ASSOCIATION OF CARBOHYDRATE AND PROTEIN IN WHEAT GLUTEN

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by

Graham John McMaster

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ABSTRACT

McMaster, Graham J., Ph.D., The University of Manitoba, May 1982. Association of Carbohydrate and Protein in Wheat Gluten. Major Professor; W. Bushuk.

Carbohydrate and protein components of bread wheat gluten were examined for potential associative properties. The levels of carbohydrate in three fractions, the alcohol-soluble fraction (gliadin), the alcohol-soluble fraction I (derived from the alcohol-soluble fraction by gel filtration), and the alcohol-insoluble fraction (glutenin) were found to be 0.6%, 2.9%, and 17.0% (w/w), respectively. The carbohydrate components of the alcohol-soluble and the alcohol-insoluble fractions were exclusively associated with the "high molecular weight" fraction obtained by gel filtration chromatography on Sephadex G-200. Partial chemical modification of the protein components (by reduction of disulfide bonds and alkylation of exposed sulfhydryl groups) did not release the carbohydrate components from the protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the peak fractions obtained by gel filtration of the reduced-alkylated gluten fractions, showed that the carbohydrate was specifically associated with aggregating protein subunits (peak I) of intermediate molecular weight ($< 68,000$). No carbohydrate was associated with the fraction (peak II) comprising subunits of highest molecular weight (68,000 to 110,000).

The SDS-PAGE patterns of two carbohydrate-containing fractions

(alcohol-soluble fraction I and alcohol-insoluble fraction) were similar. However, these fractions differed distinctly in the monosaccharide composition of the respective carbohydrate components. The alcohol-soluble fraction I contained galactose and the alcohol-insoluble fraction contained glucose as the major sugars.

Coincident stains for carbohydrate and protein after electrophoresis of the alcohol-soluble fraction I on agarose-acrylamide gels indicate that these components may be covalently linked. A galactose-hydroxyproline linkage (hydroxyproline-arabinogalactan peptide) was tested for but was not present in this fraction. A linkage involving serine is speculated but was not confirmed.

The carbohydrate component in the alcohol-insoluble fraction was released by hydrolysis of the protein component by Proteinase-K, an endopeptidase. Subsequent hydrolysis of the carbohydrate with amyloglucosidase yielded glucose, indicating that the carbohydrate of this fraction is an α-glucan. The molecular weight of the major portion of this carbohydrate component is between 6,000 and 12,000 (determined by gel filtration chromatography). Complete separation of the protein and carbohydrate in the proteolytic digest was achieved by gel filtration chromatography on Biogel P-6. Accordingly, it was concluded that the association between the carbohydrate and the protein in the alcohol-insoluble (glutenin) fraction is non-covalent.

Amino acid compositions of the peak fractions obtained by gel filtration chromatography showed that the proteins of peak I (contain carbohydrate) are more hydrophobic than peak II and III fractions (without carbohydrate). Because of the relatively high contents of proline, glycine, and hydrophobic amino acids, it is postulated that the secon-

dary structure of gluten proteins would favour the β-sheet and β-turn structure over the α-helix conformation. Secondary structure analysis of the 25 N-terminal amino acids of A_2-gliadin using the method of Chou and Fasman supports a folded β-sheet structure with a β-turn present at residues 12, 13, 14, and 15.

This study has confirmed the presence of two different low molecular weight carbohydrates in the aggregates of two gluten fractions comprising specific protein subunits. In addition, theoretical evidence was presented on the presence of the β-sheet and β-turn secondary structures in gluten proteins.

I. INTRODUCTION

Gluten is the proteinaceous, viscoelastic material that remains after the flour solubles are removed from a wheat dough by kneading in a stream of water. The unique rheological properties of bread dough are derived almost entirely from its gluten. While much has been learned in the past century about the physical and chemical nature of gluten, much more information is needed to explain precisely the functionality of gluten on the basis of the molecular structure of its components and their respective interactions.

Gluten contains approximately 80 to 85% protein, 5 to 10% lipids (dry weight basis) with the remainder of the material consisting largely of carbohydrates. Specifically, the aim of this research was to investigate the role of the carbohydrate component in gluten structure through biochemical and physical studies. Although preparations of gluten are known to contain carbohydrate, relatively little information has been obtained on this component, or its importance in gluten structure. This component has often been simply referred to as "occluded starch" (e.g. Kasarda et al. 1971 and references cited therein), and consequently its presence in gluten preparations has been relegated to that of a "contaminant", the implication, therefore, being that it is of limited functional significance. Recent work, however, has identified the presence of relatively large amounts of carbohydrate in purified glutenin fractions obtained from gluten (Khan 1977). The present study was carried out to determine whether the carbohydrate and protein components of

these fractions were "associated" and if so, whether the linkage between the two components was covalent or non-covalent. It was speculated that the carbohydrate component may be directly involved in the aggregation tendency of specific glutenin subunits and thereby contribute to the functionality of this protein in gluten, hence in dough.

In addition, the research was extended to include the gliadin (alcohol-soluble) component of gluten which also contains a characteristic carbohydrate-containing fraction as shown for the first time in the present study. Furthermore, speculation on the physical properties of gluten proteins, in the context of their interactions with carbohydrate, led to a theoretical examination of these proteins, from the point of view of their amino acid composition and possible secondary structures. The theoretical prediction method of Chou and Fasman (1978a) was used to predict a secondary structure for the 25 N-terminal amino acid residues of A_2-gliadin.

Accordingly, this thesis deals with three major aspects of gluten structure:

1. The carbohydrate-containing fraction of the alcohol-insoluble (glutenin) fraction of gluten;
2. The carbohydrate-containing fraction of the alcohol-soluble (gliadin) fraction of gluten;
3. The β-sheet and β-turn structure of gluten proteins predicted from amino acid composition and sequence.

II. REVIEW OF LITERATURE

A. Introduction

The major objective of the research project described in this thesis was to investigate and extend present knowledge on the physical and chemical properties of wheat gluten. Carbohydrate has been known to be present in preparations of wheat gluten, however, little research has been conducted into the associative properties of these components. Initially, this review of literature will cover a broad range of publications on wheat gluten, its rheology, structure, and importance in the breadmaking process. Information on carbohydrate and protein associations in wheat flour will be documented.

B. The Wheat Gluten Complex

A large amount of research has been carried out on the rheological, physical, and chemical properties of gluten proteins. Bread has become a staple in the diets of the developed countries and research into the baking properties of wheat flour has become important. Wheat flour has been separated into various components. The relative importance of these components has been evaluated by reconstitution studies. Thus, the importance of the gluten fraction in the "breadmaking potential" of wheat flour was realized.

Wheat gluten was first isolated by Becari in 1728 (Kasarda et al. 1971). The traditional method of preparation of gluten involves the mixing of flour and water to form a dough, followed by gentle washing

of the "dough ball" in water or dilute salt solution to remove starch and other soluble material. Gluten is recovered as a rubbery mass containing approximately 80% of the total protein of the flour (Dill and Alsberg 1924; Fisher and Halton 1936). Gluten mass, when formed this way, contains approximately 70% water. Lipids make up 5 to 10% of the dried gluten. They are not naturally associated with the protein, but are believed to be bound to the protein components during dough formation (Lee and Wan 1963; Olcott and Mecham 1947; Wootton 1966). Occluded starch makes up most of the remainder of gluten preparations (10 to 12%); small amounts of non-starchy carbohydrates may also be present (Udy 1957).

C. Classification of Wheat Endosperm Proteins

Wheat proteins have been classified traditionally according to their solubility properties. The first comprehensive fractionation scheme for wheat proteins was developed by Osborne in 1907 and was based on the differential solubility of the proteins in various solvents. Wheat proteins were classified into four major fractions:

i) albumins - soluble in water;

ii) globulins - soluble in salt solutions;

iii) gliadins - soluble in 70 to 90% ethyl alcohol;

iv) glutenins - soluble in dilute acid or alkali.

Gliadin and glutenin fractions comprise the main proteins of wheat gluten. Glutenin was defined as that portion of wheat proteins that is soluble in dilute acid or alkali, after sequential extraction of wheat flour with salt and alcohol solutions.

D. Functionality, Rheological Properties, and Breadbaking Potential of Wheat Proteins

The "quality" of a wheat flour is a term used to describe the performance of that flour in breadbaking. A good quality flour has a number of functional and rheological parameters that are considered suitable for baking. Some of these include mixing tolerance, dough development time, and baking performance. A moot question that has often been asked is: "What constituent(s) of wheat flour is(are) most important to the rheological properties of bread dough?".

The evaluation of these constituents in breadmaking quality has relied on the isolation of particular flour fractions and monitoring the effects of their reconstitution to "base" flours.

Initial reconstitution studies demonstrated that for a single wheat variety or a class of wheat varieties, increased loaf volume is directly proportional to increased protein content (Finney 1943 ; Finney and Barmore 1948). These studies also implicated gluten protein in the breadmaking process. Other fractions such as the water solubles, when reconstituted with a constant source of gluten and starch and baked into bread, showed no significant improvement on loaf volume. However, the presence of the water-soluble fraction was required to produce a normal loaf of bread (Hoseney et al. 1969).

The functional importance of glutenin in breadmaking was reported by Pomeranz in 1965. The dispersability of flour proteins in a solvent of urea (3 M), pyrophosphate buffer (0.01 M, pH 7.0) was examined for wheat cultivars of differing breadmaking quality. Cultivars of poor breadmaking quality contained a greater proportion of urea-soluble proteins (gliadin and soluble glutenin). Cultivars of good breadmaking

quality were found to contain a greater proportion of urea-insoluble proteins (glutenin and residue). These findings were confirmed by baking studies on reconstituted flours (Shogren et al. 1969). Various fractions of gliadin and glutenin were obtained by stepwise pH precipitation of gluten proteins dissolved in lactic acid (0.005 M). Reconstitution of individual protein fractions to base flours revealed that decreased amounts of glutenin resulted in decreased mixing time and flour water absorption. Increased amounts of gliadin proteins led to an increase of loaf volume and a large increase in the oxidation requirement for optimal loaf volume.

A comprehensive study of the relationships between solubility fractions of flours based on the Osborne procedure and breadmaking quality has been made (Orth and Bushuk 1972; Orth et al. 1972). Twenty-six spring wheats of widely differing quality were grown at four locations in western Canada. Loaf volume was found to be inversely related to the proportion of acetic acid-soluble glutenin. Conversely, the insoluble glutenin (residue protein) was positively correlated to loaf volume. It was concluded that glutenin was responsible for the variation in loaf volume among the varieties within the class of wheat investigated.

Mixing experiments have also demonstrated the functional importance of gluten proteins in breadmaking. In general, flours that show a longer mixing time to optimum dough development have better breadmaking potential than flours with a short mixing requirement. Mecham (1968) demonstrated that freeze-dried doughs contained more protein that was soluble in dilute acidic acid than the original flours. Tanaka and Bushuk (1973b, c) showed that the amount of residue protein (insoluble

glutenin) decreased with increased mixing time beyond peak dough development in the farinograph. A mechanism of depolymerization of glutenin through disulfide bond interchange reaction was postulated to explain the decrease in the amount of residue protein and the breakdown of dough structure. This hypothesis, however, was in contrast to the work of Tsen (1967), who proposed a mechanism of disaggregation for dough breakdown on prolonged mixing. Lee and MacRitchie (1971) studied the effect of flour protein fractions on the rheological properties of dough using the mixograph and alveograph. Flour was extracted sequentially with water, 2 M urea, 4 M urea, and 0.1 M sodium hydroxide. Addition of the urea (2 M) extract (mainly gliadin and soluble glutenin) to a base flour resulted in decreased mixing stability, and weaker, more extensible doughs. Addition of the urea (4 M) and sodium hydroxide (0.1 N) extracts (mainly glutenin) increased mixing stability and produced stronger doughs.

Bushuk and Wrigley (1971) investigated the possibility that glutenin may have arisen from aggregation of low molecular weight proteins during the ripening and drying stages of grain development. They showed that glutenin was present at all stages of grain development (from 12 days after anthesis to maturity) and did not form rapidly during the late stages of maturation when the moisture content of the grain decreased markedly. They also observed that a low molecular weight glutenin was present in hexaploid wheats but was not present in durum wheats in the late stages of grain maturity. It was postulated that this low molecular weight glutenin may be related to breadmaking quality. Huebner and Wall (1976) fractionated glutenin from good quality and poor quality wheat flours. Two fractions were obtained: fraction I (a high molecular weight

glutenin) and fraction II (a low molecular weight glutenin). The ratio of fraction I to fraction II was higher for flours of good breadmaking varieties.

More recently, factors responsible for the poor breadmaking quality of high yielding European wheats (e.g. Maris Huntsman) have been identified in studies using reconstitution techniques and small scale test baking (Booth and Melvin 1979). Interchanging flour components with analogous fractions of Canadian hard red spring wheat demonstrated that these factors resided in the gluten, rather than in the starch or the water-soluble fraction. The proportion of protein insoluble in lactic acid solution (residue protein) was much lower in Maris Huntsman than the Canadian wheat sample, thereby supporting the earlier work of Orth and Bushuk (1972). Baking studies showed that no improvement in loaf volume resulted when protein levels were increased with residue protein derived from Maris Huntsman. These studies also showed a lack of breadmaking quality in both the lactic acid-soluble and the lactic acid-insoluble components of gluten derived from Maris Huntsman. Recent studies on good and poor quality Australian wheats showed similar results (MacRitchie 1978).

Payne et al. (1979) used SDS polyacrylamide gel electrophoresis (SDS-PAGE) patterns to survey bread wheats of related pedigrees. A subunit of glutenin with a molecular weight of 145,000 appeared to be correlated with desirable breadmaking quality. The authors postulated two mechanisms by which this subunit may improve glutenin for breadmaking. Firstly, the subunit may form large and stable glutenin aggregates more effectively than other high molecular weight subunits present. Secondly, the addition of genes which code for this subunit to a genome

may simply increase the quantity of the specific high molecular weight subunits and thus improve breadmaking quality. However, it must be pointed out that several unrelated cultivars of good breadmaking quality in this study did not contain the high molecular weight subunit implicated in breadmaking quality. Amongst this group were the cultivars Marquis (Canada) and Gabo (Australia), both renown for good breadmaking quality.

