complex in their composition; xylans and glucomannans are important constituents. Sometimes the distinction between pectin and hemicellulose is blurred. Polysaccharides have been isolated which resemble pectin in composition, but showed typical solubility properties of hemicelluloses (Hall et al., 1974).

Composition data of cell walls have been obtained from different systems. Thimann and Bonner (1933) investigated the walls of avena coleoptiles. They found 42% cellulose, 38% hemicellulose, 8% pectin and 12% protein. Albersheim et al. (1973) described the primary cell wall of sycamore (Acer pseudoplatanus) cell-suspension cultures as consisting of one third cellulose, one third hemicellulose and one third pectin and protein. The suspension-cultured sycamore cells are believed to contain only primary wall. The data on Acer pseudoplatanus closely resemble those obtained from the bean Phaseolus vulgaris, the two being distantly-related dicotyledons.

The most recent model of the primary cell wall of sycamore cell culture reported by Albersheim and his co-workers

(1975) shows cellulose-fibers coated with a layer of xyloglucans. The latter are hydrogen bonded to the cellulose. The reducing end of each xyloglucan molecule is glycosidically bound to an arabinogalactan, which in turn is linked to a rhamnogalacturonan. The protein component is not included in this model. Albersheim (1975) depicts the arabinogalactan as consisting of a chain of arabinose units appended to a chain of galactose units. Relatively few side chains are present and have only one sugar unit. The cell walls of dicotyledons appear to be very similar to each other in composition. However, the model just described in detail does not fit data obtained on monocotyledons (Albersheim, 1975).

Burke and co-workers (1974) also investigated the primary cell walls of a suspension-cultured Douglas fir. They found them structurally more related to the walls of dicotyledons than of monocotyledons. Hydroxyproline was more abundant in Douglas fir walls than in any of the monocotyledons they examined, but less than in sycamore. The two hydroxyproline-arabinosides from sycamore were not present in Douglas fir. Lamport and Miller (1971) reported hydroxyproline-arabinosides in three other gymnospermae: Ginko biloba, Cupressus spec. and Ephedra spec..

II. THE PROTEINACEOUS CELL WALL COMPONENT EXTENSIN

Protein in the cell wall was noted as long ago as 1888 (Wiesner, 1888). It is well researched in sycamore (Acer pseudoplatanus) suspension culture (Albersheim et al., 1973), carrot root slices (Gardiner and Chrispeels, 1975), pea hypocotyl (Sadava et al., 1973), tomato stem callus (Lamport, 1970), and tobacco callus (Olson, 1964). Lamport called the protein extensin (Lamport, 1964), speculating that its role might have something to do with cell extension. This protein is part of a glycopeptide, containing arabinose and galactose. Hydroxyproline is the most abundant amino acid. For this reason, hydroxyproline has been used as a marker amino acid in tracing extensin.

It is difficult to definitely describe the structure of a cell wall component, because the polymers cannot be extracted from the wall without degradation. Extraction with acid or base resulted in simultaneous, but partial, cleavage of several types of bonds in the wall (Albersheim et al., 1973). Substructures like the hydroxyproline-rich glycopeptide extensin can only be investigated upon degradation. Most of the hydroxyproline-rich glycoproteins are covalently attached to the cell wall matrix (Chrispeels and Sadava, 1971). In addition, before degradation, the wall has to be defined and purified.

Which method leaves the walls contaminated with plasmatic parts of the cell? Which method of purification also extracts essential wall components?

In the case of carrot discs, Chrispeels (1969b) ground them with 0.5% Nonidet P40, a nonionic detergent. The insoluble part was designated wall, which he then fractionated further. Since detergent can solubilize membranes, the method seems to be adequate to clean the cell wall from cytoplasmic components; the wall of young pea stems was obtained by homogenizing segments in water, the insoluble part was further washed and sedimented by centrifuging at 1000xg for three minutes. Sycamore and bean-cells were broken by shaking a thick suspension in water with glass beads until no intact cells were present (Lamport and Northcote, 1960). A preparation, indisputably "cell walls," were obtained from Chlamydomonas gymnogama which sheds its walls during mating. Cell walls could simply be collected by differential centrifugation (Miller et al., 1974).

From cell walls liberated in that fashion, the glycoprotein complex extensin was isolated. Since it could not be purified without the use of degradative methods, it was difficult to obtain complete information about its structure. Using degradative procedures, fractions containing hydroxyproline-

arabinosides or additional galactose and serine were obtained (Lamport, 1972). Hydrolysis in 0.22 M barium hydroxide for six hours at 100°C released hydroxyproline-arabinosides from the cell wall. These have low molecular weights and react like free hydroxyproline with the specific isatin reagent (Lamport, 1971).

Glycopeptides containing arabinose, galactose, hydroxyproline, serine, tyrosin and frequently lysine and valine were obtained by degrading cell walls of dicotyledons with a crude cellulase/protease preparation from Aspergillus niger (Lamport, 1969). Hydroxyproline was the most abundant amino acid in these glycopeptides which were stable in weak alkali (0.5N potassium hydroxide, eighteen hours, 4°C), but were hydrolyzed by strong alkali yielding free amino acids plus hydroxyproline-O-arabinosides (Lamport, 1971).

It was shown by electrophoresis that both the secondary amino and the carboxyl groups in hydroxyproline were free. Thus, a hydroxyl group was the most likely point of substitution. The arabinosides did not reduce alkaline silver nitrate; hence the reducing group of arabinose was blocked. However, treatment at pH 1 for one hour at 100°C (which cleaves arabinosides) split the arabinosyl-hydroxyproline linkage. The peptide then became susceptible to proteolytic attack (Lamport, 1971).

Hydroxyproline in plant cells was generally found to be at least partially arabinosylated. In lower plants, di- or trisubstituted hydroxyproline was reported; in dicotyledons, tetraarabinosides predominated; in corn, unsubstituted hydroxyproline was followed in order of occurrence by trisubstituted hydroxyproline (Lamport, 1971).

A cytoplasmic precursor has been reported, which appears to be identical to extensin except that it is not irreversibly bound to the cell wall matrix. It can easily be extracted from homogenized cells with nondestructive reagents (Brysk and Chrispeels, 1972). Very elegant kinetic studies performed by Olson (1964) justify the extract being regarded as an extensin precursor. Tobacco stem callus suspension-cultured cells were incubated with ^{14}C-proline for half an hour and chased for different lengths of time. The ^{14}C-proline was taken up in contrast to ^{14}C-hydroxyproline which was not incorporated. The radioactivity was then determined in the protoplasm, in the wall material that was extracted from the cell wall by a salt solution, and in the residual wall. The specific activities of both proline and hydroxyproline in the protoplasmic fractions were originally high and about equal; both declined rapidly. However, in the salt-extractable wall, only some turnover occurred in proline and the activity continued to increase in hydroxyproline.

About the same effect was found in the cell wall-residual fraction, but more pronounced. Similar kinetic experiments, showing a precursor-product relationship in a glycopeptide from the protoplasm and from the wall, have been reported with carrots (Sadava and Chrispeels, 1971a; Gardiner and Chrispeels, 1972; Brysk and Chrispeels, 1972; Chrispeels, 1969a; Chrispeels et al., 1974; Gardiner and Chrispeels, 1975).

Chrispeels and others, in a series of investigations (see above), showed the steps in extensin formation: proline is enzymatically oxidized to hydroxyproline, then glycosylated by arabinose, and finally the completed glycoprotein is secreted to the cell wall. When carrot discs were incubated with labelled proline, label was found in hydroxyproline. Even after the label was removed or was chased with excess nonradioactive proline, the biosynthesis of radioactive peptidylhydroxyproline continued. Thus it was suggested that hydroxyproline can be synthesized by plant cells from proline.

The hydroxylation occurs after the proline is incorporated into the protein, since the protein synthesis inhibitor cycloheximide did not inhibit the oxidation of already incorporated proline to hydroxyproline. The chelating agent α,α-dipyridyl, which inhibits the formation of bound hydroxyproline from bound proline, but does not have any effect on

overall protein synthesis, together with ferrous ions, which cause a sudden initial increase of hydroxyproline synthesis, served as useful tools to investigate the time of the oxidation of the proline. When cycloheximide was added, just prior to ferrous ions, to carrot discs that were preincubated with dipyridyl and then labelled with ^{14}C-proline, protein synthesis stopped, but a burst of hydroxyproline synthesis took place in the cytoplasm. The latter statement is based on the finding that nearly all of the hydroxyproline formed after addition of ferrous ions was found in the cytoplasm and not in the wall (Chrispeels and Sadava, 1971). A peptidylproline hydroxylase has been isolated from carrot discs and other tissues which converted peptidylproline to peptidylhydroxyproline in vitro (Sadava and Chrispeels, 1971b).

The attachment of arabinose to hydroxyproline may also occur in the cytoplasm, since hydroxyproline arabinosides similar to the ones isolated from the wall could be found in the cytoplasm. When tissue was incubated in either ^{14}C-proline or ^{14}C-arabinose, the radioactivity first appeared in the hydroxyproline in the isolated glycopeptides and three to four minutes later in the sugar. An enzyme that catalyzes the glycosylation of extensin has been isolated by Karr (1972).

Chrispeels (1969b) and Olson (1964) described the secretion of the glycopeptide to the wall. However, the evidence for

this process is meager. For instance, in Olson's pulse-chase experiment, the loss of radioactivity in hydroxyproline in the cytoplasm is much higher than the gain in the cell wall.

III. THE CELL WALL OF GRAMINEAE

The cell walls of gramineae, as investigated on six suspension-cultured monocots (Burke et al., 1974), are remarkably similar to each other, but substantially different from the ones of dicotyledons. Arabinogalactan, xyloglucan, and rhamnogalacturonan were found in the dicotyledon cell wall in addition to cellulose. In the six monocotyledons: wheat, oats, rice, sugar cane, brome grass and rye grass, an arabinoxylan comprised 40 to 60% by weight of the wall in the cultured cells. Since the arabinoxylan had been isolated from a wide variety of monocotyledon tissues (Aspinall et al., 1963; Aspinall et al., 1956), it can be categorized as the principal component of the primary cell walls of monocotyledons. In wheat endosperm, for example, the arabinoxylan was reported to make up 85% of the polysaccharides in the cell wall, in addition to smaller amounts of glucomannan and cellulose (Mares and Stone, 1973a).

The amount of protein is in the same range as in dicotyledons. However, the amino acid composition is different, in that monocotyledons or at least gramineae are low in hydroxyproline; only very minor amounts have been found (Table 1). The cell line isolated from endosperm contained no detectable amounts of hydroxyproline. Hydroxyproline could not be detected in cell walls from barley endosperm or from barley

aleuron (Fincher, 1975; McNeil et al., 1975). However, Cleland (1967) found 60% of all the hydroxyproline in oat coleoptiles localized in the wall; also hydroxyproline arabinosides were found in Zea mays pericarp walls (Lamport and Miller, 1971).

TABLE I. PROTEIN AND HYDROXYPROLINE CONTENT IN THE WALLS OF SIX SUSPENSION-CULTURED MONOCOTYLEDONS AND SYCAMORE

Species	Cell Line Isolated from	Protein in Cell Wall*	Hydroxyproline in Cell Wall*
Wheat	root tissue	11	0.14
Oats	embryo	16	-
Rice	root tissue	17	0.13
Sugar cane	internode	15	0.14
Brome grass	embryo	14	0.16
Rye grass	endosperm	7	0.05
Sycamore	cambium	10	2.0

*Values are weight percent

These differences cannot readily be explained by the extraction methods that these authors used. Fincher (1975), as well as Cleland (1975) regarded as wall the residue that remained after extraction of tissue with aqueous ethanol.

Fincher used 70% ethanol, whereas Cleland used 80% ethanol. After the ethanolic extraction, Cleland washed the walls with water. McNeil (1975) isolated walls after an aqueous washing procedure. Note that a hydroxyproline-rich glycopeptide, which will be discussed in more detail later, was extracted from flour (Fincher and Stone, 1974a) which was precipitated in 80% ethanol, but was soluble in 70% ethanol. If it were present in oat coleoptiles, water would have washed it away. It should be mentioned that another monocotyledon, namely asparagus spec., was reported to have cell walls rich in hydroxyproline (Selvendran, 1975a).

Since the beginning of this century, research has been done on the so-called pentosans in flour, especially with respect to their industrial use for bread baking. One distinguishes water-soluble and alkali-soluble pentosans. The latter are frequently referred to as hemicelluloses. The alkali-soluble part is believed to be associated with cellulose of the wall. Once liberated from the wall, the hemicelluloses become water-soluble. The major sugars in the water-soluble pentosans are arabinose and xylose, as observed for the cell wall matrix. There is no report about hydroxyproline in the hemicelluloses.

In dicotyledons, workers have been looking for wall

precursors in the part of the cell that is soluble in non-destructive solvents. Hydroxyproline served as a marker (Cleland, 1968); however, hydroxyproline-containing fractions do not necessarily have anything to do with the cell wall. It is especially difficult to verify the origin of trace amounts which might be cytoplasmic contaminants.