E. Physical and Chemical Studies of Gluten Proteins

Physico-chemical studies have been undertaken to provide information on the structure of gluten proteins. A knowledge of the structural properties of these proteins may then provide insights into their functionality in breadmaking. The parameters studied have included amino acid composition, amino acid sequence, polyacrylamide gel electrophoresis, gel filtration, optical rotary dispersion, viscosity, molecular weight, and disulfide contents. All these studies have provided information relevant to the complex structure of functional gluten. Of the gluten fractions studied, glutenin, the alcohol-insoluble fraction, has received most attention.

Wu and Dimler (1964) studied the viscosity, sedimentation velocity, ultraviolet difference spectra, and optical rotary dispersion of glutenin and concluded that the glutenin molecules are highly asymmetric. Another study, using optical rotary dispersion, concluded that glutenin, when dispersed in urea (3 M), possessed a low α-helix (secondary structure) content (Wu and Cluskey 1965). In hydrochloric acid solution, however, the α-helical content of glutenin was found to be somewhat higher (Wu and Cluskey 1965). Applying this technique to gliadin and glutenin in

aluminum-lactate buffer, Cluskey and Wu (1966) observed that gliadin contained a greater proportion of α-helix than did glutenin. Addition of urea resulted in a decrease of α-helicity in glutenin. The authors postulated that the secondary structure of glutenin was a mixture of random coil and α-helix with the random coil being the predominant form.

Addition of small quantities of reducing agents to preparations of glutenin causes a marked increase in its solubility. Beckwith and Wall (1966), investigating the reduction and reoxidation of proteins in glutenin solutions, observed a large decrease in viscosity immediately after addition of reducing agent. However, on subsequent standing, a slight increase in viscosity was observed. The initial viscosity decrease was attributed to the cleavage of interpolypeptide disulfide bonds. The slight increase in viscosity on standing was attributed to either the unfolding of polypeptide chains after slower reduction of intra-chain disulfide bonds or to non-covalent aggregation of the products. The oxidation of reduced glutenin at a concentration of 5% (w/v) resulted in a product similar to native glutenin in viscosity, molecular weight, and elasticity. It was concluded that an appropriate ratio of intra- and inter-chain polypeptide disulfide bonds is essential for the visco-elastic properties of glutenin required for optimum functionality in the breadmaking process. Bietz and Wall (1972) studied the subunit composition of gliadin and glutenin proteins using SDS-PAGE. Fifteen subunits were identified in the glutenin fraction, ranging in molecular weight from 11,000 to 133,000 daltons. Orth and Bushuk (1973b) identified approximately the same number of subunits in bread wheat.

Bietz and Wall (1973) showed that 62% of reduced-alkylated glutenin was soluble in 70% aqueous ethanol solution. SDS-PAGE analysis of this

fraction showed that it comprised mainly "gliadin-like" subunits of 44,000 and 36,000 daltons. However, there were definite differences in the amino acid composition of these subunits from that of gliadin proteins.

Dalek-Zawistowska et al. (1975) postulated that glutenin existed as a high molecular weight aggregate in equilibrium with disaggregated, low molecular weight proteins. In this study, an acetic acid dispersion of gluten was subjected to gel filtration on Sephadex G-200. Two peaks were obtained; a high molecular weight, excluded peak (glutenin), and an included peak (gliadin). Rechromatography of the excluded peak again yielded two peaks; the second peak eluting at the same volume as the previous included peak. However, the amino acid composition of the two included peaks were different. Proteins from the included peak (obtained from rechromatography of the original excluded peak) were radioactivity labelled and incubated with unlabelled proteins from the excluded peak and again subjected to gel filtration. Radioactivity was found in the excluded peak providing the basis for the equilibrium (aggregation) hypothesis.

Apart from the physico-chemical studies of gliadin and glutenin, a number of studies using electron microscopy have provided information on the ultra structure of gluten proteins. Seckinger and Wolf (1970), using transmission electron micrographs, examined particles of gliadin proteins from both hard and soft wheats. Particle diameters of 20 to 80 Å were obtained, representing a molecular weight range of 17,000 to 216,000 daltons. Discrete particles were not formed by the glutenin fraction.

A transmission electron microscope study of mature endosperm cells demonstrated the presence of residual structures such as endoplasmic

reticulum and amyloplast membranes (Simmonds 1972). The author concluded that a proportion of the glutenin fraction was derived from the endoplasmic reticulum and the remainder from the high molecular weight protein fraction in the endosperm of developing and mature grain and from the association of protein and lipid molecules when flour is mixed with water to form dough (Bushuk and Wrigley 1971; Simmonds and Wrigley 1972).

Transmission electron microscopy was used to demonstrate that storage proteins in wheat endosperm are deposited in protein bodies within lipo-protein membranes (Buttrose 1963; Graham et al. 1962; Jennings et al. 1963). It was observed that protein bodies are formed and their number and size increases throughout grain development. These increases were accompanied by an increase in the proportion of acetic acid-soluble glutenin (Jennings 1968; Jennings and Morton 1963a, b; Jennings et al. 1963). In another study, Bernardin and Kasarda (1973a, b), using scanning and transmission electron microscopy, observed that storage proteins were deposited in a laminar form within protein bodies. The laminar structure is converted to a sheeted structure upon hydration of the protein when water is added to the flour. The authors postulate that under stress, as in dough mixing, these sheets rupture forming fibrillar webs of protein which are composed of fibrils ranging in diameter from 50 to several thousand Angstrom units. Although a great deal of research has been carried out on gluten proteins, a definite molecular structure for these proteins has not evolved. A number of models of glutenin proteins have been proposed on the basis of available data in attempts to explain the functional properties of the protein. The literature on these models is reviewed in the next section.

F. Models of the Structure of Glutenin

A number of models of glutenin have been proposed. The first models were simplistic and tended to be inflexible in nature. These were gradually modified, as new information became available. The most recent models are much more encompassing and more flexible in nature.

The basis of all the structural models of glutenin reported in the literature relies heavily on the disulfide bond as the interpolypeptide cross-linkage. This raises the question whether the disulfide bonds are inter-molecular or intra-molecular, or more realistically, the proportion of each type present in the functional glutenin structure.

Some authors, using specific chemical reagents, have attempted to distinguish between two types of disulfide bonds (Ewart 1972a; Stevens 1966, 1973). These experiments indicated that a substantial number of interpolypeptide disulfide bonds are present in glutenin. Kasarda et al. (1976) have pointed out, however, that this interpretation involves the unproven assumption that under the conditions used, inter-molecular bonds are reactive and that intra-molecular bonds are not reactive.

Ewart (1968) proposed the first model of the structure of glutenin. This model was based on the data obtained in an earlier study of the disulfide bond content of glutenin (Redman and Ewart 1967a, b). Glutenin was represented as a linear molecule of polypeptide chains (subunits) attached to each other by interchain disulfide bonds. However, it was subsequently realized that this model could not explain the rheological and functional properties of dough and gluten (Ewart 1972a). These properties included: (a) viscous flow, (b) the almost instantaneous appearance of viscoelasticity as soon as flour is wetted, and (c) dough formation in the presence of inhibitors of disulfide

exchange reactions. Accordingly, Ewart (1972b) proposed a more complex structure for glutenin. In this second model, extensively folded polypeptide chains were joined by two interchain disulfide bonds to each neighbouring subunit to form "concatenations". In a third model, Greenwood and Ewart (1975) introduced a modification to include unbranched molecules with a variable number of polypeptide chains, not necessarily of the same type, joined together by disulfide bonds to form large molecules possessing linear unbranched configuration. The authors postulated that the "rubber-like" elasticity, characteristic of glutenin, is derived from strong secondary forces at regions of interaction (or entanglement) termed nodes. Ewart (1977), in proposing a further modification of his model, placed less emphasis on these regions of interaction, and put forward the concept of sequential build up of secondary forces to explain the phenomenon of dough elasticity.

A contrasting model for glutenin proteins was proposed by Kasarda et al. (1976). This model contains no interchain disulfide bonds, but only intrachain disulfide bonds with secondary forces playing a major role in forming the highly insoluble glutenin particles. This view was based on earlier observations of the reversible aggregation of A-gliadin subunits into microfibrillar structures through secondary forces only. A-gliadin aggregated or disaggregated, depending on pH and ionic strength, to form particles with molecular (particle) weights of millions of daltons (Bernardin et al. 1967; Kasarda et al. 1967). Transmission and scanning electron microscopy of the aggregated A-gliadin showed that it possessed a microfibrillar structure, similar to structures derived from wheat endosperm protein (Bernardin and Kasarda 1973a, b; Orth et al. 1973a, b). In the model of Kasarda et al. (1976), glutenin is

depicted as a complex aggregate of subunits held together by highly specific interacting secondary forces such as ionic, hydrogen, and hydrophobic bonds. The authors suggested that the aggregate can be readily dissociated by reducing the intrachain disulfide bonds, thereby destroying the specific conformation required for the aggregation. Reagents that disrupt the ability of a protein subunit to participate in the specific interactions necessary for aggregation, should have similar effects on the viscosity of the system to those expected of reagents which cleave intermolecular disulfide bonds of a cross-linked system.

Kobrehel and Bushuk (1977) showed that the acetic acid-insoluble glutenin can be solubilized by the addition of soaps such as sodium stearate. By further demonstrating that this solubilization procedure does not involve the breakage (reduction) of disulfide bonds (Hamauzu et al. 1979) the hypothesis that glutenin may be an aggregate and not a large molecule is strongly supported.

The most recent model for the structure of "functional" glutenin, proposed by Khan and Bushuk (1979a)is inherently flexible in nature. In developing this model, the authors point out that researchers concerned with the functionality of glutenin in breadbaking (e.g. Orth and Bushuk 1973c; Shogren et al. 1969) used relatively crude preparations, while those concerned with fundamental physico-chemical studies (e.g. Jones et al. 1959; Mita and Yonezawa 1971; Orth and Bushuk 1973a; Bietz and Wall 1975) used relatively "purified" preparations.

The aim of the purification was to remove low molecular weight "contaminants" from the glutenin preparations. These "contaminants" were defined as unreduced protein components that entered a 5% SDS-

polyacrylamide gel upon electrophoresis. Khan and Bushuk (1977) suggested that there are two groups of proteins in a glutenin complex: a) those that enter the gel on PAGE (glutenin I) and those that remain at the point of application (glutenin II). It was concluded that the relative quantities of these types of glutenin molecules, or their ability to form aggregates, may be important factors in breadmaking quality. In the proposed model, glutenin I proteins are held in the structural complex by strong non-covalent forces similar to those of the Kasarda et al. (1976) model. Khan and Bushuk (1979a) showed that glutenin I components comprise mainly subunits with a molecular weight of 68,000 or less, and are soluble in acetic acid and other dissociating solvents. Glutenin II comprises larger molecules in which polypeptide subunits are joined by interpolypeptide disulfide bonds. The stability of the functional aggregates depends on the presence of both intramolecular disulfide bonds in glutenin I and the intermolecular disulfide bonds of glutenin II.

Upon reductive cleavage of its disulfide bonds, the subunits of glutenin II enter the 5% gels on SDS-PAGE and migrate at a mobility equivalent to molecular weights of 68,000 and higher. According to this model, not all of the glutenin I enters the gel on SDS-PAGE of unreduced glutenin as some subunits are tightly bound within the compactly folded glutenin II. The change in structure resulting from the reduction of disulfide bonds in glutenin II, allows the release of glutenin I components. The authors claim that the model is qualitatively consistent with the known rheological (visco-elastic) properties of hydrated glutenin. The physical interactions between glutenin I and glutenin II provide for the mobility required for viscous flow under stress, and the

disulfide cross-links of glutenin II contribute the elasticity in dough.

A comprehensive interpretation of protein interactions as the basis of gluten structure has been proposed by Bietz and Wall (1980). These authors indicate that at least six major types of polypeptides, each with distinct features, properties, and origins, contribute to the unique rheological properties of wheat gluten. These types of polypeptides are briefly described in the sections that follow.

1. High Molecular Weight Polypeptides

This group of polypeptides is found only as subunits of glutenin (MW 80,000 to 130,000). They differ from low molecular weight glutenin subunits in amino acid composition and from gliadin in that they are insoluble in 70% ethanol. The authors suggested that these polypeptides have sufficient cysteine present allowing reaction between themselves and other polypeptides forming disulfide networks of glutenin. The possibility of separate particulate origins for different glutenin subunits (Simmonds 1972), leads the authors to suggest that these high molecular weight polypeptides have an independent origin or synthetic mechanism such as endoplasmic reticulum.

2. Aggregating Polypeptides

After treatment of glutenin with hydrophobic bond-breaking solvents such as SDS or sodium dodecanoate in the absence of reducing agents, gel filtration on Sepharose CL-4B separates a number of low molecular weight proteins from the high molecular weight glutenin peak (Huebner and Wall 1976). Reduction and alkylation of glutenin and chromatography on Sephadex G-200 results in high molecular weight and low molecular weight subunits eluting as included peaks B and C. However, the

excluded peak A contains polypeptides that remain aggregated under the gel filtration conditions used (Huebner and Wall 1974; Khan and Bushuk 1979b). A membrane origin was proposed for the aggregating polypeptides.

3. 44,000 MW Polypeptides

After reductive cleavage of disulfide bonds of glutenin, this group of polypeptides are soluble in neutral 70% ethanol, thus resembling gliadin in solubility. However, unlike gliadin, they have a molecular weight of approximately 44,000 daltons. These polypeptides occur in mature wheat endosperm in the high molecular weight gliadin or as subunits of glutenin held by aggregation or by inter-chain disulfide bonding. It was suggested that these polypeptides may originate in protein bodies (Miflin and Shewry 1979).

4. 36,000 MW Non-Gliadin Polypeptides

These proteins occur as intermolecularly-disulfide bonded subunits of high molecular weight gliadin or of glutenin.

5. Albumins, Globulins, and ω-Gliadins

Albumin and globulin proteins may adsorb or non-covalently associate with other wheat proteins. ω-gliadins are unique (compared with other gliadins) in having a molecular weight of 60,000 to 80,000 daltons and in lacking the amino acids cysteine and methionine. It was suggested that ω-gliadins interact with other proteins in a non-covalent fashion.

6. Low Molecular Weight Gliadins

These proteins comprise a group of homologous polypeptides with a molecular weight of 36,000 daltons. However, they are heterogenous in charge density and thereby produce many bands on electrophoresis.

Synthesis of these proteins occurs on ribosomes associated with the endoplasmic reticulum and they are deposited as storage proteins in protein bodies (Morton et al. 1964).

It has been suggested that the ethanol-soluble 44,000 and 36,000 molecular weight non-gliadin polypeptides, which comprise a large proportion of glutenin (Bietz and Wall 1973), are linked through intermolecular disulfide bonds to form small oligomers termed high molecular weight gliadin. Similarly, with additional disulfide cross-links these proteins can form high molecular weight glutenin or they can associate strongly with aggregating polypeptides to form another type of glutenin. These authors postulated that these reactions and interactions occur during synthesis, maturation, or desiccation of the endosperm in random order, resulting from juxtaposition of individual protein particles and organelles. Upon rehydration (as occurs during dough formation), glutenin can interact with low molecular weight gliadin and albumins, globulins, and ω-gliadins in at least three ways: (a) disulfide interchange reactions promoting more stable conformations, (b) reaction between bonding sites previously inaccessible, and (c) non-covalent associations of molecules (hydrogen, hydrophobic, and ionic bonding).

The current models of glutenin structure allow a large number of different covalent linkages and non-covalent interactions between moeities. The types of interactions proposed have been developed as researchers attempt to explain the rheological properties of doughs. However, to date no consideration has been given to the possibilities of interaction between protein and other flour components such as carbohydrate in the structure of functional glutenin. The following sections of this review will deal with research that has been done on the associa-

tions of carbohydrate and protein in wheat as well as in some other plant systems as a basis for the study reported in this thesis.

G. Glycoproteins in Plant Tissues

Proteins may be conveniently divided into two groups on the basis of their composition: (a) simple proteins containing only amino acid components and (b) conjugated proteins containing non-amino acid components, sometimes termed the prosthetic group. Conjugated proteins containing covalently linked carbohydrates are referred to as glycoproteins, proteoglycans, or peptidoglycans. There appears to be no clear distinction between the meanings of these terms. However, it is common practice to use the term glycoproteins when the substituted carbohydrate moiety has a comparatively low degree of polymerization. The remaining terms are usually applied to substances in which the polysaccharide component makes up most of the molecular weight (Brown and Kimmins 1977). Sharon and Lis (1981) differentiated between glycoproteins and proteoglycans on the basis of the carbohydrate component. In glycoproteins, such as serum glycoproteins, the oligosaccharides consist of up to 20 monosaccharide units, mostly in branched sequences that do not contain repeating structures. The proteoglycans, such as chondroitin sulfate and dermatan sulfate, contain linear units of 50 to 100 monosaccharide residues as repeating disaccharides structures. Sharon and Lis (1981) noted that the size and number of oligosaccharide chains in different glycoproteins can be highly variable and it is not unusual to find glycoproteins with as little as 1% carbohydrate (e.g. collagen) or as much as 85% (soluble blood group substances).

Because of their implication in the biology of higher animals and of mammals, glycoproteins derived from secreted fluids or circulating

body fluids have been investigated in great detail. However, the study of the substances from plant tissues has been neglected. This is probably due to the incorrect belief that amino sugars, which are characteristic constituents of glycoproteins, were not detected in plant tissues (Sharon 1965). It is now known that D-glucosamine is a widespread component of plants, although a relatively minor one. Other amino sugars are rarely found in plants, if at all.

Plant glycoproteins are not confined to a single species or to a specific part of the plant, but appear to be widely distributed, both phylogenetically and anatomically. They include lectins (phytoagglutinins), enzymes, toxins, structural, and other glyco proteins for which no biological function has been found. Although the role of saccharide moieties in some animal glycoproteins is now well understood, very little is known about their function in plant glycoproteins (Sharon 1974).

For unequivocal demonstration that a protein is indeed a glycoprotein, it is necessary to isolate from its digests a low molecular weight glycopeptide containing linked amino acids and carbohydrate residues. Such glycopeptides have been isolated from only a very small number of plant glycoproteins. However, for the majority of other glycoproteins, providing satisfactory evidence of their homogeneity through various purification steps is presented, it is often assumed that the carbohydrate is indeed an integral part of the molecule, covalently linked to the polypeptide chain (Sharon 1974).

H. Carbohydrate Constituents and Carbohydrate-Protein Linkages of Plant Glycoproteins

Plant glycoproteins isolated to date have a lower carbohydrate content than that found in animal glycoproteins (Sharon 1974). Extensin

precursor in carrot tissue has approximately 40% carbohydrate content; this is the highest reported carbohydrate content in a plant glycoprotein. In animal glycoproteins, a carbohydrate content of 50% (as found in mucins) to 85% (blood group substances) is not unusual. Seed storage proteins in legume species (Derbyshire et al. 1976) and some non-legume (Goding et al. 1970) species have been shown to contain small amounts of bound carbohydrate, suggesting a glycoprotein nature. However, the low levels of carbohydrate often found in seed glycoproteins have posed difficulties in both qualitative and quantitative analysis (Eaton-Mordas and Moore 1979).

The most common carbohydrate component of plant proteoglycans and glycoproteins comprises arabinose and galactose (Knee 1975; Brown and Kimmins 1977). A group of arabino-3,-6-galactans known as Aspinall Type II (Clarke et al. 1979) is distributed widely in plant tissues and is often associated with protein. These arabinogalactans occur in seeds, leaves, roots, fruit, as well as xylem sap. They are also constituents of many exudate gums of the angiosperms (e.g. Acacias) and gymnosperms. Arabino-3,-6-galactans are also found in filtrates and plant cells produced by tissue culture (Clarke et al. 1979).

Three types of carbohydrate-protein linkages have been proposed for plant proteoglycans and glycoproteins containing arabinose and galactose. These include galactopyranosyl 4-O-hydroxyproline linkage, a galactopyranosyl-O-serine linkage, and an arabinosylfuranosyl 4-O-hydroxyproline linkage (Clarke et al. 1979). In the proteoglycans from wheat endosperm, a galactopyranosyl-4-O-hydroxyproline linkage has been demonstrated in a water-soluble arabinogalactan (Fincher et al. 1974). The same linkage was found in the proteoglycan isolated from filtrates of Acer

culture tissue (Pope 1977). Alkali labile D-galactosyl-O-serine linkages have been demonstrated in proteoglycan isolated from the leaf tissue of Cannabis sativa (Hillstead and Wold 1977; Hillstead et al. 1977a). These bond compounds are presumed to link the main arabinogalactan chains to the protein. A second glycosyl-amino acid, which is alkali stable and believed to be glycosyl-hydroxyproline, has been detected in Cannabis leaves (Hillstead et al. 1977b). A proteoglycan isolated from rice bran has been shown to have an arabinofuranosyl-4-O-hydroxyproline linkage (Yamagishi et al. 1976). More recently, arabinogalactans associated with protein have been isolated from the female reproductive tissues of Gladiolus (Gleeson and Clarke 1979; Gleeson and Clarke 1980), as well as from the stigma exudate of Lilium longiflorum (Aspinall and Rosell 1978).

Proteoglycans containing arabinose and galactose have been implicated in diverse functions such as cell-cell adhesion, nutrition of growing pollen tubes, and as a response to microbial infection. It has also been suggested that they may be markers of identity in plant tissue, variation in identity being expressed in the terminal sequences of their saccharide chains (Clarke et al. 1979).

Arabinose and galactose containing glycoproteins are characteristically associated with the cell wall fractions of both higher and lower plant species (Lamport 1970; Preston 1979). These cell wall glycoproteins are sometimes referred to as "extensin" to convey the involvement of this glycoprotein in the control of elongation of growth (Lamport and Miller 1971; Cho and Chrispeels 1976). In these polymers, arabinose oligosaccharides are linked O-glycosydically to hydroxyproline and galactose residues are linked O-glycosydically to serine. These glycoproteins

appear to be associated with the water-insoluble cell-wall polysaccharides, especially cellulose (Monro et al. 1976), but have not yet been isolated in free form. Most of the arabinose in extensin occurs as short tetrasaccharide side chains containing (1→3) and (1→4) linkages (Lamport 1970). There is also evidence that cell wall hydroxyproline-rich glycoproteins contain a polyuronide arabinogalactan attached O-glycosydically to hydroxyproline (Selvendran 1975). Single D-galactose residues have been shown to be linked O-glycosydically to serine in extensin from tomato (Lamport et al. 1973), whereas extensin from carrot contains a short chain of galactose residues linked to serine (Cho and Chrispeels 1976). The potato tuber N-acetylglucosamine binding lectin is a glycoprotein with a structure resembling the cell wall glycoprotein in its arabinose-hydroxyproline linkage, but is richer in arabinose and is water-soluble (Allen and Neuberger 1973). Murray and Northcote (1978) compared this glycoprotein with the insoluble cell wall glycoprotein from potato callus cells. Their results indicated that the insoluble (wall) and the soluble (lectin) glycoproteins contain hydroxyproline residues glycosylated by short chain arabinose oligosaccharides. Glycoproteins rich in hydroxproline, arabinose, and galactose are a major cell wall component of the green algae Chlamydomonas reinhardii and are also present in wall preparations of other green algae (Miller et al. 1972; Roberts 1979). The glycoproteins from C. reinhardii can be extracted and reassembled in vitro to give a crystalline wall lattice (Catt et al. 1976).

The occurrence of glucose in plant glycoproteins has been of considerable interest because of the potential role of such macromolecules in the initiation of starch, glycogen, and cellulose biosynthesis

(Whelan 1976; Krisman and Barengo 1975; Lavintman et al. 1974; Hawker et al. 1974). D-glucose, although it does not appear to be widespread, has been found in a number of glycoproteins (Brown and Kimmins 1977). The distribution of D-mannose in plant glycoproteins is restricted. A low content of this monosaccharide occurs in glycoproteins which usually also contain glucosamine. Soybean agglutinin, a lectin, is an example of this class of glycoprotein (Sharon 1974). The carbohydrate moeity of soybean agglutinin (containing D-mannose and N-acetyl glucosamine) has been isolated from pronase digests of the glycoprotein, together with aspartic acid as the only amino acid involved in the carbohydrate linkage (Lis et al. 1966). The carbohydrate peptide binding group is β-N acetylglucosaminyl-L-asparagine. The carbohydrate structure is Man $\alpha(1\rightarrow 3)$ [Man $\alpha(1\rightarrow 6)$] Man β $(1\rightarrow 4)$ Glc NAc $\beta(1\rightarrow 4)$ Glc NAc-Asn, previously found in many animal glycoproteins as well as in those from fungi and yeast (Lis and Sharon 1978).

I. Glycoproteins in Wheat Tissue

Although a number of glycoproteins and proteoglycans have been reported in preparations of wheat tissue, few have been isolated and the glycopeptide characterized. A water-soluble proteoglycan, an arabinogalactan, that is associated with protein has been isolated from wheat endosperm (Fincher and Stone 1974). This arabinogalactan peptide is predominantly polysaccharide (92% w/w) with galactose and arabinose present in a ratio of approximately 1.5:1 (w/w). The arabinogalactan is covalently linked with a peptide (8% w/w) which contains 16 to 20% (on a molar basis) of hydroxyproline (Fincher and Stone 1974). The chemical and physical properties of this peptide have been closely

examined (Fincher et al. 1974). The arabinogalactan peptide is a non-associating, polydisperse macromolecule with a molecular weight of approximately 22,000. Examination of the products of partial acid hydrolysis of the polysaccharide component showed that arabinose is present in the α-L-arabinofuranosyl configuration. Infra red-absorption spectra and optical-rotation studies suggest that the D-galactopyranose residues are linked by glycosydic linkages in the β-anomeric configuration. Partial degradation of the substance by successive treatment with oxalic acid and Na OH showed that the linkage between the polysaccharide and the peptide involves galactose and hydroxyproline residues and is glycosydic in nature (Fincher et al. 1974). The intracellular location and function of this arabinogalactan-peptide has not been determined. A similar galactose- and hydroxyproline-rich polymer has been found in barley and Lolium perenne seeds (Fincher and Stone 1974). This observation lends support to the suggestion that the substance has a storage function. However, other studies, on the changes occurring in the water-soluble non-starchy polymers from wheat endosperm, suggest that the arabinogalactan peptide is not significantly altered during germination (Fincher and Stone 1974). Kundig et al. (1961) fractionated the water-soluble wheat flour pentosans on DEAE-cellulose columns and determined the chemical composition and characteristics of the fractions. Fraction 1 was a pure arabinoxylan and fractions 2 to 5 were reported to be glycoproteins. The polysaccharide constituents of three of the glycoprotein fractions (2, 4, and 5) were composed of xylose, arabinose, and galactose. Fraction 3 contained galactose and arabinose. It was shown that the galactose residues in the latter fraction are a part of the arabinogalactan, a polysaccharide distinctly different from the

arabinoxylans (Neukom and Markwalder 1975). This fraction contained 6 to 8% protein. The high content of hydroxyproline (16 to 20%) indicated that it is most likely identical with the arabinogalactan peptide isolated by Fincher et al. (1974).