Since this thesis deals with water-soluble, non-starchy polysaccharides, literature concerning water-soluble pentosans and the probability of their relation to the cell wall will be reviewed.

IV. WATER-SOLUBLE PENTOSANS

Water-soluble pentosans comprise less than 1% of wheat endosperm flour (Kakuda, 1973). They are very heterogeneous with respect to molecular weight and charge, as judged from chromatography on columns of DEAE-cellulose. Kakuda (1973) estimated the molecular weights of water-soluble pentosans using dextran standards for pentosan fractions containing mainly carbohydrates, and globular protein standards for fractions predominantly composed of protein. Weights varied from 26,000 to 140,000 using carbohydrate standards, and from 2,150 to 150,000 when calculated from protein standards.

Kündig et al. (1961) fractionated water-soluble pentosans from wheat endosperm into five fractions on a column of

DEAE-cellulose column; the column eluted with borate. Fraction I, the biggest fraction, contained arabinoxylan only. Fractions II to V contained various amounts of protein, in addition to arabinose, xylose, and galactose. Fraction II, a glycoprotein, could be split, on digestion by pronase, into two high molecular weight fractions (Neukom, et al., 1967): an alcohol-insoluble arabinoxylan containing no galactose, and an arabinogalactan, containing some protein, composed of 70% galactose and 30% arabinose. Kakuda (1973) subdivided peak II into two subfractions using Kündig's (1961) method: Peak IIa, low in protein, and Peak IIb, containing two to three times as much protein, depending on the flour used. The carbohydrate data agree with Kündig's observations for peak II, insofar that both fractions of peak II contained arabinose, xylose, and galactose as the only sugars present.

Neukom's results from digestion with Pronase suggest that galactose was not linked directly to the main xylan chain, but rather occurred as an arabinogalactan which then was connected via a polypeptide bridge to arabinose in the arabinoxylan.

Several authors have speculated about the nature of the carbohydrate-protein linkage. The amino-group of an amino acid could be bound to a sugar. Rehfeld (1963) mentioned the presence of an amino-sugar in rye-pentosans; he stained a

pentosan hydrolysate on a thin-layer plate with a reagent specific for amino-sugars. Wrench (1966) found an amino-sugar in wheat pentosans by proteolytic digestion of a water-soluble glycoprotein obtained by salt extraction. Hydroxylproline has been suggested as the linking amino acid (Fincher and Stone, 1974a).

V. HYDROXYPROLINE IN WATER-SOLUBLE GLYCOPEPTIDES IN CEREALS

It is difficult to decide which part of the pentosans is a cytoplasmic component. The term "pentosan" is defined by composition and not by biological function. At least in wheat, water-soluble pentosans are composed of a straight chain of xylopyranosyl residues, linked beta-1, 4, to which are attached arabinofuranosyl residues at the 2 or 3 position of individual xylose units (D'Appolonia, 1973). In general, it is assumed that polysaccharides which are soluble in 70% ethanol have a low molecular weight and have either cytoplasmic origin or stem from the middle lamella (Fincher et al., 1974). Fincher et al. (1974) and Cleland (1968) tried to locate the hydroxyproline-containing glycopeptides in the cell. Fincher et al. (1974) concluded from the fact that the arabinogalactan was isolated without recourse to degradative enzymes or chemical extraction procedures that it might not be associated with plant cell wall polysaccharides. Cleland distinguished

cytoplasmic fractions from cell wall fractions using solubility criteria. The portion soluble in 80% ethanol was designated cytoplasmic and the portion insoluble as cell wall.

Fincher et al. (1974) and Fincher and Stone (1974a) examined flour extract, from wheat endosperm, which was precipitated by 80% cold ethanol, but soluble in 70% ethanol. They separated it further into a fraction soluble in saturated ammonium sulfate (an arabinogalactan-peptide) and a fraction insoluble in saturated ammonium sulfate (an arabinoxylan). Table V (p. 51) shows the composition of these fractions. Fifty-nine percent of the total carbohydrate in the arabinogalactan-peptide was galactose and 41% was arabinose. This is in the same range as Neukom's arabinogalactan-peptide mentioned above, with its 70% galactose and 30% arabinose. The most abundant amino acids are glutamic acid, glutamine and alanine (Fincher and Stone, 1974a; Neukom, 1967). Hydroxyproline was present in a higher percentage in Fincher and Stone's preparation than the three amino acids both research groups listed as very frequently occurring. Unfortunately, Neukom and co-workers did not list hydroxyproline at all.

The composition of the arabinogalactan-peptide and its structure were determined in considerable detail (Fincher et al., 1974): 92% polysaccharide, 8% protein, of which 16 to 20% per 100 moles of amino acid was hydroxyproline. The

molecular weight was 22,000 as calculated from the sedimentation coefficient, the intrinsic viscosity and the diffusion coefficient. The structure suggested from partial degradation by acid, hydrazine, or barium hydroxide was a galactose backbone with arabinose side chains, and with hydroxyproline being the amino acid residue involved in the linkage of polysaccharide to protein. Cell walls from endosperm were found to be practically free of hydroxyproline, but it was found in the cytoplasm.

Cleland (1968) found hydroxyproline-containing cell-constituents in oat coleoptiles. He found 20% of all the cell hydroxyproline in a trichloroacetic acid-soluble, non-dialysable fraction, referred to as cytoplasmic, and 60% in the wall. He incubated avena-coleoptile sections in a medium ^{14}C-proline and studied the uptake of radioactivity in proline and hydroxyproline. Pulse-chase experiments did not show a product-precursor relationship between the cytoplasmic hydroxyproline-containing glycopeptide and the hydroxyproline in the wall. Cleland did not publish composition data of this cytoplasmic glycopeptide. He concluded that there are at least two classes of hydroxyproline-proteins; the wall-concentrated extensins and a second group which are located in the cytoplasm and are never transported to the wall.

Actually, nothing is known about the function of the

arabinogalactan-peptide and very little about the arabinoxylan. A study (Fincher and Stone, 1974b) of how gibberellic acid changes the amount or appearance of both in stimulating germination shows an approximately threefold increase of arabinose- and xylose-containing polymers. Their molecular size decreases. The increase in water-soluble pentosans is probably due to cell wall degradation. What happens to the arabinogalactan-peptide during endosperm modification could not clearly be observed because of the large increases in soluble arabinoxylan. These studies do not answer the question about the function of non-starchy cytoplasmic polysaccharides in the endosperm either.

VI. HYDROXYPROLINE IN THE PRIMARY CELL WALL QUESTIONED

After having reviewed the literature which showed the occurrence of extensin in very many dicotyledons, but not in monocotyledons, it should be mentioned that a group of researchers (Steward et al., 1974) doubted the occurrence of a hydroxyproline-rich protein in primary walls in general. This school especially criticized the experiments on sycamore-suspension cultures mentioned previously, because the cells were harvested in bulk without regard to their morphological and functional differences. Steward et al. (1974) distinguished between living, flaccid, and senescing cells. In autoradiographic experiments in which the cells were grown in ^{14}C-proline

a high number of silver grains could be localized in the cell walls of senescing cells, which lacked any protoplasmic organization whatsoever. In the walls of actively dividing cells, very little label was noted. The senescing cells had thickened walls, so that the label could be regarded as being incorporated into the secondary wall. Actually, these findings agree with Sadava et al. (1973) who reported an increased synthesis and accumulation of cell wall hydroxyproline which coincides with the cessation of elongation growth in pea-epicotyl. Lamport (1970) also mentioned the possibility that protein is involved in secondary thickening of the cell wall.

MATERIALS AND METHODS

I. MATERIALS

A. Wheat Flour, regional baking standard, a mixture of good milling and baking quality samples from nine varieties (Buckskin, Trison, Homestead, Scout, Cloud, Eagle, Sage, Centurk, Kirwin) grown in Newton, Hays and Garden City, Kansas.

B. Origin of the Wheat Root Extract. This material was available from preliminary experiments done in this laboratory (Nordin, 1975) using Shawnee variety wheat.

C. TLC Plates. Brinkman pre-coated silica gel 60 (E. Merck) thin-layer plates (0.25 mm x 20 cm x 20 cm).

D. Enzymes. Hog pancreatic α-amylase was purchased from SIGMA Chemical Company, St. Louis, Mo. Glucoamylase was obtained from the Miles Laboratories, Inc., Kankakee, Iowa.

II. METHODS

A. Analyses:

1) Total carbohydrate was determined by using the phenol-sulfuric acid method (Dubois et al., 1956). Arabinose served as a standard for both pentoses and hexoses. Absorbance was determined at 480 nm. When mainly galactose was present, the data from the arabinose standard were multiplied by 1.35. When xylose was to be determined, the arabinose data were divided by 1.4.

2) Protein was determined by the Folin-Ciocalteau method using bovine serum albumin (Schwarz/Mann Company, Orangeburg, NY) as a standard (Lowry et al., 1951).

3) Hydroxyproline was determined by the method of Kivirikko and Liesmaa (1963). In preliminary experiments, the method of Neumann and Logan (1950) was used, which is fifteen times less sensitive. Both methods are based on the same principle: hydroxyproline is oxidized to pyrrole which further reacts with p-dimethylaminobenzaldehyde to form a complex having a pink color. Neumann and Logan used sodium peroxide for oxidation and Kivirikko and Liesmaa, sodium hypobromide.

4) Amino Acids were determined after hydrolysis of 50 mg of a protein containing fraction in 6 ml of 6N hydrochloric acid under reduced pressure for 12 hrs. at 110°C. The hydrochloric acid was evaporated, the residue redissolved in water several times. Finally the residue was solved in 5 ml of water o

which .5 ml were applied to the amino and analyzer column accelerated system (Beckman Model 120C). The mole percent amino acid per 100 moles was calculated from the height and the width of the peaks according to standards.

B. Hydrolyses:

1) Hydrolysis for sugar identification: Minute amounts of sample were hydrolyzed in tubes sealed under reduced pressure in 2N trifluoroacetic acid for one hour at 110°C. Trifluoroacetic acid was evaporated under a nitrogen stream, and the residue dissolved in water.

2) Hydrolysis for sugar analysis: Samples were hydrolyzed in tubes sealed under reduced pressure in 2ml 1N hydrochloric acid for two hours at 100°C. The acid was evaporated at 95°C under a stream of nitrogen. The hydrolysates were redissolved twice in 1ml of water, which then again was evaporated.

3) Hydrolysis for hydroxyproline determination: Samples (1-3mg) were hydrolyzed in a sealed evacuated tube in 6N hydrochloric acid at 110°C for six hours. The hydrolysates were filtered through sintered glass and then dried on a steam cone. If no humin was visible, the filtering step was omitted.

C. Thin Layer Chromatography:

1) Identification of sugars: The sugar hydrolysates were applied to TLC plates. The plates were developed in one

dimension by three ascents of butanol-acetone-water (40:50:10); plates were air-dried after each ascent.

Staining: Plates were stained by dipping them into a dioxane-methanol solution containing two grams triphenyl-tetrazoliumchloride per 118 ml solvent. The plates were air dried, exposed to ammonia vapors and then heated at 80° for ten minutes in an ammonia atmosphere. Sugars stain red; there is practically no background staining.

2) Quantitative sugar analysis: The sugar residues were dissolved in water and applied in a line to two thin-layer plates. Sugar standards were applied along one edge which was separated from the main part of the plate by scraping off a thin band of silica gel. The plates were chromatographed in butanol: acetone: water (40:50:10), three ascents. Sugar components were located, after covering the portion of the plate containing the sample with a glass-plate, by spraying the portion containing the standards with the reagent used for sugar identification. After the standards were visibly stained, corresponding portions were scraped from the portion of the plate containing the sample, and the silica gel eluted with hot water, concentrated and assayed for carbohydrate using the phenol-sulfuric acid method.

3) Identification of amino acids: Protein hydrolysates were applied to TLC plates. The plates were chromatographed

using 96% ethanol, 28-30% ammonia (70:30), and 96% ethanol-water (70:30) in the second dimension.

Staining: a) Plates were dipped into an acetone solution containing 0.3 percent ninhydrin and three percent glacial acetic acid. The color was developed in an oven at 90°C for several minutes. Proline and hydroxyproline give yellow spots, while other amino acids form blue spots.

Staining: b) Plates were dipped into an n-butanol containing 0.2 percent isatin and four percent glacial acetic acid. Then the plates were heated for five to ten minutes at 90°C. Proline and hydroxyproline give blue spots, while other amino acids stain pink (Acher et al., 1950).

D. Isolation of Crude Water-Soluble Pentosans:

1) According to the procedure of Kündig et al. (1961) except that precipitation with trichloroacetic acid and digestion with α-amylase were omitted (See Fig. 1). Throughout this thesis, this preparation will be referred to as pentosan 1.

2) Isolation of crude water-soluble pentosans by trichloroacetic acid extraction. To compare with Cleland's (1968) results, flour was extracted with twice the weight of fifteen percent trichloroacetic acid below 2°C (See Fig. 2). The low temperature was maintained during the whole extraction

procedure until the solution had a neutral pH, since trichloroacetic acid possibly could degrade acid labile linkages. These pentosans will be called pentosan 2.