Neukom et al. (1967) studied gelation of pentosan solutions and flour extracts upon addition of oxidizing agents. This gelation is unusual and not exhibited by other natural polysaccharides. It has been shown that soluble arabinoxylans (Fausch et al. 1963) and insoluble arabinoxylans (Geissmann and Neukom 1973) contain small amounts of ferulic acid which can be released by careful saponification with alkali under nitrogen. The ferulate is most likely bound by ester linkages to the xylan chain. Both arabinoxylans were similar in composition, however, the reason for the insolubility of the larger part of the pentosans is not known. It had been suggested that the ferulic acid residues are dimerised by oxidative coupling to form di-ferulic cross-links which would insolubilize the pentosans (Geissmann and Neukom 1973). This view is supported by the detection and isolation of small amounts of diferulic acid from the water-insoluble pentosans of wheat endosperm (Markwalder and Neukom 1976). The gels formed by oxidation can be liquified by proteolytic enzymes. Treatment of the flour extract with pronase before oxidation prevented gelation. A coupling of a tyrosine residue with a ferulic acid residue forming a tyrosine-ferulic acid cross-link was postulated as the mechanism of the oxidative gelation (Neukom 1976).

Wrench (1966) reported the presence of a glycopeptide in sodium pyrophosphate extracts of wheat flour. The hexose, pentose, and hexosamine contents of the glycopeptide are 44.0%, 5.6%, and 0.2% (w/w), respectively. The glycopeptide is heterogenous with respect to molecular weight. The

nature of the linkage between protein and carbohydrate was not determined. Rohrlich et al. (1963) reported the presence of a glycoprotein in wheat flour. Paper electrophoresis yielded one band that contained both protein and carbohydrate. The fraction (approximately 21% protein) was unstable below pH 4.6 and separated into protein and carbohydrate moieties. This would suggest that the linkage between carbohydrate and protein was non-covalent. Inouye et al. (1974) isolated two carbohydrate-rich fractions (A and B) from wheat gluten. Fraction B contained more lipid than fraction A. The lipid portion of fraction B consisted mainly of glycolipid, the main two components being galactolipid and glucolipid. After digestion with pronase, defatted fraction A was isolated by chromatography on DEAE-cellulose and Sephadex G-25. Arabinose, galactose, and glucose were present in almost equal amounts in the glycopeptide. The authors did not determine the nature of the carbohydrate-protein linkage.

Graveland et al. (1979) reported the presence of six glycoprotein fractions in wheat flour. These fractions accounted for 70% of wheat endosperm protein and appear to be highly glycosylated, containing up to 40% carbohydrate. Apart from reporting the presence of carbohydrate in wheat flour fractions, the authors did not present any evidence for a covalent linkage between carbohydrate and protein. Subsequently, the same workers (Graveland et al. 1980) elaborated a functional role for these fractions in the rheological properties of dough.

J. Interaction of Proteins and Polysaccharides in Wheat Dough Systems

Interaction of proteins and polysaccharides of wheat flour was first noted in studies of the viscosity of dispersions and extracts of wheat flour. Udy (1956) reported that soluble polysaccharides accounted

for approximately two-thirds of the intrinsic viscosity of dilute acid extracts of flours, even though they represented only 1% of the sample weight. The authors reasoned that because wheat proteins have much lower intrinsic viscosities than do soluble polysaccharides, any increase in the ratio of proteins to polysaccharides should decrease the observed intrinsic viscosity. However, viscosities of dilute acid dispersions of flour increased approximately 10 times the value expected from the increase of protein content (Bresson and Barmore 1955). Further viscometric studies showed that the flow properties of dispersions of wheat flour in dilute acids are influenced primarily by an interaction between dispersed polysaccharides and gluten proteins (Udy 1957). Addition of sodium salicyclate (8% solution) and urea (2 M) prevented these interactions. Urea also caused firm gels from polysaccharides and gluten to liquify when shaken gently. These results support the view that weak secondary molecular forces were involved in the interaction between gluten proteins and soluble polysaccharides of wheat. Udy (1957) also suggested that molecular size of the polysaccharide may be the most important single factor in the interaction.

Wilham et al. (1959) studied the effects of addition of neutral polysaccharides to wheat flour on farinograph mixing curves. Addition of 2% native B-2 dextran produced an increase of 10% in water absorption and modified the farinogram. These effects were attributed to protein-polysaccharide interactions. However, not all of the dextrans that were added produced these effects. Addition of the more highly branched dextran B-742 increased water absorption value by 5% and had less influence on the shape of the farinogram than did the native B-512 dextran. Addition of clinical B-512 dextran (MW $\sim$ 70,000) caused a slight

decrease in water absorption and had no effect on the mixing curve. It was concluded that the effect of added dextrans appeared to be related to the molecular size of the polysaccharide. An interesting demonstration of the effect of native B-512 dextran on the gluten complex was provided by kneading a gluten ball in a solution of 5% native dextran. The partial disintegration of the gluten ball that was observed suggested that the dextran caused a marked change in the solubility properties of the gluten proteins. Clinical B-512 dextran and the highly branched B-742 dextran did not give this effect.

Jones and Erlander (1967) extended studies on the interaction of dextrans and wheat proteins by measuring the turbity of hydrocolloids in solution and by monitoring effects on dough mixing curves. Dextrans with high intrinsic viscosities and high percentages of α 1,6 glycosidic linkages produced a large effect on mixing curves. The effect of viscosity, however, was found to predominate over the effect of α 1,6-bonds. Dextrans containing a high percentage of α 1,6-bonds did not react if they possessed low intrinsic viscosity. In solution, some dextrans interacted with gluten, gliadin, and glutenin in acetic acid (0.01 M) producing either turbidity or a precipitate. The interaction was dependent upon protein concentration. It was found that not all dextrans or dextran fractions interact to the same degree and that some do not interact at all. The authors stated that the effect of dextran on mixing curves and on solution turbidity may not be due to the same phenomenon; dextrans which produce an effect in the mixer sometime have no effect in solution. In general, dextrans that had no effect on dough mixing curves were of high molecular weight, had high intrinsic viscosity, and contained relatively few branches. The dextran fraction that produced the greatest

turbidity with gluten proteins was the low molecular weight clinical dextran.

Huebner and Wall (1979) studied the effect of additions of polysaccharides to flour mixed into doughs in the farinograph. Two microbial polysaccharides increased peak time and dough stability. Variable effects on the mixing characteristics were obtained by the addition of certain microbial polysaccharides and small amounts of commercial carrageenan gum. The authors also investigated polysaccharide-protein interactions by adding polysaccharide to partially purified gluten solutions and determining the turbidity and viscosity of the resulting mixtures. The effects varied from no apparent interaction to strong association and precipitation. The authors suggested that the mechanism of the interaction involves ionic (electrostatic) and hydrogen bonding. The configuration of the polysaccharide molecule and the availability of charged groups on the molecule were found to be important.

Finney (1943), using reconstitution techniques, observed various responses to the addition of flour solubles to gluten-starch doughs depending on the wheat variety used as the source of the solubles. In further studies, it was demonstrated that the protein component, rather than polysaccharide, was responsible for the effect of flour solubles on loaf volume (Pence et al. 1950). However, the work of Tracey (1964) focused attention on the pentosan component of the water-soluble flour fraction. Addition of snail digestive juice, a known source of polysaccharases, but not proteases, to the flour solubles caused a reduction in loaf volume. Without addition of enzyme, the water-solubles increased loaf volume. The authors presented evidence that this effect was due to pentosanases rather than other polysaccharases in the enzyme preparation.

Cawley (1964) studied the effects of addition of various viscous gums on the loaf volume of bread from gluten-starch blends. Addition of agar, sodium alginate, amylose, chondroitin sulfate, dextran, Ficoll, pectin, DEAE-cellulose, and insoluble carboxymethyl-cellulose had no effect on loaf volume. Addition of hydroxypropylmethyl-cellulose, methyl cellulose, guar gum, amylopectin, soluble carboxymethyl cellulose, and carob bean gum increased loaf volume. Polymers with larger degrees of substitution appeared to be most effective in increasing loaf volume. Polysaccharides with charged groups present were found to be ineffective with the exception of dextran sulfate (which has a high degree of substitution), and soluble carboxymethyl cellulose in which the charge is on the bulky side group.

The effect of differences in starch properties in baking has been noted by a number of researchers (Sandstedt et al. 1939; Harris and Sibbitt 1941). Starches isolated from corn, rice, and potato (Harris 1942; Sandstedt 1961) lack the baking quality of wheat starch. Pence et al. (1959) reported that protein, starch, and protein-starch interaction effects were all significant factors governing flour quality. D'Appolonia and Gilles (1971) isolated the starch from 12 varieties of hard red spring wheat. These preparations were reconstituted with common preparations of water solubles and dry gluten and the blends tested for baking quality. This study showed some differences in loaf volume depending on the varietal source of the starch. On the basis of this and similar previous studies, it has been concluded that the starch component of the wheat flour contributes to the overall baking quality of the flour. Experimental evidence has been obtained on the binding of native wheat proteins to gelatinized wheat starch (Dahle 1971). This

binding was impaired by heat denaturation of the protein and high pH conditions. Soy protein isolates added to wheat starch did not exhibit the unique binding properties of wheat proteins (Dahle et al. 1975).

Whilst the literature contains numerous publications on the role of carbohydrate and protein interaction and of glycoproteins in breadmaking quality, explicit details on the nature of the interactions and the constituents involved in the interactions is lacking. The present study was designed to obtain information on the possible carbohydrate-protein interactions in gluten preparations.

III. MATERIALS AND METHODS

A. Material

The wheat cultivar Napayo was chosen for the investigations in this research project. Napayo is representative of the hard red spring class of wheat produced in Canada. The sample was a part of the 1977 Bread Wheat Co-operative Test in which Napayo is one of the secondary standard cultivars.

Tempered wheat of this cultivar (15.5% moisture) was milled into flour on a Buhler pneumatic experimental mill to a flour extraction of 73.5%.

B. Defatting of Flour Samples

Flour (60 g) was extracted by dispersion and magnetic stirring in 1-butanol (300 ml, 20 min, 20° C). The mixture was then filtered on a Buchner funnel at reduced pressure. The process was repeated three times. This was followed by one extraction with acetone (5 volumes) and finally with petroleum ether (5 volumes). The defatted flour was air dried and stored at 4° C.

C. Fractionation Procedure for Wheat Gluten

The fractionation procedure used was basically that of Orth and Bushuk (1973a) developed for the preparation of glutenin. A gluten ball was washed from flour (20 g) using a steady stream of distilled water. The wet gluten was divided into small pieces and solubilized by magnetic stirring (16 hr, 4° C) in an aqueous solvent (160 ml) containing acetic

acid (0.1 N), urea (3 M), and a detergent, cetyltrimethyl ammonium bromide (0.1% w/v). The preparation was centrifuged (12,000 g, 4° C, 30 min). Ethanol was added to the supernatant to a concentration of 70% v/v and the pH was adjusted to 6.4 with dropwise addition of NaOH (1.0 M). The suspension was stored (16 hr, 2° C) and then centrifuged (12,000 g, 15 min, 4° C). The precipitate was dialyzed extensively against acetic acid (0.01 N), frozen, and freeze dried to provide an alcohol-insoluble fraction. This fraction will be referred to as glutenin.

The supernatant was reduced to 50% in volume by rotary evaporation under reduced pressure at 30° C and dialyzed against 0.01 N acetic acid. The retentate was frozen and freeze dried to provide an alcohol-soluble fraction.

D. Dialysis of Wheat Protein Fractions

Solutions were placed in dialysis tubing (exclusion limit - 8,000 daltons) which had been previously soaked and washed in distilled water. Dialysis was carried out in 4-litre glass beakers at 4° C. Magnetic stirring provided continuous mixing of the solutions. The ratio of sample volume to external dialysis solution was no greater than 1:40. The dialysis solution was changed at least 10 times at regular intervals to ensure complete removal of low molecular weight substances such as urea. Following the dialysis, the retentates were frozen and freeze dried.

E. Determination of Protein Content

A number of methods were used for the determination of protein content of flour, defatted flour, and various fractions obtained from the gluten preparations.

1. Total Nitrogen

Nitrogen content of the flour was determined using the Kjeldahl method (American Association of Cereal Chemists 1970). Protein content of flour was obtained by multiplying the percentage nitrogen content by the conversion factor 5.7 (Tkachuk 1969).

2. Amino Acid Analysis

When the sample size was limited, the protein content was determined by amino acid analysis. In this analysis, the amino acid data was normalized to a theoretical nitrogen recovery of 90%. In practice, nitrogen recoveries varied slightly in the range of 88 to 92%. The protein content was expressed as a percentage of the total weight of amino acids compared to the weight of sample before hydrolysis.

3. Absorbance at 280 nm

Protein content was also expressed as absorbance at 280 nm. Two amino acids, tryptophan and tyrosine, absorb at this wavelength. This procedure was routinely used to monitor protein content of fractions in all gel filtration experiments.

4. Determination of Protein Content with Ninhydrin Reagent

The content of α-amino groups was determined with ninhydrin reagent according to the method of Mertz et al. (1974). This assay was used to estimate the amount of amino acids and peptides in eluates from the gel filtration of the various protein hydrolysates.

F. Estimation of Carbohydrate Content

Carbohydrate content was estimated as "total carbohydrate" (pentoses and hexoses), using the phenol-sulfuric acid method (Dubois

et al. 1956). Glucose was used as the standard sugar. Because of the insoluble nature of the gluten fractions, the assays were performed in a variety of solvents, other than water, including acetic acid (0.1 M), acetic acid (0.1 M) + urea (3 M), acetic acid (0.1 M) + urea (8 M), Tris-HCl buffer, and ethanol (70%). In routine assays, 1.0 ml of phenol (5%) was added to 1.0 ml of solvent containing a sample in a Pyrex boiling tube. The mixture was vortexed thoroughly and 5.0 ml of concentrated sulfuric acid was added in a direct stream to the liquid surface. After cooling (15 min), the absorbance was measured at 490 nm on an Hitachi 40-100 spectrophotometer.