3. <u>According to Preece and McKenzie's procedure (1952) with slight variations.</u>

The procedure of Fincher and Stone (1974a) was used (See Fig. 3.) to isolate a crude pentosan fraction referred to as pentosan 3 in this thesis. The isolation procedure of Fincher and Stone (1974a) is based upon one by Preece and McKenzie (1952).

Neither Fincher and Stone nor Preece and McKenzie reported whether the ethanolic extract was decanted hot or after it cooled. This is relatively important since the major glycopeptide is reported to be soluble in 70% ethanol, but insoluble in cold 80% ethanol. I extracted in both ways. In one preparation, I decanted the ethanolic solution still hot. My mode of isolation varied from Fincher and Stone's in three points: 1. I extracted the ethanol-insoluble residue at room temperature and not at 40°C. 2. I precipitated the proteins from the water-soluble fraction before and after enzymatic digestion by heat coagulation. 3. The enzyme used was glucoamylase instead of salivary amylase. The water-soluble pentosans obtained from this procedure were designated as pentosan 4.

Water-soluble pentosans were isolated in a manner identical to the pentosan 3 preparation, but the ethanolic solution was decanted while hot; this will be called pentosan 5. When the ethanol was decanted after the suspension was cooled, the preparation was designated pentosan 6.

Pentosans 2 and 3 were fractionated with saturated ammonium sulfate, according to Fincher and Stone (1974a), into a fraction soluble in ammonium sulfate and one insoluble in ammonium sulfate.

E. Isolation of Crude Water-Insoluble Pentosans:

Flour (100g) was made into a doughball and allowed to rest for six hours. It was washed under a stream of water, and the water-dispersible part was centrifuged at low speed. The upper brown layer containing the desired pentosans was mechanically removed with a spatula, dispersed in water and again centrifuged at low speed. The washing was repeated until most of the starch that was visible as a white layer had been removed. The crude pentosans were dissolved in 80 ml dimethyl sulfoxide, and then diluted approximately ten-fold with water and α-amylase (1800 units) was added. The mixture was dialyzed for six days until it no longer stained blue with iodine. Then the dialyzed pentosans were lyophilized.

F. Isolation of Water-Soluble Polysaccharides from Wheat Roots:

The wheat root extract (Nordin, 1975) was extracted

with ice-cold, fifteen-percent trichloroacetic acid. The soluble part was shaken with ether as in the TCA extraction (see Fig. 2), dialyzed at 0°C against ice water and freeze dried. The total yield was 32 mg from 100 mg root extract. The final extract gave a positive test for hydroxyproline; the insoluble residue gave a negative test.

G. Gel Chromatography:

The freeze-dried materials were dissolved in water, 0.01 M phosphate buffer, pH 7 or Tris buffer, pH 7, and applied to a Column (75.5 x 1.6 cm) of Sepharose. Components were eluted with the solvent used for extraction of the sample. Fractions (1 ml or 1.25 ml) were automatically collected. V_o was determined with dextran blue and V_t with glucose.

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

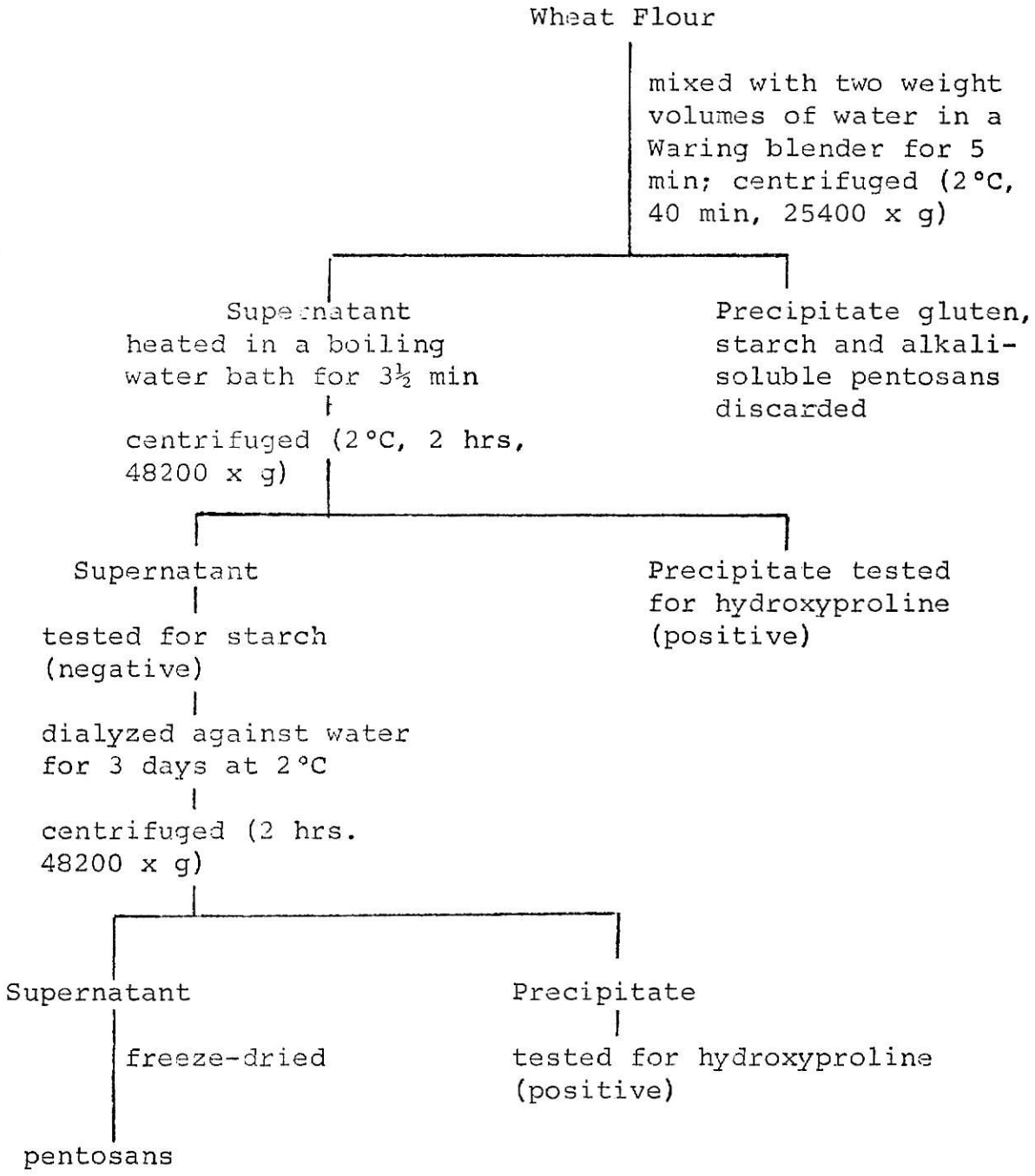

Fig. 1. Isolation of water-soluble pentosans (pentosan 1) by the method of Kündig, et al. (1961).

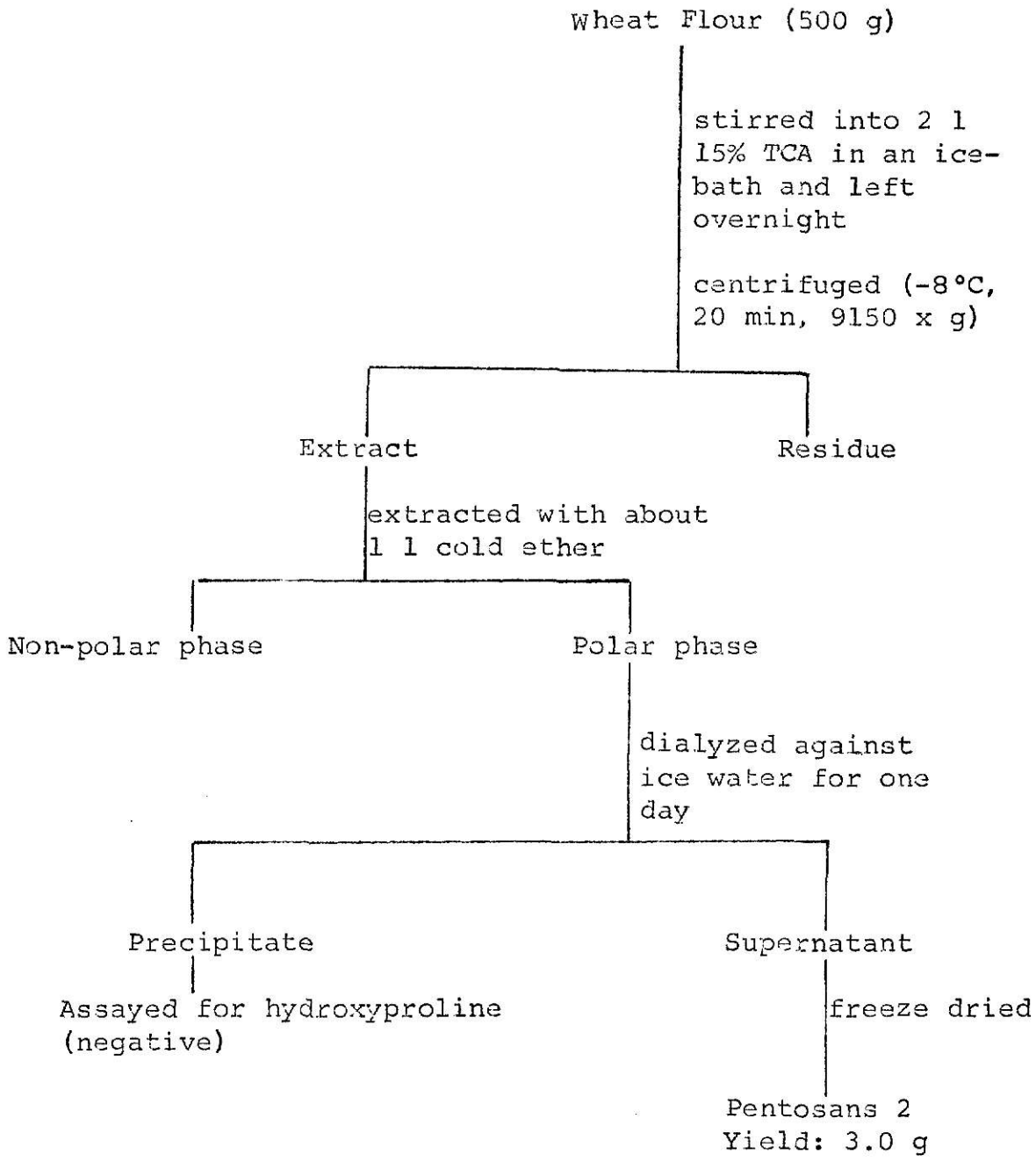

Fig. 2. TCA Extraction of Wheat Flour.

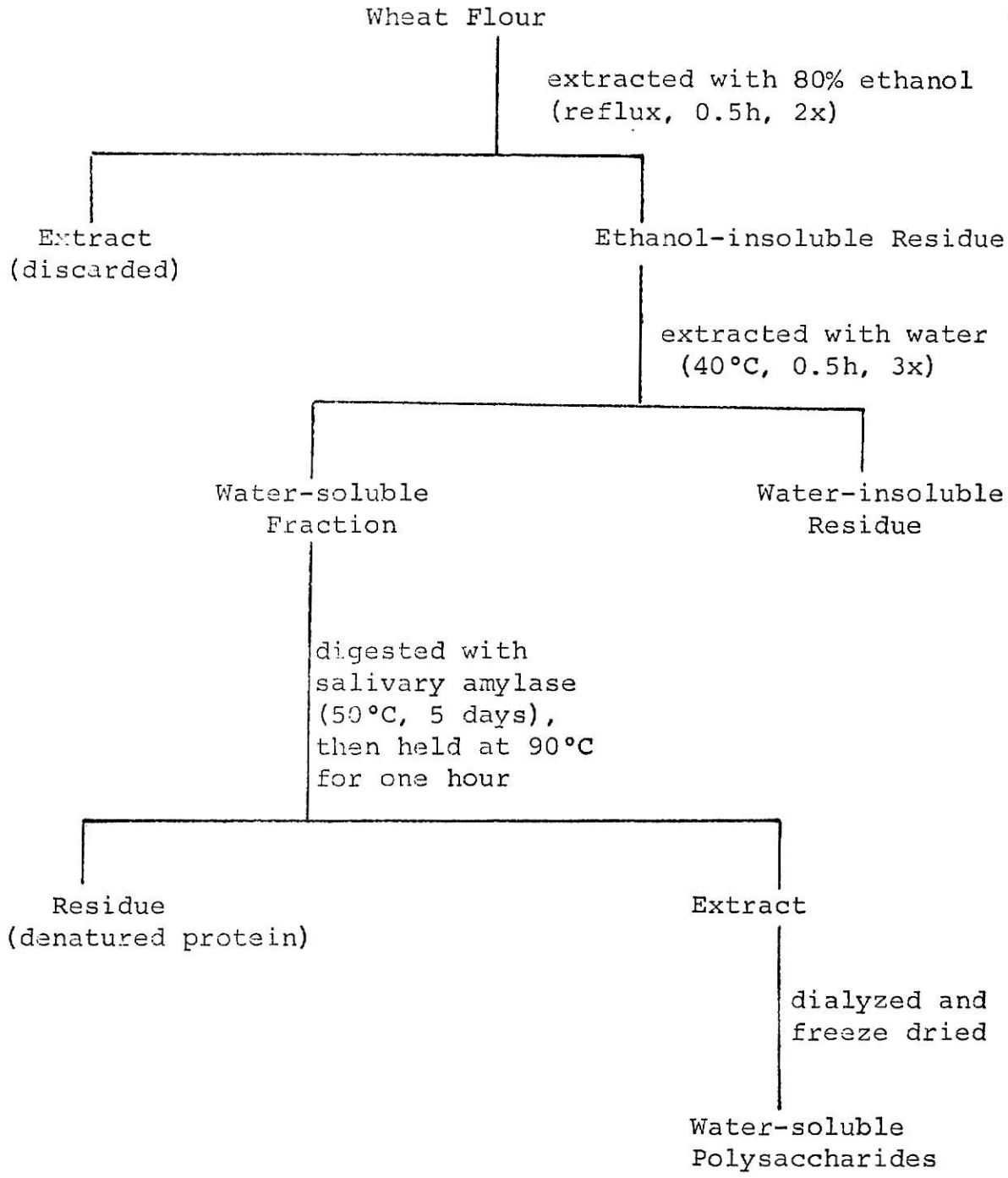

Fig. 3. Procedure for Extraction of Water-Soluble Polysaccharides According to Fincher and Stone (1974a).
(Pentosans 3; Pentosans 4, 5 and 6 involved minor modifications of this procedure.)