G. Gel Filtration Chromatography of Wheat Proteins

1. Sephadex G-200 and G-15

The Sephadex (G-200 and G-15) matrices were prepared by gentle dispersion in distilled water containing sodium azide (0.02%) for 3 days at room temperature. The swollen matrices were then equilibrated with a number of changes of the desired buffer (solvent) and de-aerated by aspirating at reduced pressure. The prepared Sephadex beads were decanted into a column as outlined in the booklet entitled "Gel Filtration in Theory and Practice" supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. The columns were packed with manufacturer's recommended head pressures and were operated, using the downward flow technique. In order to minimize the gradual compression of the matrix, the operating pressure was always less than that of the packing pressure.

Flow rates were maintained constant by using a marriotte flask as the solvent reservoir. Eluent was monitored continuously with a UV recorder and fractions collected at regular intervals of time with a

fraction collector. Protein and carbohydrate determinations were made on individual fractions and appropriate fractions were pooled, dialyzed against acetic acid (0.01 M), and freeze dried for further analyses or rechromatography.

2. Estimation of Molecular Weight by Gel Filtration

Molecular weights of fractionated proteins were determined using a calibration curve obtained by plotting elution volume versus molecular weight for proteins of known molecular weight (Andrews 1964, 1965). In some instances potassium chloride (20 mM) was included in the solvent to minimize adsorptive effects of proteins on the Sephadex matrices (Janson 1967). The elution volumes of the standard proteins were determined by chromatography of each protein separately. The proteins used in this study as calibration standards were as follows:

Protein	Molecular weight (MW) (daltons)	Source
γ-Globulin	160,000	Sigma Chemical Co.
Bovine serum albumin	68,000	Sigma Chemical Co.
Ovalbumin	45,000	Sigma Chemical Co.
α-Chymotrypsinogen	23,000	Sigma Chemical Co.
Lysozyme	14,300	Sigma Chemical Co.

The excluded (void) volume (V_o) of the Sephadex columns was determined as the elution volume of blue dextran. Bovine serum albumin was chromatographed following blue dextran to remove any residual dextran bound to the Sephadex matrix. The total (included) volume (V_t) was determined by chromatographing tryptophan and glucose.

3. Pre-Conditioning of Sephadex Columns

Sephadex columns were washed continuously with solvent to reduce "background" amounts of glucose released from the matrix. This pre-treatment reduced the carbohydrate levels in the column eluent to less than 1 μg ml^{-1}.

4. Biogel P-6

Biogel P-6 matrix was added gradually to the buffer solution and the resulting suspension allowed to hydrate (4 hr, 20° C). The beads were de-aerated by aspirating at reduced pressure. The column was packed according to manufacturer's recommendations and was operated using the downward flow technique. Fractions (2.0 ml) were collected with a fraction collector. The excluded volume (V_o) of the column was determined by the chromatography of bovine serum albumin. The total volume (V_t) of the column was estimated by the chromatography of tryptophan, glucose, and glutamic acid.

H. Reduction of Disulfide Bonds and Alkylation of Sulfhydryl Groups in Wheat Proteins

Protein disulfide bonds were reduced with β-mercaptoethanol and the exposed sulfhydryl groups were alkylated with 4-vinyl pyridine (Friedman et al. 1970). The protein (1.0 g) was dispersed in 100 ml of buffer solution (pH 7.5) containing Tris (16.11 g), HNO_3 (7.12 g), KCl (0.75 g), urea (484.8 g), and Na_2EDTA (1 mg) per litre. Nitrogen gas was bubbled through this mixture (15 min) to minimize re-oxidation of exposed sulfhydryl group by atmospheric oxygen. β-Mercaptoethanol (1.0 ml) was added and the mixture stirred with a magnetic stirrer (16 hr, 20° C). Alkylation of sulfhydryl groups was achieved by reaction with 4-vinyl pyridine (1.5 ml) with magnetic stirring (120 min, 20° C).

The pH of the solution was adjusted to 3.0 by dropwise addition of glacial acetic acid. The acidified mixture was dialyzed against acetic acid (0.01 M), frozen, and freeze dried. The final product of this alkylation is S- 4-pyridylethyl -L-cysteinyl protein derivative.

I. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Technique

SDS-PAGE was carried out at pH 8.4 according to the method of Koenig et al. (1970) as modified by Khan and Bushuk (1977). A 5% polyacrylamide gel was prepared by dissolving acrylamide (10.2 g) and bisacrylamide (0.27 g) in 200 ml of Tris-borate buffer (0.125 M, pH 8.4) containing SDS (0.1%). Sodium sulfite (50 mg) and dimethylaminopropionitrile (0.5 ml) were added and the solution was de-aerated by aspiration at reduced pressure. Ammonium persulfate (2%, 4.0 ml) was added and the solution was poured into the electrophoresis apparatus to form a slab approximately 6 mm thick.

2. Sample Preparation

Wheat proteins were subjected to SDS-PAGE in both the reduced and non-reduced form. Reduction was achieved by incubation of 10 mg protein in 1.0 ml of Tris-borate buffer (0.125 M, pH 8.4) containing SDS (1% w/v) and β-mercaptoethanol (1% v/v). When reduction was not required, the β-mercaptoethanol was omitted from the solvent. Bromphenol blue was added to the samples as a tracking dye. Sucrose was added to increase the density and thereby facilitate loading of the samples on the gel before electrophoresis (5 hr, 110 V, and 50 mA). Both electrode buffers contained Tris-borate buffer (0.125 M, pH 8.4) and SDS (0.1% w/w).

3. Staining and Destaining of Gels for Protein

After electrophoresis, the gels were rinsed in distilled water to remove excess buffer and were immersed in staining solution overnight. The staining solution consisted of Coomassie Brilliant Blue (R-form, 1.6 g) in ethanol (800 ml), water (800 ml), and glacial acidic acid (176 ml). The gels were destained using frequent changes of a solution comprising 400 ml aqueous acetic acid (10%) and 100 ml methanol.

4. Staining and Destaining Gels Containing SDS for Carbohydrate

The periodic acid-schiff (PAS) method was used to detect carbohydrate in SDS-polyacrylamide gels (Fairbanks et al. 1971). SDS was removed from the gels by washing consecutively with the following solutions:

- isopropanol (25%) + acetic acid (10%), 24 hr
- isopropanol (10%) + acetic acid (10%), 24 hr
- acetic acid (10%), 12 hr.

To facilitate removal of SDS from polyacrylamide gels, and to aid in the staining and destaining procedures, the thickness of the slab gels was reduced from 6 mm to 2.5 mm.

Consecutive treatments of the gels with the following reagents were used for the detection of carbohydrate:

- 0.5% periodic acid (2 hr)
- 0.5% sodium arsenite, 5% acetic acid (1 hr)
- 0.1% sodium arsenite, 5% acetic acid (20 min)
- 0.1% sodium arsenite, 5% acetic acid (20 min)
- Schiff reagent (overnight)
- 0.1% sodium metabisulfite, 0.01 N HCl (4 hr).

Bands containing carbohydrate stained a bright pink colour against a transparent background.

5. Estimation of Molecular Weight of Wheat Proteins by SDS-PAGE

The molecular weight of wheat proteins by SDS-PAGE was estimated by the procedure of Weber and Osborn (1969). The relative mobility of protein bands was calculated as follows:

$$\text{Relative Mobility} = \frac{\text{Protein migration (cm)}}{\text{Dye migration (cm)}} \times \frac{\text{Length of gel before staining (cm)}}{\text{Length of gel after destaining (cm)}}$$

The following proteins of known molecular weight were used to derive a calibration curve of the relationship between relative mobility and log molecular weight:

Protein	Subunit molecular weight (MW) (daltons)	Source
Lysozyme	14,300	Sigma Chemical Co.
Phosphorylase 'a'		Sigma Chemical Co.
- monomer	90,000	
- dimer	180,000	
Bovine serum albumin		Sigma Chemical Co.
- monomer	68,000	
- dimer	136,000	
X-linked bovine serum albumin		Sigma Chemical Co.
- monomer	68,000	
- dimer	136,000	
- trimer	204,000	
Ovalbumin	45,000	Sigma Chemical Co.
Trysinogen	21,600	Sigma Chemical Co.
Pepsin	35,000	Sigma Chemical Co.
Cytochrome C	13,000	Sigma Chemical Co.
β-Lactoglobulin	18,500	Sigma Chemical Co.

J. Electrophoresis of Wheat Protein Fractions Using Polyacrylamide Gels and Agarose-Polyacrylamide Gels

1. PAGE of Alcohol-Soluble Protein Fractions in Aluminum-Lactate Buffer (pH 3.1)

Electrophoresis of alcohol-soluble protein fractions was performed using a flat-bed apparatus as described by Bushuk and Zillman (1978). Polyacrylamide gels (6%) were prepared and aluminum lactate buffer pH 3.1 was used for electrophoresis. Protein bands were stained using Coomassie Blue (24 hr) and were destained with 12% trichloroacetic acid (48 hr).

2. PAGE of Alcohol-Soluble Protein Fractions Using Disc Gels

Polyacrylamide disc gels (4%) were prepared according to the method of MacGregor and Meredith (1971). β-Alanine was used as the buffer at pH 4.5. This method provides for the formation of a large pore stacking gel (2.5% acrylamide), polymerized using riboflavin in the presence of light. The smaller pore running gel (4% acrylamide) was polymerized using ammonium persulfate as the catalyst.

Protein was detected using the Coomassie Blue dye as described previously. Carbohydrate was detected using the periodic acid-schiff reagent, also previously described.

3. Electrophoresis of High Molecular Weight Alcohol-Soluble Gluten Fractions in Agarose-Acrylamide Disc Gels

The high molecular weight alcohol-soluble gluten fractions which did not enter 4.0% acrylamide gels were subjected to electrophoresis on a large pore gel system comprising 0.5% agarose and 2.0% acrylamide. The agarose used was Type II Agarose (Sigma Chemical Co.), which has a low content of charged sulfate groups and exhibits medium endo-osmosis

during electrophoresis. The method used was basically that used for the separation of nucleic acids by Perret et al. (1979). A number of modifications that were made are included in the following procedure.

Stock solutions:

A Acrylamide solution: 3.6 g acrylamide + 0.4 g bisacrylamide in β-alanine buffer (50 ml).

B TEMED solution: 0.5 ml TEMED + 24 ml KOH (1.0 N) pH adjusted to 4.3 with acetic acid. Final volume 100 ml.

C Catalyst: 10 mg riboflavin + 600 mg of ammonium persulfate in 10 ml water.

D Agarose: 1% agarose in β-alanine buffer (pH 4.3).

Procedure:

1. Equal volumes of A and B were combined and mixed thoroughly.
2. Solution de-aerated by aspiration at reduced pressure.
3. 100 µl of catalyst solution (C) was added for each 10 ml of A + B.
4. Equal volume of hot 1% agarose solution D added.
5. Disc gels were loaded and layered with water to form a horizontal interface.
6. Gels could be stored in β-alanine buffer at 4° C.

Protein bands were stained with Coomassie Blue and destained as previously described. Carbohydrate was detected using the periodic acid-schiff reagent (Fairbanks et al. 1971).

K. Determination of Amino Acid Composition of Protein

1. Preparation of Sample

The hydrolysates for amino acid composition analyses of flour,

gluten, and protein fractions were prepared as follows. The sample (5 mg) was weighed out directly into a hydrolysis tube and 4.0 ml of 6 N HCl (3 times distilled) containing 1 drop of caprilic acid (anti-foaming agent) was added to the sample. The mixture was flushed with nitrogen (5 min) and the tube was sealed. Hydrolysis was carried out at 110° C for 24 hr. The hydrolysate was cooled, frozen with liquid nitrogen, placed in a desiccator containing solid NaOH, evacuated, and left overnight to dry. The dried sample was taken up in sodium citrate buffer (8.0 ml, 0.2 M, pH 2.2) and centrifuged to remove insoluble material. The supernatant was used for the analysis.

2. Amino Acid Analysis

The Beckman Model 121 Automatic Amino Acid Analyzer equipped with an Infotronic Integrator was the instrument used for the composition analyses. The procedure was that of Spackman et al. (1958).

L. Qualitative Analysis of Monosaccharide Composition of Carbohydrate Components by Paper Chromatography

1. Hydrolysis of Carbohydrate Components for Monosaccharide Analysis

Samples (10 mg) were hydrolyzed with H_2SO_4 (2 N, 1.0 ml) in a boiling water bath (2 hr), cooled, and neutralized by addition of $BaCO_3$. The resulting suspension was centrifuged and the supernatant retained. The $BaSO_4$ pellet was washed twice with distilled water (0.5 ml) and the supernatants combined. Sugar content of the combined supernatants was estimated by the phenol-sulfuric acid method of Dubois et al. (1956).

2. Paper Chromatography

Descending paper chromatography of monosaccharides was performed according to the method of Partridge and Westall (1948). Neutralized

carbohydrate hydrolysates (2 μl) were spotted on Whatman No. 1 chromatography paper. Separate spots of standard sugar mixtures were co-chromatographed for identification of unknown sugars by their R_G values. The standard mixtures included galactose, mannose, arabinose, glucose, and xylose. Other standards included glucuronic acid, galacturonic acid, fucose, N-acetylglucosamine, maltose maltotriose, and glucosamine. The solvent system used as the moving phase for the separation of hexoses and pentoses contained a mixture of ethyl acetate, pyridine, and water in the ratio of 8:2:1 (v/v). The separation of glucose, maltose, maltotriose, and lower oligosaccharides was achieved using a solvent system of n-propanol, ethyl acetate, water in a ratio of 14:2:7 (v/v) as the moving phase.

After chromatography, the papers were removed from the chromatography tank, dried, and the sugars detected using $AgNO_3$ as described by Trevelyan et al. (1950). The chromatogram was rapidly passed through a reagent solution of $AgNO_3$ in acetone, dried, and dipped in a solution of ethanolic NaOH. Sugar spots were visible within a few minutes. Excess silver oxide was dissolved by washing the chromatogram in 5% sodium thiosulfate. The chromatogram was finally washed in running water for 1 hr and air dried for keeping as a permanent record.

M. Quantitative Analysis of Monosaccharides by Gas Liquid Chromatography (GLC)

1. Preparation of Sugar Derivatives for GLC

Gluten fractions (10 mg) were hydrolyzed as described previously, and the neutral sugars in the carbohydrate hydrolysates were converted to their respective alditol acetates using the method of Jones and Albersheim (1972). Sodium borohydride solution was freshly prepared by

dissolving sodium borohydride (80 mg) in ammonium hydroxide (0.1 N, 2.0 ml). To reduce the sugars, hydrolysates (approx. 2.0 ml) were treated with 0.5 ml of the prepared sodium borohydride for 12 hr. After reduction of the sugars, Dowex resin (H^+ form) was added until evolution of gas ceased to bind aldonic acids present that may be formed by reduction of any uronic acids in the original sample. The resin was removed by filtering on sintered glass and washed with aliquots of distilled water. The water used in the washing was combined with the filtrate and the sample was then frozen and freeze dried. Borate produced by the decomposition of the borohydride was removed by five separate 1.0 ml washings of the freeze dried sample with glacial acetic acid (10%) in methanol. The presence of borate retards acetylation of the alditols (Albersheim et al. 1967). Acetylation of the alditols (formed from the neutral sugars) was effected by the addition of acetic anhydride (1.0 ml) and pyridine, and heating in sealed tubes in a boiling water bath for 20 min. Excess pyridine was then removed from the acetylation mixture by five separate 5.0 ml washings with toluene. The resulting material representing the alditol acetate derivative of the neutral sugars, was dried in a vacuum oven (30° C, 12 hr), taken up in acetone (0.1 ml), and stored in a self-sealing 1.0 ml serum vial.

2. Gas-Liquid Chromatography of Alditol Acetate Derivatives

The alditol acetates (1 μl in acetone) were separated and identified by gas chromatography using a Hewlett Packard model 5700A gas chromatograph equipped with a hydrogen flame ionization detector. Sample injection was performed automatically using a Hewlett Packard model 7671A Automatic Sampler. Chromatography was monitored by a strip chart recorder and the areas under the peaks were calculated instantly with a

Hewlett Packard model 3373B Integrator, and printed on a printout strip.

Separation of the alditol acetate derivatives was achieved using a glass column (4 ft in length, 1/16 inch internal diameter) packed with a matrix of OV225 (3%) on Gas Chrom Q (100 to 200 mesh). The temperature program involved an isothermal oven temperature of 200° C. The injection port and detector temperature was 250° C. Nitrogen was used as the carrier gas and applied at a flow rate of 40 ml min^{-1} and at a pressure of 30 lb in^{-2}. The flow rate of the hydrogen gas was 30 ml min^{-1} at a pressure of 15 lb in^{-2}. The air flow rate was 210 ml min^{-1} at a pressure of 24 lb in^{-2}.

The sugar derivatives were identified from the retention times of equivalent derivatives of known sugars. An internal standard (the alditol acetate of 2-deoxyglucose) was included to check for any major losses during the derivatization procedure. Results will be presented as relative percentages of alditol acetate derivatives of the total detected.

N. Determination of α-amylase Activity

Alpha-amylase activity was determined using the chromogenic substrate Phadebas (Pharmacia Fine Chemicals) following the method of Barnes and Blakeney (1974). For the assay of α-amylase activity of commercial protease enzymes, the assay conditions chosen were similar to those described for the proteolytic digestion of gluten protein fractions. Four ml of thermolysin solution (0.5 mg ml^{-1}) in Tris-HCl buffer (100 mM, 5 mM $CaCl_2$, pH 8.3) was incubated with one tablet of Phadebas substrate for 2 hr at 50° C. Release of dye from the substrate was measured as absorbance at 620 nm and enzyme activity was expressed as m EU per mg protein.

Two ml of Proteinase K (0.34 mg ml^{-1}) in Tris-HCl buffer (100 mM, 5 mM, $CaCl_2$, pH 8.3) was incubated (40° C, 24 hr) with one Phadebas tablet. Enzyme activity was reported as above.

O. Measurement of Reducing Sugar Content of Carbohydrate-Protein Fractions

Reducing sugar contents of the carbohydrates present in gluten fractions were determined by the copper-reduction method of Nelson (1944), as modified by Roybt and Whelan (1968). For these determinations, fractions (5 to 10 mg) were solubilized in acetic acid (0.1 N) or aqueous ethanol (70%). Fractions that remained insoluble in these solvents were hydrolyzed by protease enzyme and the determination carried out on the released carbohydrate. Glucose was used as the standard sugar and results were expressed as percentage reducing sugar present in the carbohydrate by weight.

P. Determination of α 1,4; α 1,6 Glucan Hydrolytic Activity

Alpha 1,4; α 1,6 glucan hydrolytic activity was also determined in commercial preparations of protease enzymes. The conditions of the assay were chosen such that they were similar to those employed for the proteolytic digestion of gluten fractions. Soluble starch was used as the substrate and was prepared by pouring a cold slurry into boiling Tris-HCl buffer (100 mM, 5 mM $CaCl_2$). The solution was cooled and diluted with buffer to give a substrate concentration of 1.0% w/v. The pH was adjusted to 8.3 by addition of HCl (1.0 N). Protease solutions were made up in buffer as previously described. Enzyme solution (5.0 ml) was added to an equal volume of prepared substrate and the mixture incubated at 40° C (Proteinase K) and 50° C (Thermolysin). Aliquots (1.0 ml) were withdrawn at appropriate time intervals and assayed for reducing sugar

content. Appropriate enzyme solution and substrate blanks were also assayed. Enzyme activity was expressed as μg glucose produced per ml of enzyme solution per minute.

Q. Determination of Hydroxyproline

Hydroxyproline content of protein hydrolysates was determined colorimetrically by the method of Neuman and Logan (1950) as modified by Leach (1960). In order to assay low levels of hydroxyproline in column fractions from the chromatography of protein hydrolysates on Biogel P-6, three consecutive fractions were pooled and evaporated to dryness. The dried hydrolysate was taken up in 0.5 ml of distilled water and assayed for hydroxyproline. Results were expressed as percentage hydroxyproline of the protein by weight.

R. Proteolytic Hydrolysis of the Protein Component of Gluten Fractions

Proteinase K, an endoprotease, was used to hydrolyze the protein component of gluten fractions. The gluten fractions were incubated with a solution of Proteinase K (1.0 ml) made up in Tris-HCl buffer (100 mM, 5 mM $CaCl_2$, pH 8.3) for 16 hr at 40° C. An enzyme protein to substrate protein ratio of approximately 1 to 30 was used. The enzyme solution was carefully layered beneath the freeze dried gluten fraction in the test tube and the digestion allowed to proceed without initial shaking of the contents. This procedure prevented "clumping" of the gluten fraction and facilitated the proteolytic digestion. After 1 hr the mixture was shaken at regular intervals. A further 0.2 ml of enzyme solution was added after 16 hr and the incubation continued for 4 hr. The digest was then centrifuged (12,000 x g, 15 min, 20° C) and the solubilized material retained for further analysis. The pellet was washed with

buffer and recentrifuged, and the supernatant retained. The pellet was dispersed in a solvent containing acetic acid (0.1 N) and urea (3 M). Carbohydrate was determined on these preparations by the method of Dubois et al. (1956). The dispersed material in acetic acid and urea was again centrifuged and the carbohydrate content of the supernatant determined by the above method.

S. Linkage Analysis of the Carbohydrate Component of the Alcohol-Insoluble Gluten Fraction

The carbohydrate component of the alcohol-insoluble gluten fraction, after being released from the gluten fraction by protease digestion, was incubated with the enzyme amyloglucosidase. A breakdown of the carbohydrate component by this enzyme would, therefore, indicate that the component was an α-glucan. After incubation with amyloglucosidase, the mixture was analyzed by paper chromatography for the appearance of carbohydrate breakdown products.

The supernatant of the Proteinase K digestion was first treated with an Amberlite MB-IA mixed-bed ion exchange resin to remove buffer salts (Tris-HCl and $CaCl_2$). The resin was removed from the mixture by filtering on sintered glass and the filtrate analyzed for carbohydrate by the phenol-sulfuric acid method of Dubois et al. (1956). The volume of the supernatant was reduced to 0.2 ml and 15 μl of the concentrate was spotted on Whatman No. 3 paper for chromatographic analysis of sugars.

Amyloglucosidase enzyme was solubilized in sodium acetate buffer (25 mM, pH 4.5) at a concentration of 0.2 mg enzyme per ml. One ml of this solution was added to the desalted supernatant (approximately 0.2 ml, see above), and the mixture incubated for 1 hr at 40° C. Buffer salts were removed by addition of Amberlite MB-IA mixed bed ion exchange

resin and the resin removed by filtering on sintered glass. The carbohydrate content of the filtrate was again determined by the phenol-sulfuric acid method. The volume of the filtrate was reduced to 0.2 ml and 15 µl of the solution was spotted on the paper chromatogram.

One ml of the enzyme solution was also concentrated to a volume of 0.2 ml and 15 µl spotted on the paper chromatogram to check for extraneous sugars in the preparation. A standard solution of glucose, maltose, and maltotriose was also spotted.

The solvent for the chromatography procedure consisted of a mixture of n-propanol, ethyl acetate, and water in a ratio of 14:2:7, respectively. The chromatography was carried out for 26 hr using the downward flow technique. The remaining procedures for the identification of sugars and oligosaccharides were as previously described.

T. Alkaline Hydrolysis of Gluten Fractions

Gluten fractions were subjected to alkaline hydrolysis with solutions of barium and sodium hydroxides. This was to test for the presence of hydroxyproline-galactose linkages that may be present in the gluten fractions. Because these linkages, found in arabinogalactan peptides isolated from plants (Clarke et al. 1979) are resistant to hot alkaline digestion, carbohydrate contents of the gluten fractions were monitored before and after the treatment with alkali.

1. Hydrolysis with Barium Hydroxide

Gluten fractions (10 to 25 mg) were hydrolyzed with 1.0 ml of saturated aqueous solution of $Ba(OH)_2$ (approximately 0.22 M) in sealed tubes at 100° C for 8 hr (Lamport 1969). Hydrolysates were cooled and neutralized with H_2SO_4 (0.4 M) to a phenol red endpoint. The mixture was

centrifuged (10,000 x g, 10 min, 20° C) to remove precipitated barium sulfate and the supernatant evaporated to dryness by rotary evaporation. The dried hydrolysate was taken up in 1.0 ml of sodium chloride solution (0.1% w/v) containing sodium azide (0.02%) to prevent bacterial growth. The carbohydrate content of the resulting solution was determined by the phenol-sulfuric acid method and protein was estimated using ninhydrin reagent. Hydroxyproline was determined by the method of Leach (1960).

The prepared alkaline hydrolysate was then subjected to gel filtration chromatography on Biogel P-6.

2. Hydrolysis with Sodium Hydroxide

Gluten fractions (10 to 25 mg) were hydrolyzed with 1.0 ml of NaOH (2.5 M) in sealed tubes at 100° C for periods of 8 or 12 hr. Hydrolysates were cooled and neutralized with HCl (0.1 M) to a phenol-red endpoint. Sodium silicate which was formed by the action of alkali on borosilicate glass during the digestion, precipitate upon neutralization, and was removed by centrifugation (10,000 x g, 10 min). The sedimented silicate was washed with water to remove any residual digest material and the combined washings added to the prior supernatant. The digested material was then evaporated to dryness and the dried hydrolysate taken up in 1.0 ml of sodium chloride (0.1%) containing sodium azide (0.02%). The carbohydrate content of the resulting solution was determined using the phenol-sulfuric acid method. Protein was determined using ninhydrin reagent. Hydroxyproline was determined by the method of Leach (1960).

The prepared alkaline digest was subjected to gel filtration chromatography on Biogel P-6.

U. Partial Acid Hydrolysis of Alcohol-Insoluble Gluten Fractions

The alcohol-insoluble gluten fraction was partially modified by mild acid digestion with solutions of oxalic acid, hydrochloric acid, and sulfuric acid. After the acid treatments, the mixtures were centrifuged and the carbohydrate content of the supernatant monitored to ascertain release of the carbohydrate component from the gluten fraction complex.

1. Hydrolysis with Oxalic Acid

Samples of the alcohol-insoluble gluten fraction (20 mg) were heated with 1.0 ml of a weak solution of oxalic acid (0.0125 M) at 110° C for 3 hr. The hydrolysates were cooled and centrifuged (12,000 x g, 10 min, 20° C). The carbohydrate content of the supernatant was determined using the phenol-sulfuric acid procedure.

2. Hydrolysis with Hydrochloric Acid

Samples of alcohol-insoluble gluten fraction (20 mg) were heated with 1.0 ml of 0.1 N, 0.25 N, and 1.0 N hydrochloric acid at 110° C for 4 hr. The cooled hydrolysates were neutralized by addition of NaOH and centrifuged (12,000 x g, 10 min, 20° C). The carbohydrate content of the supernatant was measured using the phenol-sulfuric acid method. The sugar present in the hydrolysates were identified by paper chromatography. The digests were also subjected to gel filtration chromatography on Biogel P-6.

3. Hydrolysis with Sulfuric Acid

A sample of the alcohol-insoluble gluten fraction (50 mg) was incubated with 5.0 ml of 0.3 N sulfuric acid at 110° C. Aliquots (300 μl) were withdrawn at various times up to 2 hr, rapidly cooled, and

neutralized with 15 1 of 3 N NaOH. The resulting mixture was centrifuged (8,000 x g, 5 min, 20° C) to remove any insoluble material. The supernatants were assayed for total carbohydrate and reducing sugar.