RESULTS

I. NONSTARCHY POLYSACCHARIDES FROM WHEAT ENDOSPERM

A. Analyses of Pentosan Preparations

1) Hydroxyproline

The classical way to obtain water-insoluble pentosans from wheat flour is the dough-kneading procedure (Materials and Methods) while the method of Kündig et al. (1961) has been widely used to isolate water-soluble pentosans. Since hydroxyproline has been reported to be present in cell walls (Cleland, 1967; Lamport, 1964; Olson, 1964; Albersheim et al., 1973), I expected to find it predominately in the water-insoluble pentosans or hemicelluloses which are believed to represent a part of the cell wall matrix (Hall et al., 1974). These pentosan preparations (the water-insolubles, and the water-solubles referred to as pentosans 1) were assayed for hydroxyproline by Neumann and Logan's (1950) method, and by the more accurate method of Kivirikko and Liesmaa (1963). The water-insoluble pentosans contained 0.12% hydroxyproline when assayed with Kivirikko's method and 0.31% with Neumann and Logan's method. Pentosans 1 were found to contain 0.24% hydroxyproline with Kivirikko's method and 0.75% using Neuman and Logan's method. The former method consistently gave results about one third the magnitude of the latter method.

In addition, hydroxyproline was identified, after hydrolysis of the pentosans, by two-dimensional thin-layer chromatography. Samples were applied to thin-layer plates after hydrolysis in 3N hydrochloric acid. Acid did not interfere with the solvent or the rate of migration to any recognizable extent. Proline and hydroxyproline both stain blue with isatin, whereas other amino acids give a pink color. The two solvent systems used (Materials and Methods) clearly separated those two amino acids. Hydroxyproline had R_f values of 0.4-0.57 and proline 0.83-0.90. Proline stained darker blue than hydroxyproline, so it was always more clearly visible. The most common method for chromatographic identification of amino acids is to stain with ninhydrin. The yellow color which proline and hydroxyproline yield with ninhydrin is not sufficiently clear or visible to positively identify hydroxyproline. Finally, hydroxyproline was found using a combination of thin-layer chromatography and Kivirikko and Liesmaa's (1963) method. Chromatography served to concentrate this amino acid. A hydrolysate from 100 mg purified pentosans was applied as a band to a thin-layer plate and chromatographed in one dimension in solvent 1 (Materials and Methods). After staining, bands were visible at R_f values 0.89, 0.68, 0.60, 0.54, 0.42, 0.35, 0.15, 0.115, and at the origin. The bands at R_f 0.42, 0.54, 0.60 and 0.68 were scraped off, the silical gel soaked in water,

blended in a Sorvall Omnimix for three minutes and filtered. The filtrates were dried at an elevated temperature under normal pressure, dissolved in water, and assayed for hydroxyproline with Kivirikko's method. Only one band R_f 0.60 showed the characteristic pink color of hydroxyproline. Thus, hydroxyproline was positively identified in water-soluble pentosans.

Subsequent to our establishing the presence of hydroxyproline in wheat flour, the work from Stone's laboratory (Fincher and Stone, 1974a; Fincher et al., 1974) appeared, describing an arabinogalactan-peptide in wheat endosperm which was very rich in hydroxyproline. Fincher and Stone reported a shorter isolation procedure originating in work reported by Preece and McKenzie (1952). The procedure is based on the insolubility of the hydroxyproline-rich molecule in 80% ethanol, whereas many cereal proteins are soluble in this solvent and hence can be eliminated. Since Fincher and Stone's results (1974a) will be mentioned frequently, their pentosan preparation, from which they obtain the hydroxyproline-rich arabinogalactan-peptide, will be designated pentosan 3. Using Fincher and Stone's procedure, we obtained a preparation (pentosan 4) which did not contain any hydroxyproline when analyzed on the amino acid analyzer and only a trace when assayed with Kivirikko and Liesmaa's method. The major difference in the procedures seemed to be that I used glucoamylase,

whereas Fincher and Stone used salivary amylase. To have a better comparison two pentosan preparations were made with amylase as the digesting enzyme. I also noticed that the ammonium sulfate-soluble part of the trichloroacetic acid-soluble material, described in the next paragraph, was soluble in hot 80% ethanol, but precipitated after the ethanol had cooled. Fincher and Stone described their arabinogalactan-peptide as soluble in 70% cold ethanol, and thus it is probably at least partially soluble in hot 80% ethanol. Unfortunately, neither Fincher and Stone nor Preece and McKenzie reported whether they decanted the ethanolic solution from the flour residue while it was still hot or after cooling. Hence, two flour extractions were done; in one, the ethanolic solution was decanted while still hot, in the other it was not decanted until it had cooled to room temperature. The pentosans were designated pentosans 5 and 6, respectively. The hydroxyproline content of pentosan 6 was ten times higher than that of pentosan 5, but still only represented one tenth of the hydroxyproline in pentosan 3 (Table II).

Lamport (1970) and Cleland (1968) used cold 15% trichloroacetic acid to extract hydroxyproline-rich material. Their procedure had the advantage that it is believed to yield cytoplasmic fractions, enabling a rough estimate of the possible location of the pentosan to be obtained. Their procedure was

Table II. HYDROXYPROLINE[a] IN PENTOSAN FRACTIONS

Pentosan	Hydroxy-proline w/w (%)	Subfraction obtained by precipitation with saturated ammonium sulfate	Hydroxy-proline w/w (%)
Water-insoluble Pentosans	0.12		
Pentosan 1	0.24		
Pentosan 2	0.023	soluble fraction	0.13
		insoluble fraction	0.000
Pentosan 3	0.42	soluble fraction	1.34
		insoluble fraction	0.000
Pentosan 4	trace		
Pentosan 5	0.004		
Pentosan 6	0.038		

a All values were obtained by Kivirikko's and Liesmaa's method (1963)

used to yield a preparation designated pentosan 2. Its hydroxyproline content was 0.023% which is miniscule in comparison to the hydroxyproline content of pentosan 3 (Table II). Pentosan 2 was fractionated into two subfractions with saturated ammonium sulfate solution, in order to compare my results further with those of Fincher and Stone (1974a). The portion insoluble in saturated ammonium sulfate was found to be free of hydroxyproline, whereas the portion soluble in saturated ammonium sulfate contained 0.13% hydroxyproline. Fincher and Stone reported 1.34% hydroxyproline in a similar preparation. The hydroxyproline content of fractions discarded during the isolation of pentosans 1 was determined. The proteinaceous precipitate obtained by boiling the water-soluble flour extract contained 0.25% hydroxyproline which is about the same amount that was found in pentosan 1. The protein-precipitate obtained after dialysis was somewhat lower in its hydroxyproline content, 0.19%.

2) Protein-Assays of Water-Soluble Pentosan Preparations

The water-soluble pentosans 1 and 2 and the ammonium sulfate subfractions of pentosan 2 were assayed for protein by the common Folin method (Lowry et al., 1951). Pentosan 1 was found to contain 40% protein (range = 38% to 43%). This concentration is higher than previous literature values. Kakuda (1973) investigated three different flour types and found 8 to

22.5% protein. Kündig et al. (1961) reported 8.8% protein in wheat flour before the α-amylase digestion and trichloroacetic acid precipitation.

Pentosan 2 was found to contain 3.8% protein (Table V). The soluble and insoluble fractions obtained from pentosan 2 upon fractionation with saturated ammonium sulfate contained 4.4% and 0.7% protein (Table V), respectively.

Fincher and Stone (1974a) found 2-5% protein in the equivalent pentosan preparation, 0-3% in the insoluble fraction from ammonium sulfate fractionation, and 8% in the soluble fraction (Table V).

3) Carbohydrate Composition of Pentosan Preparations

Pentosan 2 was hydrolyzed with trifluoroacetic acid and 1N hydrochloric acid. The sugar composition was determined qualitatively or quantitatively as described in "Materials and Methods." Pentosan 2 contained arabinose, xylose, galactose and a trace of glucose. The same was true for pentosan 4, in agreement with Fincher and Stone (1974a). The fraction soluble in ammonium sulfate contained 21% arabinose and 79% galactose. Xylose and glucose were not present. Interpretation of these data is difficult, since arabinose is destroyed more readily by hydrochloric acid than galactose (Nordin, 1975). There is some qualitative similarity to Fincher and Stone's arabinogalactan-peptide, reported to contain 40.8% arabinose, 59.2%

galactose, no xylose and only a trace of glucose. Fincher and Stone determined the carbohydrate in hydrolyzates by gas-liquid chromatography of alditol acetate derivatives. The ammonium sulfate-soluble fraction from pentosan 2 more closely resembled in carbohydrate composition the arabinogalactan-peptide reported by Neukom et al. (1967) to contain 70% galactose, 30% arabinose, and no other sugars.

The ammonium sulfate-insoluble fraction of pentosan 2 was found to contain only arabinose and xylose, agreeing with Fincher and Stone's data on the arabinoxylan from pentosan 3.

B. Behavior of Pentosan Preparations on Gel Chromatography

Pentosan 1 was fractionated on a column (90 x 1.6cm) of Sepharose 6B; components were eluted with 0.05 M Tris buffer containing 0.05% sodium azide. Four carbohydrate-containing components appeared to be present (Fig. 4) while the protein was not separated into components. Peaks I and II eluted near the void volume (V_o) and were not completely separated, peak III eluted at $V_e/V_o = 1.6$, and peak IV eluted just before V_t. Since the proteins did not form any clear peak with this method but rather were evenly distributed over the whole elution profile, no further chromatographic experiments with the water extract on Sepharose were done. In this particular extraction, enzymes were not inactivated by heating; hence, enzymatic degradation might have occurred.

Pentosan 4 was chromatographed on a column (75.5 x 1.6cm) Sepharose 6B and components were eluted with water. Fig. 5a shows the profile of the polysaccharide and the protein components. The elution profile obtained by Fincher and Stone (1974a) from pentosan 3, using 0.3% sodium chloride containing 0.05% sodium azide as the solvent, is shown in Fig. 5b. In both cases, two carbohydrate peaks were obtained, one at V_o and the other near the V_t. Fincher and Stone did not detect protein in the component near the V_o. This could be due to their use of a very much lower sample concentration than the one I used. The apparent carbohydrate content of the component near the V_t from the pentosan 4 preparation is much lower than peak I, whereas both are of equal height in pentosan 3 (Fincher and Stone, 1974a). These differences could either be concentration effects or an indication that the preparations are entirely different. According to the amino acid composition (Table IV), both preparations do indeed differ. Therefore, the former possibility was not investigated any further. Both peaks from pentosan 4 were assayed for their sugar composition. Arabinose, galactose, and xylose were found to be present in both peaks; however, more xylose than galactose was found in peak I and more galactose than xylose was present in the low molecular weight peak.

When pentosan 2 was chromatographed on Sepharose 6B

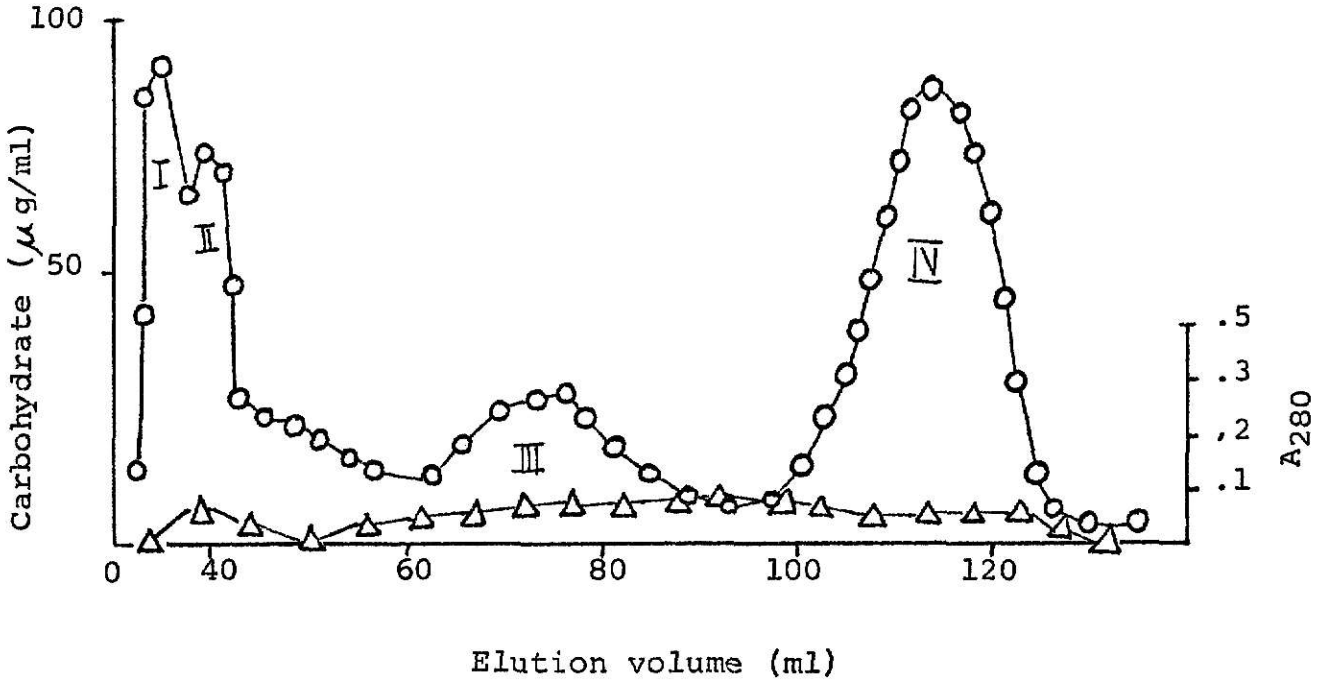

Fig. 4. Fractionation of Pentosan 1 on a column (90 x 1.6 cm) of Sepharose 6B

1 ml of sample was applied to the column and eluted with 0.05 M Tris-buffer containing 0.05% sodium-azide

o carbohydrate (μg/ml), Δ A_{280}

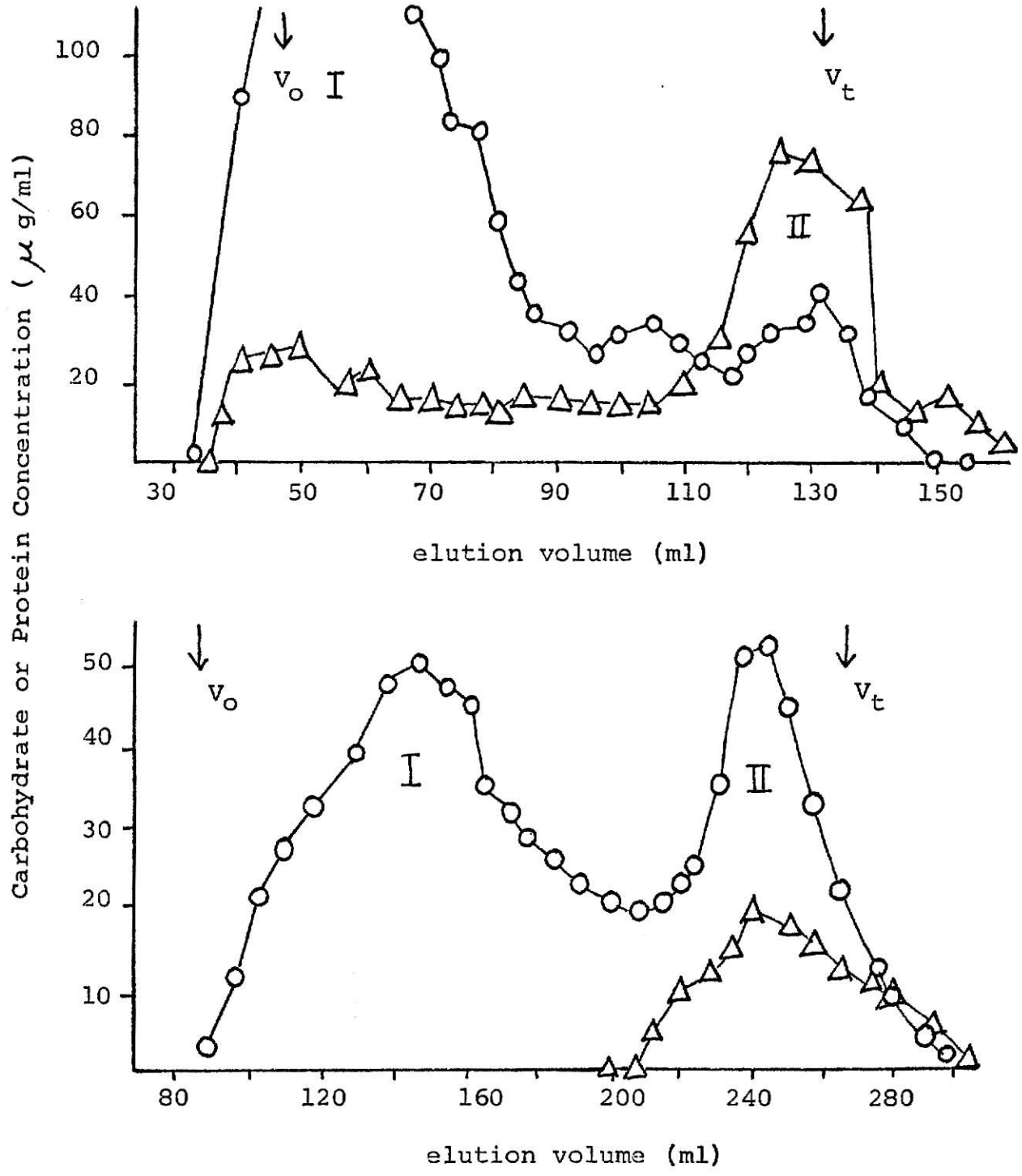

Fig. 5. Chromatography of Pentosan 4 (a) and 3 (b) on a column (75.5 x 1.6 cm) of Sepharose

a) Pentosan 4 eluted with water from Sepharose 6B
b) Fincher and Stone's data (1974a)

o carbohydrate concentration Δ protein concentration

two separate carbohydrate peaks were eluted with water, but only one protein peak was observed (Fig. 6). Carbohydrates and protein elute together at V_o. The second small carbohydrate peak at V_t does not contain any protein.

The subfraction of pentosan 2 which was soluble in saturated ammonium sulfate was also chromatographed on Sepharose 6B (Fig. 7). The elution profile resembled closely the one obtained from pentosan 2 (Fig. 6): a major carbohydrate peak at V_o and an ill-defined smaller peak. The protein peak coincided with the carbohydrate peak at V_o. The arabinogalactan-peptide obtained by Fincher and Stone from pentosan 3 eluted from Sepharose 4B as one low molecular weight peak (Fig. 7b). Thus, my preparation contained higher molecular weight material than Fincher and Stone's glycopeptide.

The high and the low molecular weight components eluted at 30-65 and 80-115 ml, respectively, were assayed for protein and hydroxyproline. Table III shows that peak I and peak II were higher in protein and hydroxyproline than the ammonium sulfate-soluble fraction from which they were obtained. The probable explanation is that some carbohydrate was lost during the Sepharose fractionation because water was used as eluant, which causes absorption of some carbohydrate to the column.

C. Comparison of Different Pentosan Preparations.

The amino acid composition of the low molecular-weight

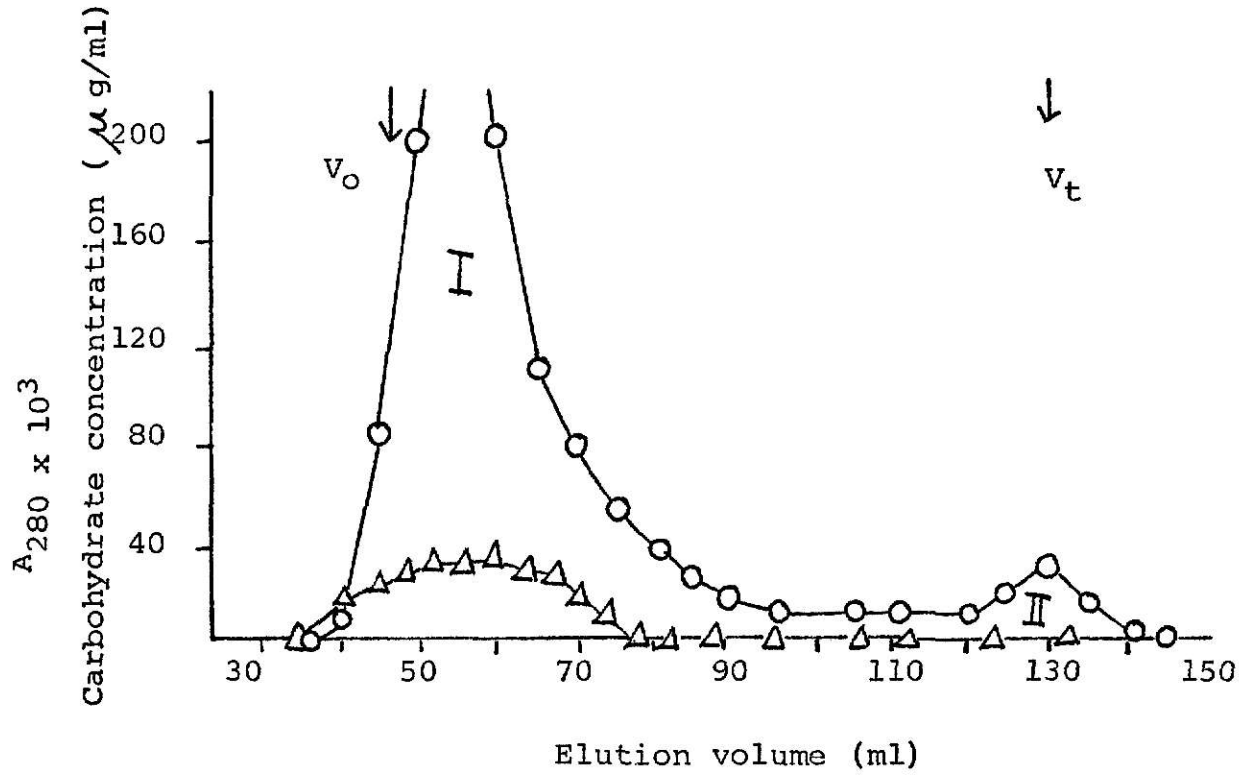

Fig. 6. Fractionation of the TCA Wheat Flour Extract on Sepharose 6B.

100 mg of pentosan 2 was applied to a Sepharose 6B column (75.5 x 1.6 cm) and components were eluted with water at 20°C.

o carbohydrate concentration

Δ absorbance at 280 nm x 10^3

Table III. PROTEIN AND HYDROXYPROLINE CONTENTS OF THE AMMONIUM SULFATE SOLUBLE FRACTION OF PENTOSAN 2 AND OF FRACTIONS OBTAINED FROM CHROMATOGRAPHY ON SEPHAROSE 6B

Polysaccharide Fraction	Hydroxyproline % w/w	Protein % w/w	Hydroxyproline as % protein w/w
Ammonium sulfate-soluble fraction	0.13	4.4	3
Peak I	0.23	6	3.8
Peak II	0.23	15.2	1.5

peak (Fig. 5, 110-135 ml) of pentosan 4 is compared with the amino acid composition of Fincher and Stone's arabinogalactan-peptide in Table IV. The amino acid compositions differ so strikingly that the protein fraction of the two extracts must be basically different. Pentosans 5 and 6, in which α-amylase was used in following exactly Fincher and Stone's procedure, also did not have hydroxyproline contents as high as Fincher and Stone report (Table II). Pentosans 5 was the preparation obtained by decanting the ethanol after it had cooled. Pentosan 6 had less than one tenth the hydroxyproline content found by Fincher and Stone (1974a).