V. Ultracentrifugation of a Purified Alcohol-Soluble Gluten Protein

Ultracentrifugation of protein samples was performed with a Beckman Model E Analytical Ultracentrifuge. The rotor used was a Beckman titanium rotor (model AN-H-181). Two samples were centrifuged simultaneously in a 12 mm double-sector cell with a synthetic boundary centrepiece containing 0.15 ml sample and 0.45 ml solvent. The centrifugation speed was 48,000 rpm. Photographs of the schlieren patterns were taken at 8 min intervals. The Schlieren bar angle was 70 in for all photographs. Sedimentation rates were calculated and reported in Svedbergs (S). The solvent used in this study was a solution of acetic acid (0.1 N) and urea (3 M). Sedimentation coefficients were determined under conditions of zero ionic strength (I = 0) and also low ionic strength by addition of potassium chloride to the solvent (20 mM, I = 0.02). To provide further information on the proteins under study, the sedimentation coefficients were also determined at a number of protein concentrations.

W. Reagents and Chemicals

Unless otherwise mentioned, biochemicals were purchased from Sigma Chemical Company.

Freshly prepared solutions of 4-vinylpyridine were purchased and stored at -18° C.

Chemicals, reagents, and solvents were mostly obtained from Fisher Scientific and were of analytical grade.

IV. RESULTS AND DISCUSSION

The first section of the Results and Discussion (parts A to G) will describe the fractionation of wheat gluten. The content of the protein and carbohydrate components in the respective fractions will be determined. The behavior of these components on gel filtration chromatography will be examined and the proteins characterized using electrophoretic techniques. The amino acid composition of the protein fractions will be determined and average hydrophobicities and charge potentials calculated from the data obtained. Possible secondary structures for the protein components will be discussed and the secondary structure of A_2-gliadin will be predicted using the method of Chou and Fasman (1978a, b) from available sequence data. The monosaccharide composition of the carbohydrate components will be determined. The characteristics exhibited by the protein and carbohydrate will be discussed in terms of possible functional roles in gluten structure.

The second section of the Results and Discussion (parts H to J) will describe the more detailed investigations into the nature of the association of the protein and carbohydrate components in wheat gluten fractions.

A. Cereal Chemistry Parameters of the Cultivar Napayo

The Canadian hard red spring cultivar Napayo was chosen for the detailed investigations described in this thesis. The sample of Napayo selected was obtained from the 1977 Central Bread Wheat Co-operative Test samples which were tested at the Department of Plant Science,

University of Manitoba. The chemical and rheological parameters of the sample were determined and compared with those of four other standard cultivars (Sinton, Manitou, Neepawa, and Marquis), which were grown in the same Test. The data obtained is given in Table 1. The hectoliter weight of Napayo (81.4 kg hl^{-1}) was satisfactory, equal to that of Manitou, and only slightly lower than that of the other cultivars. The grain protein percentage (14.5%) is higher than the value for Marquis (13.4%) and equal to that of the other cultivars. It should be noted that, although Napayo exhibited a higher flour yield, the flour ash value remained at a lower level than the values for three of the other cultivars. Napayo flour had the highest protein percentage (14.0%) of the four standard cultivars. An amylograph viscosity value of 770 BU indicated that the wheat sample was sound and did not contain excessive levels of α-amylase. The sedimentation value of 69 ml was similar to that of Sinton, Manitou, and Neepawa. The farinograph absorption of Napayo (64.2%) was satisfactory, being higher than that of Marquis and Manitou and slightly lower than the values for Sinton and Neepawa. The farinograph development time of Napayo (5.0 min) was similar to that of the other cultivars. Finally, the baking performance of Napayo was excellent. On both baking tests the sample showed the highest loaf volume of the cultivars examined.

Thus, the sample of Napayo chosen for this study exhibited typical technological properties of the cultivar and compared satisfactorily, if not favourably, with other high quality hard red spring wheats.

B. Fractionation Procedure

The choice of a suitable preparation and fractionation procedure for study of wheat gluten proteins is very important. The interpretation

TABLE 1. Comparison of some cereal chemistry parameters of the cultivar Napayo with standard Canadian hard red spring wheat cultivars.

Parameters	Cultivars				
	Napayo	Marquis	Manitou	Neepawa	Sinton
Kilogram weight ($kghl^{-1}$)	81.4	81.8	81.4	81.8	82.1
Grain protein[a] (%)	14.5	13.4	14.3	14.7	14.7
Flour yield (%)	73.5	71.8	72.6	72.5	73.0
Flour ash (%)	0.41	0.44	0.40	0.42	0.43
Flour protein (%)	14.0	12.9	13.6	13.7	13.6
Amylograph viscosity (BU)	770	640	790	680	800
Sedimentation value (ml)	69	60	69	68	69
Remix loaf volume (cc)	980	785	930	925	905
Remix blend volume (cc)	700	650	655	700	675
Farinograph absorption (%)	64.2	62.9	63.9	65.5	65.2
Development time (min)	5.0	4.5	5.0	5.0	5.0

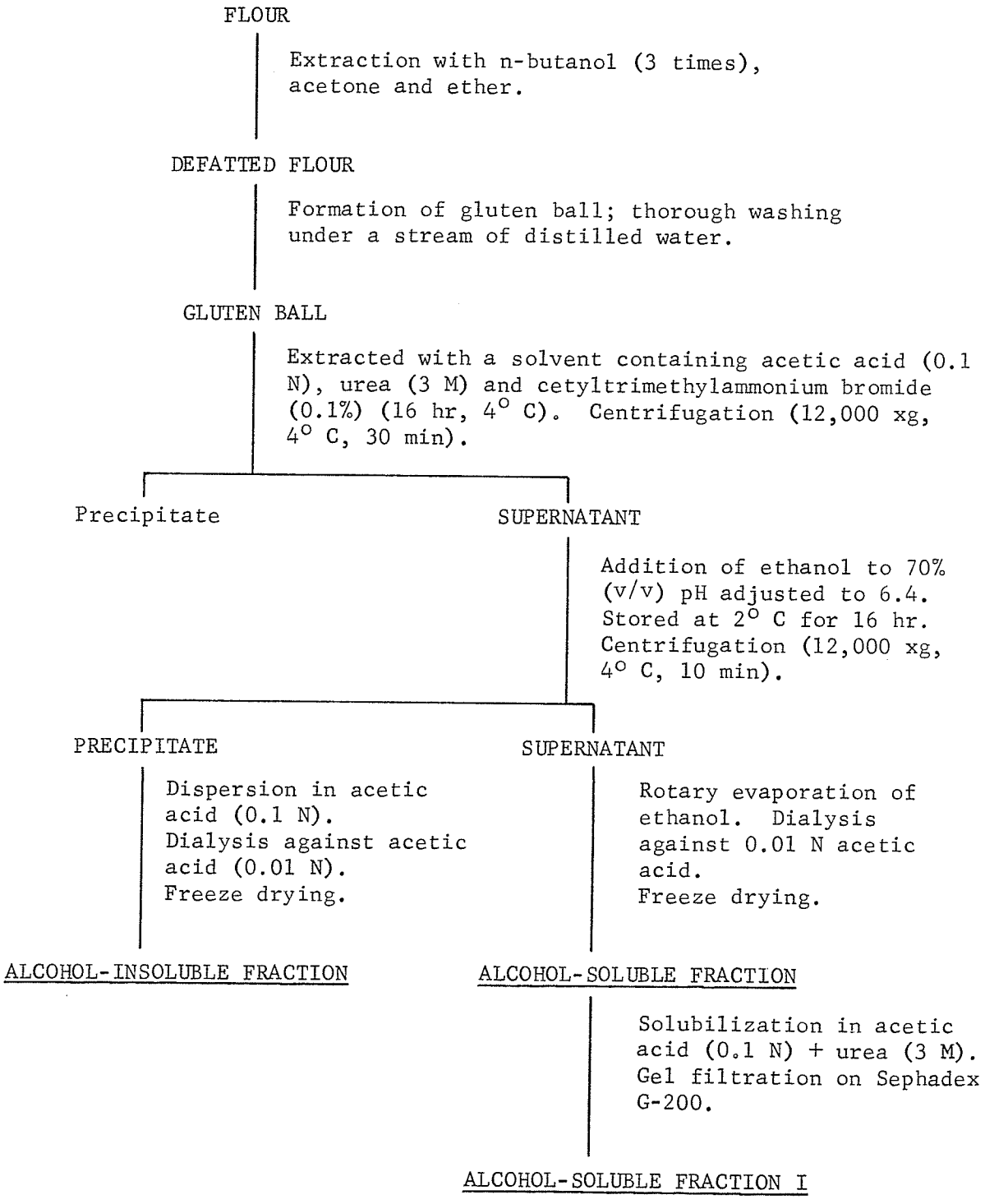
[a]Protein as N x 5.7.

of the results obtained is often limited by the possible effects that a particular fractionation procedure may have exerted on the final protein obtained. A good example of this is the effect of ethanol on proteins in baking studies. Flours that have been reconstituted with proteins that have been previously exposed to ethanol during fractionation do not bake satisfactorily (Booth and Melvin 1979).

In this study, investigations were centred on the possible associations of carbohydrate and protein in gluten. Thus, the preparation and fractionation procedure that was chosen had to satisfy at least two conditions, in order to allow reasonable interpretation of the data. The first condition was that the fractionation procedure would provide for easy removal of the flour polysaccharides that were not directly involved or associated with a developed gluten structure. The second condition was that the fractionation procedure be such that the data obtained on derived fractions could be interpreted in the light of the functional significance of any protein-carbohydrate association in the native gluten structure in a bread dough.

A summary of the fractionation procedure employed is given in Figure 1. Napayo flour was defatted by successive washings of n-butanol, followed by removal of butanol by washing with acetone,and then ether and air drying. A gluten ball was formed and washed thoroughly under a stream of distilled water. The formation of a gluten ball provided a relatively easy method to remove starchy and non-starchy polysaccharides as well as water-soluble proteins that were not directly involved in the developed gluten structure. Efficient removal of the polysaccharides that were not associated with the gluten proteins satisfied the first condition mentioned above. It is generally assumed by cereal chemists

Figure 1. Fractionation scheme employed for wheat gluten.


FLOUR
Extraction with n-butanol (3 times), acetone and ether.
DEFATTED FLOUR
Formation of gluten ball; thorough washing under a stream of distilled water.
GLUTEN BALL
Extracted with a solvent containing acetic acid (0.1 N), urea (3 M) and cetyltrimethylammonium bromide (0.1%) (16 hr, 4° C). Centrifugation (12,000 xg, 4° C, 30 min).
Precipitate
SUPERNATANT
Addition of ethanol to 70% (v/v) pH adjusted to 6.4. Stored at 2° C for 16 hr. Centrifugation (12,000 xg, 4° C, 10 min).
PRECIPITATE
SUPERNATANT
Dispersion in acetic acid (0.1 N). Dialysis against acetic acid (0.01 N). Freeze drying.
Rotary evaporation of ethanol. Dialysis against 0.01 N acetic acid. Freeze drying.
ALCOHOL-INSOLUBLE FRACTION
ALCOHOL-SOLUBLE FRACTION
Solubilization in acetic acid (0.1 N) + urea (3 M). Gel filtration on Sephadex G-200.
ALCOHOL-SOLUBLE FRACTION I

that when a gluten ball is formed, the interacting molecules providing the "vital gluten" required to make a bread dough are retained. This satisfies the second condition of the fractionation procedure. The gluten mass remaining after thorough washing in distilled water comprised 16% of the original defatted flour of Napayo on a dry weight basis.

The wet gluten was then finely divided and dissolved in a solvent containing acetic (0.1 N), urea (3 M), and a positively charged detergent, 0.01 M cetyltrimethyl ammonium bromide (AUC). Meredith and Wren (1966) used this solvent to extract 95% of flour proteins. This solvent was subsequently used by Orth and Bushuk (1973a) in the preparation of "purified" glutenin from wet gluten. The AUC dissolved gluten material was then centrifuged (22,000xg, 4° C, 30 min), the small amount of precipitate discarded, and the supernatant retained. The solubilized gluten material was fractionated by addition of ethanol to 70% v/v. The pH of the solution was adjusted to 6.4 by dropwise addition of NaOH (2 M). The mixture was stored at low temperature 2° overnight, allowing a precipitate to form. The alcohol-insoluble pellet was dispersed in acetic acid 0.1 N, dialyzed extensively against distilled water to remove solvent, frozen, and freeze dried to yield the alcohol-insoluble fraction. Alcohol in the supernatant was removed by rotary evaporation under reduced pressure. The remaining solution was then dialyzed extensively against acetic acid (0.01 N) to remove solvent, frozen, and freeze dried to yield the alcohol-soluble fraction. The alcohol-insoluble fraction contained 53% of the dry weight and the alcohol-soluble fraction 47% of the dry weight of the AUC supernatant. This procedure is similar to the method described by Orth and Bushuk (1973a) for the purification of glutenin. However, the pH precipitation step in ethanol was carried out

once only. Also, the final step in the Orth and Bushuk procedure involving ion-exchange chromatography was omitted. This step was introduced by the authors to remove low molecular weight "contaminating" proteins from the high molecular weight glutenin proteins. Subsequently, it was pointed out that these lower molecular weight components form an integral part of the glutenin (hence gluten) complex (Khan and Bushuk 1979a). For the purpose of studying possible carbohydrate protein interactions in wheat gluten proteins, extensive "purification" of the protein component at this stage was unnecessary.

The alcohol-soluble fraction was soluble in a solvent of acetic acid (0.1 N) and urea (3 M), and therefore could be further fractionated by gel filtration chromatography on Sephadex G-200 (see gel filtration profile given in Figure 4). This step provided an excluded fraction of high molecular weight, containing both carbohydrate and protein and was termed alcohol-soluble fraction I.

In summary, the three main fractions derived from the AUC-soluble fraction of gluten and used in this study are: a) the alcohol-soluble fraction; b) the alcohol-soluble fraction I - obtained from the gel filtration of the alcohol-soluble fraction, and c) the alcohol-insoluble fraction.

C. Composition of Flour and Gluten Fractions

1. Determination of Protein and Carbohydrate

The protein content of the various samples was determined from the amino acid composition data. This method of protein determination precludes the possibility of any over-estimation of protein content if small amounts of urea remain in the protein samples after dialysis.