Pentosan 2, extracted by trichloroacetic acid, and the

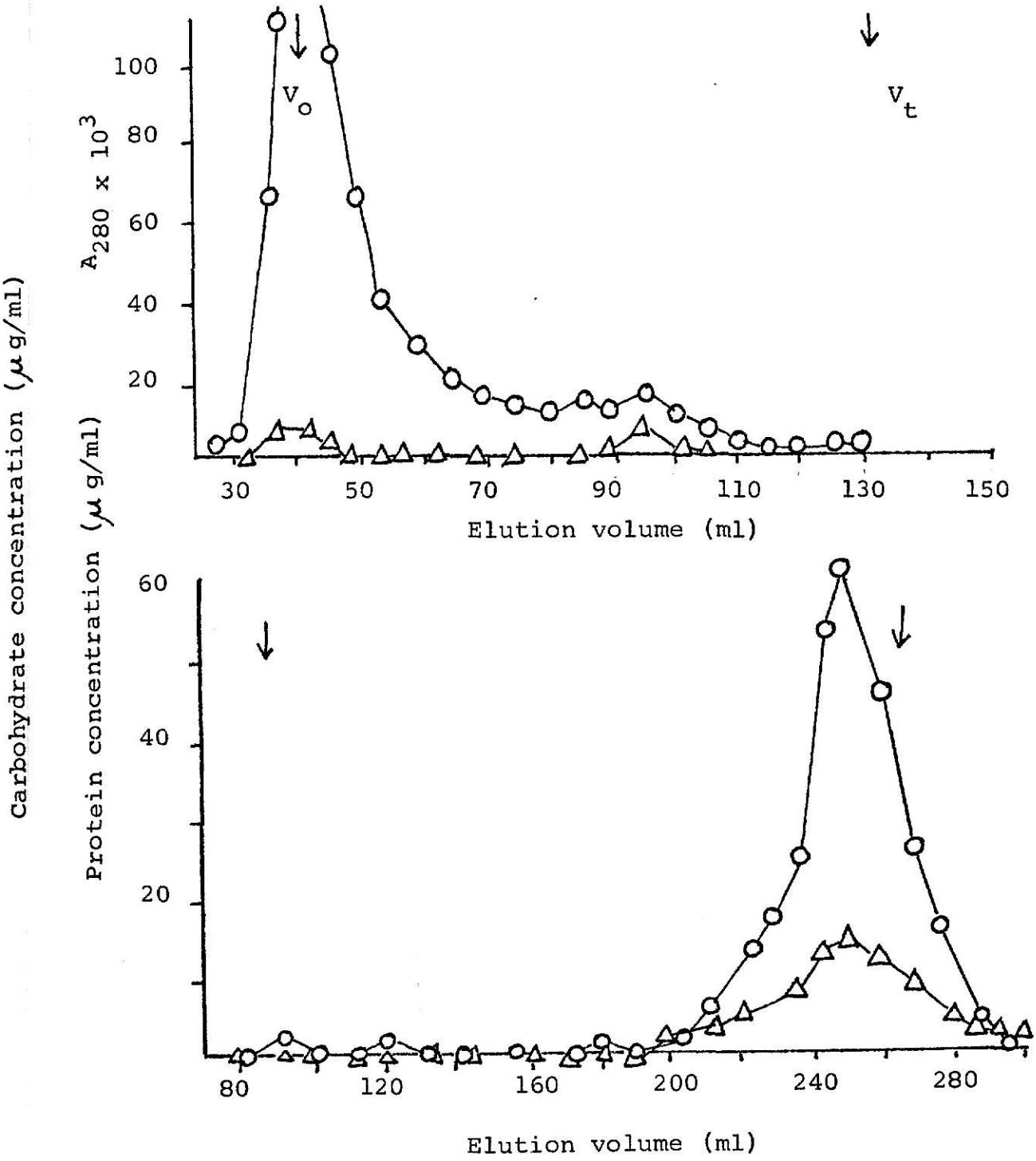

Fig. 7. Chromatography of Pentosan Fractions Soluble in Saturated Ammonium Sulfate on Sepharose.

a) Isolated from pentosan 2, chromatographed on Sepharose 6B (75.5 x 1.6 cm)
b) Fincher and Stone's data (1974a)

○carbohydrate concentration △absorbance at 280 nm or protein concentration

ammonium sulfate subfractions are compared to pentosan 3 in Table V.

Table IV. AMINO ACID COMPOSITION OF PROTEINS IN WATER EXTRACTS FROM WHEAT FLOUR [a]

Amino acid	Preparation 3	Preparation 4
Lysine	1.9	2.9
Histidine	0.6	2.3
Arginine	1.1	2.5
Hydroxyproline	18.9	0
Aspartic acid and asparagine	6.0	4.2
Threonine	6.5	2.1
Serine	8.4	7.3
Glutamic acid and glutamine	12.8	34.5
Proline	3.6	15.1
Glycine	5.6	3.8
Alanine	18.5	4.6
1/2 Cystine	0	3.9
Valine	5.8	1.9
Methionine	1.0	1.0
Isoleucine	1.7	2.9
Leucine	2.1	6.4
Tyrosine	2.8	1.7
Phenylalanine	1.2	3.8
Tryptophane	not determined	not determined
Hexosamine	1.4	not determined

[a]Fincher and Stone's (1974a) data - pentosan 3; my data - pentosan 4. Values are expressed as moles/100 moles of amino acid.

ammonium sulfate subfractions are compared to pentosan 3 in Table V.

Table V and the elution profiles from Sepharose (Fig.6) clearly show that the trichloroacetic acid extract is not iden-

TABLE V. PERCENT COMPOSITION OF PENTOSANS 2 AND 3[a] AND THEIR FRACTIONS SEPARATED BY AMMONIUM SULFATE PRECIPITATION

Measurement	Total extract		Ammonium sulfate insoluble		Ammonium sulfate soluble	
	Pentosan 3	Pentosan 2	Pentosan 3	Pentosan 2	Pentosan 3	Pentosan 2
Yield (% of total extract)	-	-	58	54	25	23
Protein %	2-5[b,a]	3-8[c]	0-3[b]	0-7[c]	8[b]	4.4[c]
Arabinose %[d]	44.1	++	34.1	++	40.8	21
Xylose %[d]	44.7	++	65.9	++	0	0
Galactose %[d]	15.2	++	0	0	59.2	79
Glucose %[d]	+	+	0	0	+	0
% Hydroxyproline (protein basis)	12.1	++	0	0	16.7	3

[b]Assayed by micro-Kjehldahl

[c]Assayed by Folin's method (Lowry et al., 1951)

[d]Assayed by gas-liquid chromatography for pentosan 3 (Fincher and Stone, 1974a) and by eluting from thin-layer plates as described in "Material and Methods" for pentosan 2

[a]Pentosan 2 is a trichloroacetic acid extract and pentosan 3 was prepared by Fincher and Stone (1974 a and b).

tical to Fincher and Stone's pentosan 3. The portion of the trichloroacetic acid extract soluble in ammonium sulfate differed in molecular size, in galactose content, in protein content, and in hydroxyproline content.

D. Degradation Study of the Arabinogalactan from the Ammonium Sulfate Soluble Part of Pentosan 2.

Since Fincher and Stone's preparation had lower molecular weight and higher arabinose and hydroxyproline concentrations than the trichloroacetic acid-extracted arabinogalactan, the possibility was investigated that hot ethanol might have partially degraded the macromolecule or removed side chains. Degradation of the high molecular-weight-protein-containing arabinogalactan obtained by trichloroacetic acid extraction was studied. Ten mg of the trichloroacetic acid soluble arabinogalactan from the ammonium sulfate fraction were applied to the Sepharose 6B column and eluted. A series of sealed tubes, each containing 10 mg of the high molecular weight arabinogalactan were heated at 100°C at the following time intervals: one, six, and twenty-four hours. Then the contents were diluted with water and dialyzed against water for two hours at 2°C to eliminate low molecular weight degradation products. Each was fractionated on Sepharose. The high molecular weight fraction from chromatography on Sepharose was heated in sealed tubes for different periods of time and then chromatographed again on Sepharose. Fig. 8 shows the elution profiles. Heating in ethanol clearly degraded the arabinogalactan, as indicated by the decrease in peak I and the disappearance of peak II. However, even prolonged treatment did not cause complete disappearance of peak I.

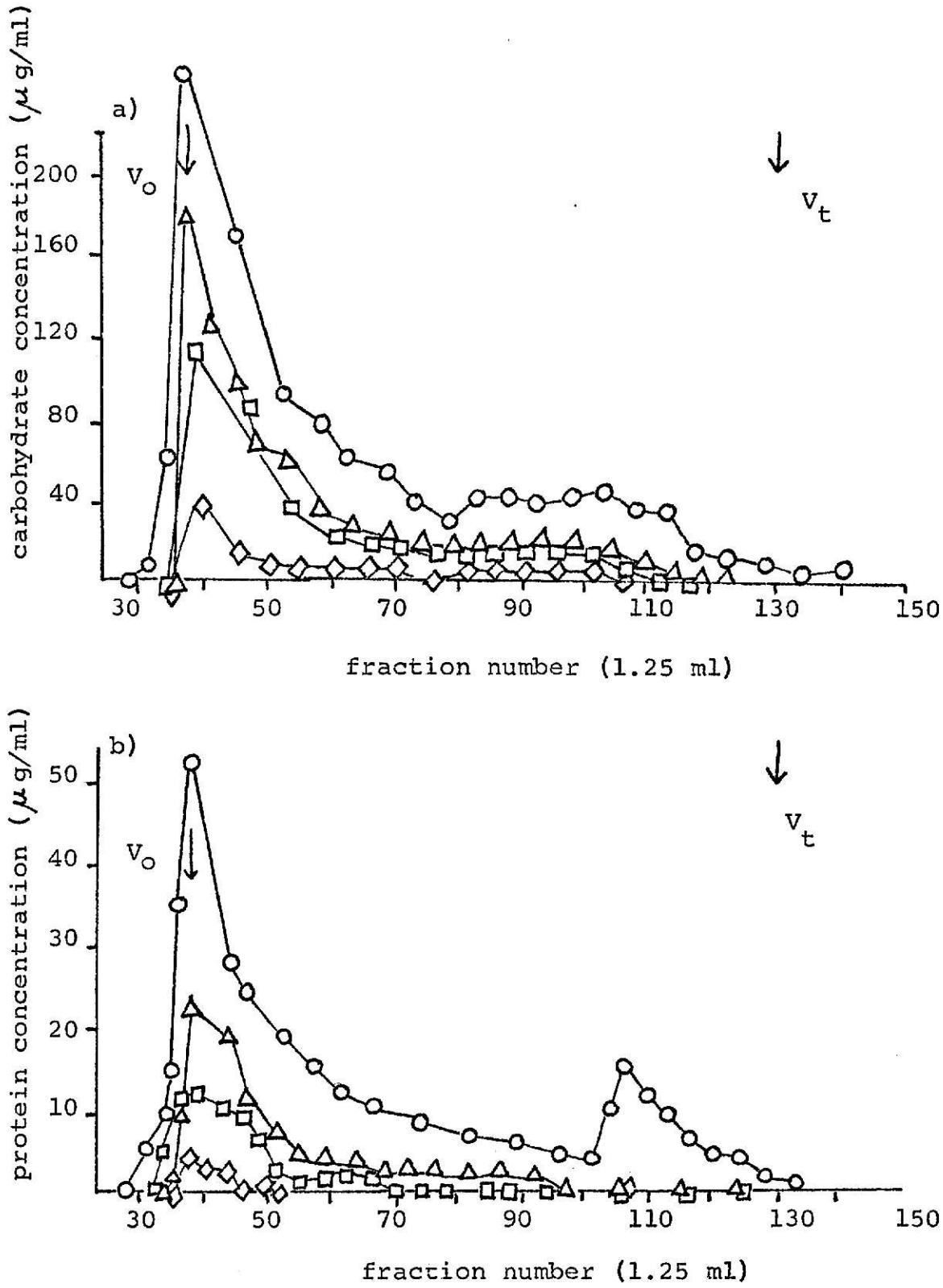

Fig. 8. Fractionation of 10 mg heat treated arabinogalactan on Sepharose 6B (75.5 x 1.6 cm).

Samples were heated at 100°C in 80% ethanol in sealed evacuated tubes and dialyzed before chromatography.

o - not heated, Δ - one hour, □ - six hours, ◇ - twenty-four hours

All degradation products were dialyzable.

Table VI shows the amounts of carbohydrate or protein present in peak I (tubes 30-76) after degradation. Both carbohydrates and protein decreased equally, as indicated by a content carbohydrate:protein ratio of about six.

Table VI. CARBOHYDRATE AND PROTEIN CONTENTS IN THE TCA-SOLUBLE ARABINOGALACTAN

Hours of heating in 80% ethanol	mg Carbohydrate in tubes 30-76	mg Protein in tubes 30-76	Ratio Carbohydrate/ Protein
0	4.38	0.77	5.7
1	2.04	0.34	6
6	1.40	0.23	6
24	0.29	0.044	6.7

The degradation is time dependent. A plot 1/c versus time yields a reasonably straight line (Fig. 9).

From this degradation study, it appears unlikely that Fincher and Stone's arabinogalactan-peptide extracted from the ethanol-pretreated flour was a degradation product. Rather the trichloroacetic acid extract is basically different. Fincher and Stone heated the flour for only one hour; their arabinogalactan-peptide did not show more than a trace of a peak at V_o on Sepharose 4B (Fig. 7b).

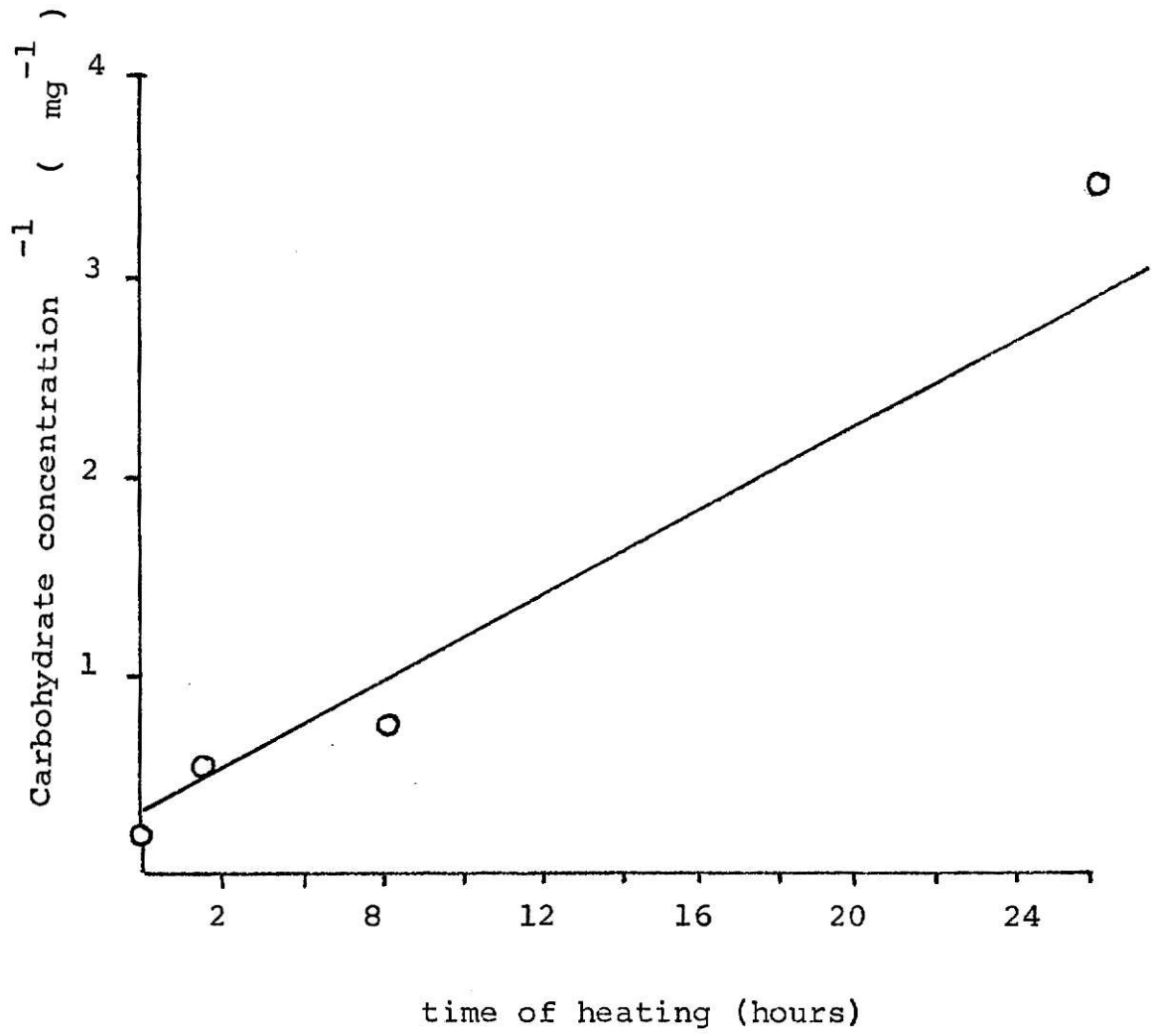

Fig. 9. Reciprocal Plot of 1/C of residual carbohydrate after heat treatment. Arabinogalactan vs. heating time.

II. HYDROXYPROLINE IN THE SEEDLING ROOTS

A lyophilized, sodium dodecyl sulfate extract from wheat seedling roots was extracted with 15% trichloroacetic acid at 0°C to obtain a fraction comparable to the endosperm extract. In addition, the trichloroacetic acid extract could be dissolved in water very easily. The yield was low, only 32%. The residue insoluble in trichloroacetic acid was analyzed for hydroxyproline. The results were negative, so all the hydroxyproline was present in the trichloroacetic acid-soluble portion. Sodium dodecyl sulfate is not known to extract the cell wall; rather it is generally used to obtain cytoplasmic fractions (Mascarenhas, 1970). Hence, this trichloroacetic acid-soluble fraction should be from the cytoplasm too. It was further fractionated on a column of Sepharose 6B (Fig. 10). The extract distributed into three peaks: peak I at V_o, peak II at $\frac{V_e}{V_o} = 1.5$ or $= \frac{V_e - V_o}{V_t - V_o} = .21$, and peak III at V_t. This profile compared very well with results found previously in this laboratory (Nordin, 1975), except that peak II was lower than in the earlier work. Because of the small quantities under each peak, it was not possible to do more than one assay on the collected peaks. Thus the total carbohydrates were calculated by determining the area under the peaks. Peak I contained 3.7 times as much carbohydrate as peak II. Peak II showed a relatively higher absorption at 280 nm than other

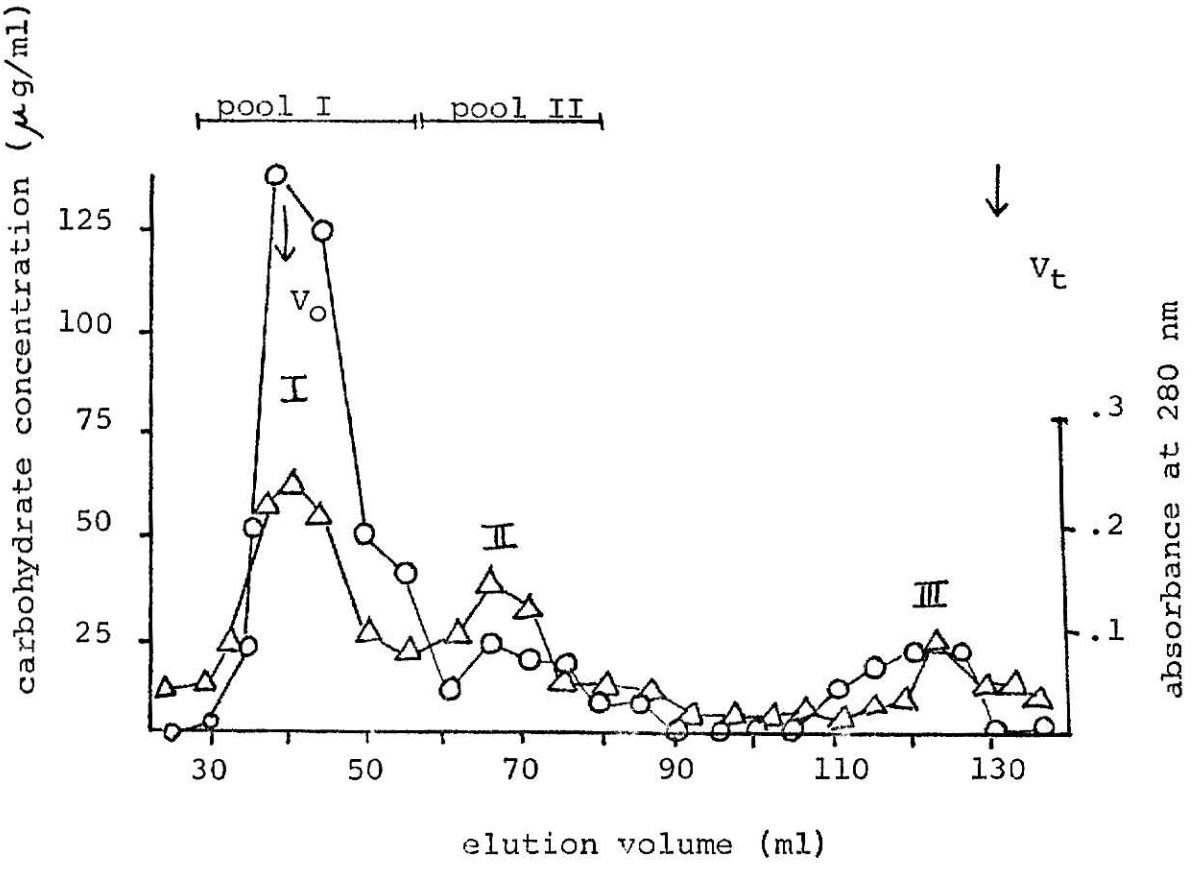

Fig. 10. Fractionation of the TCA Wheat Seedling Root Extract on Sepharose 6B

Freeze-dried TCA wheat seedling root extract (20 mg) was dissolved in 1 ml water, applied to the column (75.5 x 1.6 cm) of Sepharose 6B and eluted with water.

o carbohydrate concentration
Δ absorbance at 280 nm

peaks with respect to the carbohydrate concentration. In Fig. 10, peak I has higher A_{280} than peak II. Lyophilized peaks I and II were assayed quantitatively for hydroxyproline from one preparation and for sugar composition from a second separation. Peak III was too small for quantitative assay. It was analyzed qualitatively for hydroxyproline, which was present.

The major sugars were galactose and arabinose (Table VII). The high molecular-weight peak (peak I) contained relatively more arabinose and the low molecular weight peak (peak II) more galactose. The hydroxyproline content was about the same in peak I (2.5μg) and in peak II (2.2μg). However, with respect to the higher concentration of carbohydrates in peak I, the relative amount of hydroxyproline in peak II was much higher. Hydroxyproline of the wheat root extract was 0.4 to 0.5%. Most of this amino acid was hence found in peak II.

The recovery of carbohydrates in xylose, arabinose, glucose, and galactose was very low, only 17% of the carbohydrates in the trichloroacetic acid extract before gel chromatography. Xylose and glucose were higher in the trichloroacetic acid solubles than in the sodium dodecyl sulfate extract. A possible explanation would be that some arabinose and galactose were attached to proteins which were precipitated by trichloroacetic acid. The very low yield of trichloroacetic acid extract from the sodium dodecyl sulfate extract (only 32%) could be due to the

Table VII. CARBOHYDRATE, PROTEIN, AND HYDROXYPROLINE COMPOSITION OF THE SDS WHEAT ROOT EXTRACT, ITS TCA SOLUBLES AND PEAKS I AND II FROM SEPHAROSE 6B

Component	SDS Extract	TCA Extract	Peak I	Peak II
Total Carbohydrate (%) w/w	not reported	75	not quantized	
Total Protein (%) w/w	not reported	32	not quantized	
Arabinose (%)	46.7*	44.4	46**	27.6**
Xylose (%)	2.3*	7.4	7.4**	7.9**
Glucose (%)	5.6*	12.3	11.2**	15.6**
Galactose (%)	44.9*	37.3	33.6**	48.6**
Hydroxyproline (%) w/w	not reported	0.5	0.3	1.05

* Values from Nordin (1975)
** 17% of the carbohydrates in the unfractionated trichloroacetic acid extract were recovered in these four sugars.

relatively high percentage of these proteins.

III. COMPARISON OF THE TRICHLOROACETIC ACID SOLUBLES FROM ENDOSPERM AND FROM THE SEEDLING ROOTS

Table VIII summarizes the data concerning sugar, protein and hydroxyproline composition of the major peaks into which the trichloroacetic acid extracts from the endosperm and from the

seedling roots separated on gel chromatography. The similarities are only qualitative. In both peaks from the root extract, arabinose and galactose were the predominant sugars; in the major peak from the endosperm extract, these two sugars were the only ones visible on thin-layer chromatograms. Hydroxyproline was found in all three peaks. The endosperm extract resembled the high molecular-weight peak (peak I) with respect to its elution at V_o and with respect to the amount of hydroxyproline. However, it was similar in sugar composition to peak II rather than to peak I of the root extract.

Table VIII. ANALYSIS OF THE MAJOR PEAKS FROM SEPHAROSE 6B OF THE TRICHLOROACETIC ACID-SOLUBLE WHEAT ROOT EXTRACT AND THE TRICHLOROACETIC ACID-SOLUBLE ENDOSPERM EXTRACT

	Root Extract		Endosperm Extract
	Peak I	Peak II	Peak I
Protein (%) w/w	not quantized (lower than in peak II)	not quantized (higher than in peak I)	6
Hydroxyproline (%) w/w	0.3	1.05	0.23
Sugar Composition %			
Arabinose	46	27.6	21
Galactose	33.6	48.6	79
Glucose	11.2	15.6	0
Xylose	7.4	7.9	0

DISCUSSION

The original purpose of this work was to determine if hydroxyproline was present in pentosans from white wheat flour. It was present in both water-soluble and water-insoluble fractions. More hydroxyproline was found in the water-soluble pentosans isolated according to Kündig et al. (1961) than in any other pentosan preparation from this flour. However, this pentosan 1 contained 40% protein; all other methods yielded pentosans that had much lower protein content. Fincher and Stone (1974a) did not detect any hydroxyproline in pentosans prepared by Kündig's method. The former workers suggested that their failure to detect hydroxyproline was due to the low level of hydroxyproline-rich glycoprotein in relation to free protein in the preparation. It is also possible that hot ethanol used in their isolation denatured some of the endosperm proteins, rendering them insoluble in the subsequent water extraction. Pentosan 2, extracted by 15% trichloroacetic acid, as expected, had a lower protein content than pentosan 1, since trichloroacetic acid is one of the most effective reagents for protein precipitation.

Why I was not able to reproduce Fincher and Stone's results is not clear. Whether the ethanol used to extract the flour was decanted hot or cold seems to be crucial. Ten times as much hydroxyproline was found when the ethanol was cooled before decanting than when it was decanted hot. However, this did not account

completely for the failure to isolate Fincher and Stone's arabinogalactan-peptide. The possibility that Fincher and Stone obtained a degradation product cannot be ruled out, although it is highly unlikely since boiling ethanol did not degrade the arabinogalactan from pentosan 2 completely in a time period as long as twenty-four hours. Another cause of the discrepancies could be that botanically different wheat varieties have different constituents. The latter argument also could explain why Fincher and Stone did not find hydroxyproline in water-soluble pentosans extracted with Kündig's method.

In addition, it is obvious that wheat endosperm contains more than one arabinogalactan, since the ammonium sulfate-soluble polysaccharide with some protein separated into two peaks on Sepharose 6B. Selvendran (1975b) established the existence of more than one type of wall glycoprotein in Phaseolus coccineus. This could perhaps account for the different results of flour extraction with Kündig's et al. (1961), the trichloroacetic acid, or Preece and McKenzie's (1952) methods. Different methods could extract different glycopeptides.

Finally, it is possible that the wheat which Fincher and Stone used was harvested when less mature than the Kansas wheat. During the process of ripening, low molecular-weight arabinogalactan-peptides could have polymerized with other carbohydrates and/or

with themselves, thus accounting for the high molecular size of my preparation. It would be interesting to investigate glycoproteins from a series of wheat samples that differ in degree of maturity.

Lamport and Miller (1971) indicated that extensin is widespread in the plant kingdom and thus that it should be found in different parts of the same plant. My results show clearly that a hydroxyproline-rich arabinogalactan is present in the seedling roots. However, it was not sufficiently purified to allow a quantitative comparison. The root extract yielded an additional carbohydrate and protein peak from Sepharose 6B which had a lower molecular weight, contained more protein, more hydroxyproline and more galactose than the peak at elution volume V_o. This points to the conclusion that the lower molecular-weight material might be a precursor of the high molecular-weight material; it differed from the V_o peak insofar that it was not as extensively arabinosylated.

The hydroxyproline content of wheat flour and wheat seedling roots was found to be very low. This agrees with Burke et al. (1974) who reported a much lower hydroxyproline content in suspension-cultured gramineae than in Acer pseudoplatanus. This led them to the conclusion that hydroxyproline-rich glycopeptides do not have a structural function in the cell walls of gramineas.

Despite the vast number of publications on extensin (Lamport, 1971; Lamport, 1970; Karr, 1972; Sadava and Chrispeels, 1973), its

function is unknown. It is well established that hydroxyproline is a part of the cell walls of higher plants (Lamport, 1965; Lamport, 1970; Northcote, 1972; Selvendran et al., 1975; Selvendran, 1975b). Northcote (1972) reported hydroxyproline oligosaccharides closely bound to ∝-cellulose. Selvendran (1975a) also reported

> "that the HP-rich glycoprotein is actually associated with the cellulose fraction."

Selvendran et al. (1975) found hydroxyproline apparently associated with the lignin fraction, since it was released during delignification. They essentially extracted all hydroxyproline from the cell wall with the chlorite-acetic acid procedure. Selvendran (1975a) described the hydroxyproline content of mature runner beans as higher than of immature ones. These results indicate that the hydroxyproline-rich glycopeptides are associated with the secondary wall, rather than the primary wall. This agrees with Chrispeels (1969) and Chrispeels et al. (1974) observations on carrot discs, which increased their hydroxyproline content upon aging.

Most of the research on hydroxyproline has been done on dicotyledons. The work thus far reported on monocotyledons was on gramineae, which were found to be extremely low in hydroxyproline (Burke et al., 1974; Fincher, 1975). The only other monocotyledon examined (Selvendran, 1975a) is asparagus, which contained more hydroxyproline in the wall than gramineae, but less than typical dicotyledons. Extensin is found (Lamport, 1971) in the cell walls of phylogenetically very old plants to phylogenetically

young plants like the class of the dicotyledons. One can hypothesize that at some time in plant history, an enzymatic pathway leading to cell wall extensin was shut off in the gramineae. Arabinogalactan-peptides such as reported here probably are not from the cell wall, suggesting that it must have been present in older related plants. According to this hypothesis, the arabinogalactan which I isolated is a vestigial extensin.

REFERENCES

Acher, R., Fromageat, C., and Justisz, M. (1950); Biochim. Biophys. Acta 5:81

Albersheim, P., Bauer, W. D., Keegstra, K., and Talmadge, K. W. (1973); In Loewus, Biogenesis of Plant Cell Polysaccharides. p. 117, Academic Press, New York and London.

Albersheim, P. (1975); Sci. Am. 232: April 80

Aspinall, G. O., Carincross, I. M., and Ross, K. M. (1963); J. Chem. Soc.: 1721

Aspinall, G. O., and Wilkie, K. C. B. (1956); J. Chem. Soc.: 1072.

Brysk, M. M., and Chrispeels, M. J. (1972); Biochim. Biophys. Acta 257:421.

Burke, D., Kaufmann, P., McNeil, M., and Albersheim, P., (1974); Plant Physiol. 54:109.

Chrispeels, M. J. (1969a); J. Inst. Brew. 81:116.

Chrispeels, M. J. (1969b); Plant Physiol. 44:1187.

Chrispeels, M. J., and Sadava, D. E. (1971); 30th Symp. Soc. Dev. Biol., p. 131, June 17-19

Chrispeels, M. J., Sadava, D. E., and Cho, Y. P. (1974); J. Exp. Bot. 25:1157.

Cleland, R. (1968); Plant Physiol. 43:865.

Cleland, R., and Karlsness, A. M. (1967); Plant Physiol. 42:669.

D'Appolonia, B. L. (1973); In Y. Pomeranz, Industrial Uses of Cereals. p. 138, St. Paul, Minnesota

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebens, P. A., and Smith, F. (1956); Anal. Chem. 28:350.

Fincher, G. B. (1975); J. Inst. Brew. 81:116.

Fincher, G. B., Sawyer, W. H., and Stone, B. A. (1974); Biochem. J. 139,535.

Fincher, G. B., and Stone, B. A. (1974a); Aust. J. Biol. Sci. 27:117.

Fincher, G. B., and Stone, B. A. (1974b); Aust. J. Plant Physiol. 1:297.

Gardiner, M., and Chrispeels, M. J. (1975); Plant Physiol. (1975); 55:536.

Gardiner, M., and Chrispeels, M. J. (1973); Plant Physiol. 51:8.

Hall, J. L., Flowers, T. L., and Roberts, R. M. (1974); Plant Cell Structure and Metabolism, Longman, London.

Hochstrasser, K. (1961); Z. Physiol. Chem. 324:250.

Kakuda, N. S. (1973); Ph.D. Dissertation, Kansas State University.

Karr, A. L. (1972); Plant Physiol. 50:275.

Kivirikko, K. J., and Liesmaa, M. (1963); Acta Physiol. Scand. 60:1.

Kulp, K. (1968); Cereal Science Today 13:414.

Kündig, W., and Neukom, H. (1963); Helv. Chim. Acta 46:1423.

Kündig, W., Neukom, H., and Deuel, H. (1961); Helv. Chim. Acta 46:1423

Lamport, D.T.A. (1970); Ann. Rev. Plant Physiol. 21:235.

Lamport, D.T.A. (1971); 30th Symp. Soc. Dev. Biol. 113, June 17-19

Lamport, D.T.A. (1967); Nature 216:1322.

Lamport, D.T.A. (1964); Exp. Cell Res. 33:195.

Lamport, D.T.A. (1965); in Advances in Botanical Research 2 (Preston R. D., ed.,) p. 151, Academic Press, London.

Lamport, D.T.A. (1969); Biochemistry 8:1155.

Lamport, D.T.A. (1972); Colloques internationaux C.N.R.S.N° 212, 11th - 15th Sept. 1972.

Lamport, D.T.A., and Miller, D. H. (1971); Plant Physiol. 48:454.

Lamport, D.T.A., and Northcote, D. H. (1960); Nature 188:665.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J. (1951); J. Biol. Chem. 193:265.

Mares, D. J., and Stone, B. A. (1973a); Aust. J. Biol. Sci. 26:793.

Mares, D. J., and Stone, B. A. (1973b); Aust. J. Biol. Sci. 26:813.

Mares, D. J., and Stone, B. A. (1973c); Aust. J. Biol. Sci. 26:1005.

Mascarenhas, J. P. (1970); Biochim. Biophys. Res. Commun. 41:142.

McNeil, M., Albersheim, P., Taiz, L., and Jones, R. L. (1975); Plant Physiol. 55:64.

Miller, D. H., Mellman, J. S., Lamport, D.T.A., and Miller, M. (1974); J. Cell Biol. 63:420.

Morre', D. J., and Van Der Woude, W. J. (1971); 30th Symp. Soc. Dev. Biol. June 17-19, p. 81-111

Neumann, R. E., and Logan, V. A. (1950); J. Biol. Chem. 184:299.

Neukom, H., Kundig, W., and Deuel, M. (1962); Cereal Sci. Today, 7:112.

Neukom, H., Providoli, L., Gremli, H., and Hui, P. A. (1967); Cereal Chem. 44:238.

Nordin, P., Jilka, R., and Whitlock, L. (1975); Phytochem. 14:1355.

Northcote, D. H. (1969); Essays in Biochemistry 5:90.

Northcote, D. H. (1972); Ann. Rev. Plant Physiol. 23:113.

Olson, A. C. (1964); Plant Physiol. 39:543.

Preece, J. A., and McKenzie, K. G. (1952); J. Inst. Brew. 58:457.

Rehfeld, G. (1963); Ernahrungsforschung 7:704.

Sadava, D., and Chrispeels, M. J. (1971); Biochemistry 10:4290.

Sadava, D., and Chrispeels, M. J. (1971b); Biochim. Biophys. Acta 227:278.

Sadava D., and Chrispeels, M. J. (1973); in Biogenesis of Plant Cell Wall Polysaccharides, p.165, Academic Press, New York and London

Selvendran, R. R. (1975a); Phytochem 14:1011.

Selvendran, R. R. (1975b); Phytochem 14:2175.

Selvendran, R. R., Davies, A. M. C., and Tidder, E. (1975); Phytochem. 14:2169.

Steward, F. C., Israel, M. M., and Salpeter, M. M. (1974); J. Cell Biol. 60:695.

Thimann, K. V., and Bonner, J. (1933); Proc. Roy. Soc., London, B, 113:126.

Wiesner, J. (1888); Ber. d. Deutsch Bot. Yes. 6:187.

ACKNOWLEDGMENT

I wish to express my deepest gratitude to my major professor, Dr. Philip Nordin, for his advice and guidance before and during the course of this work and his help in preparation of this thesis.

I wish to thank Dr. David Cox for his encouragement while Dr. Nordin was on sabbatical leave.

I am also grateful to Dr. Y. Pomeranz for administering the Research Assistantship from the Grain Marketing Research Center in Manhattan and for the Research assistantship which was provided by the Biochemistry Department.

HYDROXYPROLINE IN WHEAT ENDOSPERM AND WHEAT SEEDLING ROOTS

by

Annemarie Heyne

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas 66506

1976

ABSTRACT

The water-soluble non-starchy polysaccharides or pentosans of wheat endosperm were extracted by three different methods: water-extraction, water-extraction of wheat flour that had first been treated with hot 80% ethanol, and trichloroacetic acid extraction. The chemical composition has been studied with special attention to hydroxyproline content and sugar composition. The trichloroacetic acid preparation was fractionated by ammonium sulfate precipitation into an arabinoxylan with only 0.7% protein and an arabinogalactan with 4.4% protein (galactose:arabinose 79:21). The latter contained appreciable amounts of hydroxyproline.

Polysaccharides that eluted together with protein were also isolated from wheat seedling roots with trichloroacetic acid. The extract was further separated by gel chromatography into two major peaks which were analyzed for hydroxyproline and sugar composition. Arabinose and galactose predominated.