The carbohydrate content of the samples was estimated as "total carbohydrate" using the phenol-sulfuric acid method of Dubois et al. (1956). A calibration curve of absorbance at 490 nm versus micrograms of glucose, the standard sugar used, is shown in Figure 2. Due to the insolubility of some gluten fractions in aqueous solvents, the carbohydrate content of these samples was measured with the sample dissolved in 70% ethanol or dissociating solvents such as urea solutions. The standard curves obtained in Figure 2 indicate that the presence of urea (3 M) or aqueous ethanol (70% v/v) in the sample mixture did not affect or interfere with the assay for carbohydrate. This also allowed the direct assay for carbohydrate in fractions obtained from column chromatography where the eluant contained acetic acid and urea.

Lipid contents were not directly determined but were equated to the material remaining after subtraction of the protein and carbohydrate contents from the total weight of material.

2. Composition of Gluten Fractions

The protein contents of the defatted flour, gluten ball, and gluten fractions are given in Table 2. The carbohydrate contents of the gluten fractions are also given in Table 2. The defatted flour contained 16.3% protein. The protein content of the gluten ball was 89.7%. Protein was found to be the major component in each of the three gluten fractions. The alcohol-soluble fraction contained 90.0% protein and 0.6% carbohydrate. The lipid content of this fraction (estimated by difference) was 9.4%. The alcohol-soluble fraction I contained 80.9% protein, 2.9% carbohydrate, and 16.2% lipid. It appears that most of the lipids in the gluten preparation are concentrated in this fraction. The alcohol-insoluble fraction comprised 83.0% protein and 17.0% carbohydrate.

Figure 2. Calibration curve showing absorbance at 490 nm versus micrograms of glucose for the determination of carbohydrate by the phenol-sulfuric acid method of Dubois *et al.* (1956).

Solvents:

70% aqueous ethanol : o———o
Acetic acid (0.1 N) : ●———●
Acetic acid (0.1 N) + urea (3 M): △———△

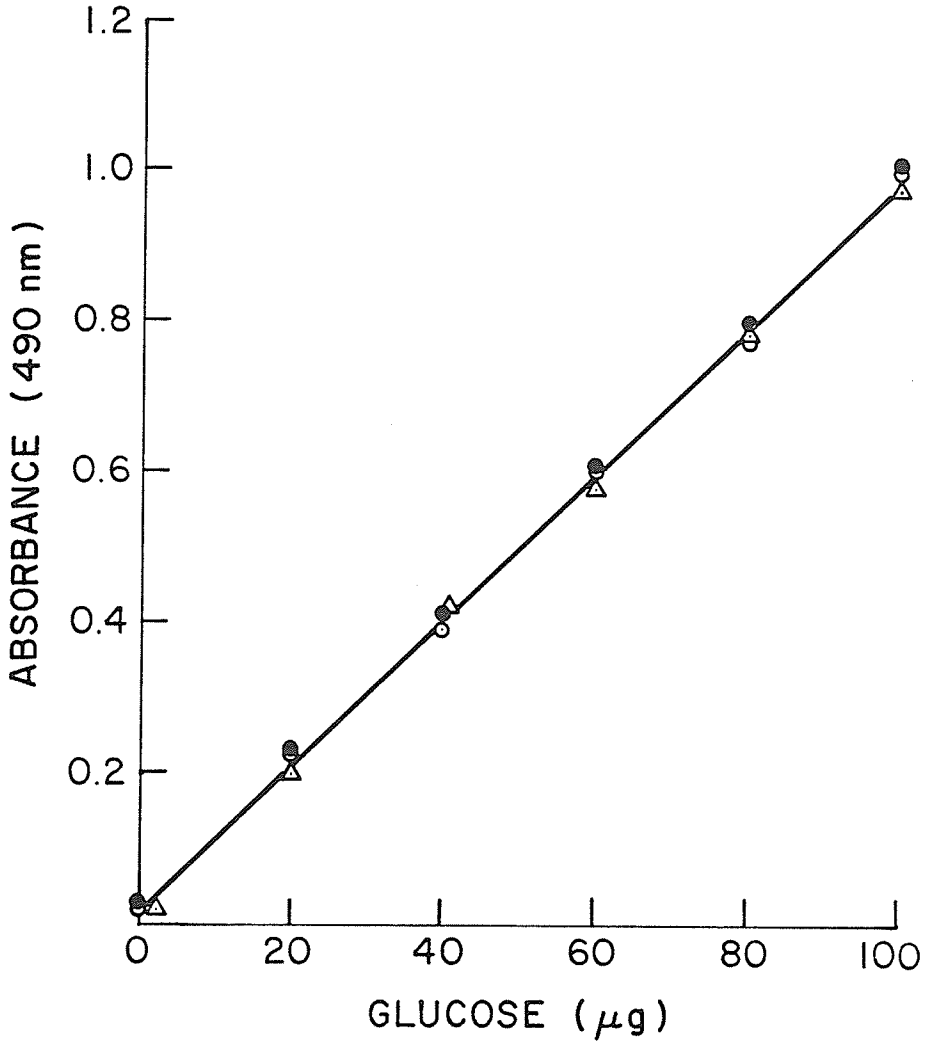

1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE (490 nm)
0
20
40
60
80
100
GLUCOSE (μg)

TABLE 2. Composition of defatted flour and gluten fractions.

Fraction	Composition[a] (%)		
	Protein[b]	Carbohydrate[c]	Lipid (by difference)
Defatted flour	16.3	nd[d]	-
Gluten ball	89.7	nd	-
Alcohol-soluble fraction	90.0	0.6	9.4
Alcohol-soluble fraction I	80.9	2.9	16.2
Alcohol-insoluble fraction	83.0	17.0	0.0

[a]Dry weight basis.

[b]From amino acid composition data.

[c]Determined by phenol-sulfuric acid method of Dubois et al. (1956).

[d]nd = not determined.

Although lipids have been reported to be associated with wheat gluten proteins (Pomeranz 1971), the present study deals only with the carbohydrates associated with the major gluten proteins.

D. Gel Filtration Chromatography and Electrophoretic Studies of Gluten Fractions

The preparations obtained from the fractionation of a gluten ball, identified in Figure 1, were subjected to further studies using chromatographic and electrophoretic techniques. These studies were undertaken to provide background information on the behaviour of both the protein and the carbohydrate components in particular fractions. The gel filtration chromatography was performed on Sephadex G-200 because of its high molecular weight exclusion limit. Another advantage in using this matrix is the wide fractionation range that it provides viz 200,000 to 12,000 daltons. The Sephadex matrices have generally become established as suitable for the chromatography of gluten proteins. Agarose gels (Sepharose 4B, 2B, 6B), because of their very high exclusion limits, were also considered, however, preliminary results indicated substantial binding of gluten proteins to the matrix. Total elution of the protein can be achieved only by the use of dissociating agents such as sodium dodecyl sulfate in the solvent system. The binding of gluten protein to the Sepharose matrix was presumed to be due to the presence of sulfate groups on the galactose residues.

1. Calibration of the Sephadex G-200 Column

Proteins of known molecular weight were individually chromatographed on the Sephadex G-200 column using the downward flow technique. The conditions used were the same for these proteins as for the gluten proteins. The partition coefficient (K_{AV}) values were determined from the

formula:

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume of protein.

V_o = void volume of column.

V_t = total volume of column

The void volume of the column (V_o) was determined by chromatographing blue dextran. Under conditions of low ionic strength in the solvent such as acetic acid (0.1 N), urea (3 M), some blue dextran was found to bind to the Sephadex matrix. Although this binding was not visible to the naked eye initially, the dextran could be removed and eluted by subsequent chromatography of a protein sample.

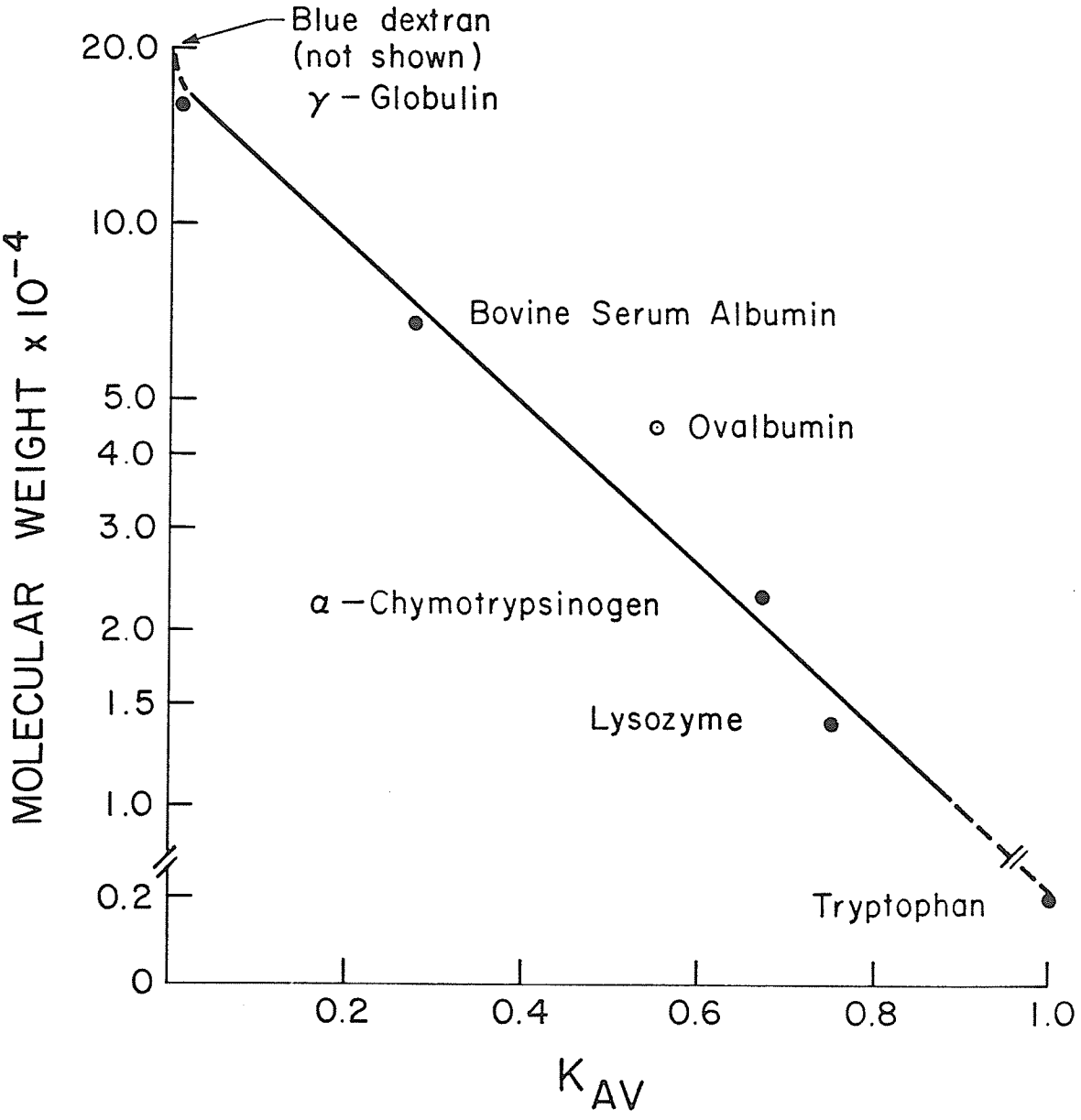
The total volume of the column was taken as equal to the elution volume of tryptophan. It was noted, however, that tryptophan may be slightly retarded on Sephadex matrix as glucose was found to elute 75 ml earlier than tryptophan. The retardation of the tryptophan may be due to an hydrophobic binding which has been observed for some other compounds on Sephadex matrix (Janson 1967).

Reference proteins used to obtain the data of Figure 3 gave a reasonably good fit to a straight line over the entire fractionation range, indicating that the column was functioning properly. The data point for ovalbumin was slightly above the line of best fit; apparently this chromatographic behaviour is characteristic for this glycoprotein (Andrews 1964, 1965). The elution profiles for the reference proteins were slightly skewed when the eluant was of low ionic strength. The positions of peak absorbance were taken as the elution volumes of the reference proteins. Addition of KCl (20 mM) to the solvent provided sufficient ionic strength (I = 0.02) to render the elution profile of

Figure 3. Plot of log (molecular weight) versus partition coefficient values (K_{AV}) on Sephadex G-200 for various proteins of known molecular weight, blue dextran and tryptophan.

Conditions:

Column size : 5.0 x 100 cm
Bed height : 80 cm
Solvent : acetic acid (0.1 N) + urea (3 M)
Flow rate : 28 ml hr^{-1}
Fraction size: 10.0 ml


Blue dextran
(not shown)
γ – Globulin
Bovine Serum Albumin
Ovalbumin
α – Chymotrypsinogen
Lysozyme
Tryptophan
MOLECULAR WEIGHT x 10⁻⁴
20.0
10.0
5.0
4.0
3.0
2.0
1.5
1.0
0.2
0
0.2
0.4
0.6
0.8
1.0
K_AV

the reference proteins symmetrical. These interesting experimental aspects are discussed in greater detail in Appendix 1.

2. Gel Filtration Chromatography of the Alcohol-Soluble Fraction

Freeze dried alcohol-soluble gluten protein (640 mg) was dissolved in a solvent (32.0 ml) containing acetic acid (0.1 N) and urea (3 M), and subjected to gel filtration chromatography on Sephadex G-200. The elution profile obtained is shown in Figure 4. The protein content of the eluted fractions was estimated by the absorbance at 280 nm and carbohydrate content by the phenol-sulfuric acid method.

Protein eluted in five individual peaks. These peaks were labelled I to V according to the order of elution in Figure 4. The first protein peak (peak I) eluted in the void volume of the column, indicating that it was excluded from the Sephadex matrix and, therefore, has a molecular weight greater than 200,000 daltons. This peak, when fractionated on Sephadex G-200 from the alcohol-soluble proteins, exhibited visible opalescence. The first included peak (peak II) was relatively small and eluted at 650 ml to 770 ml.

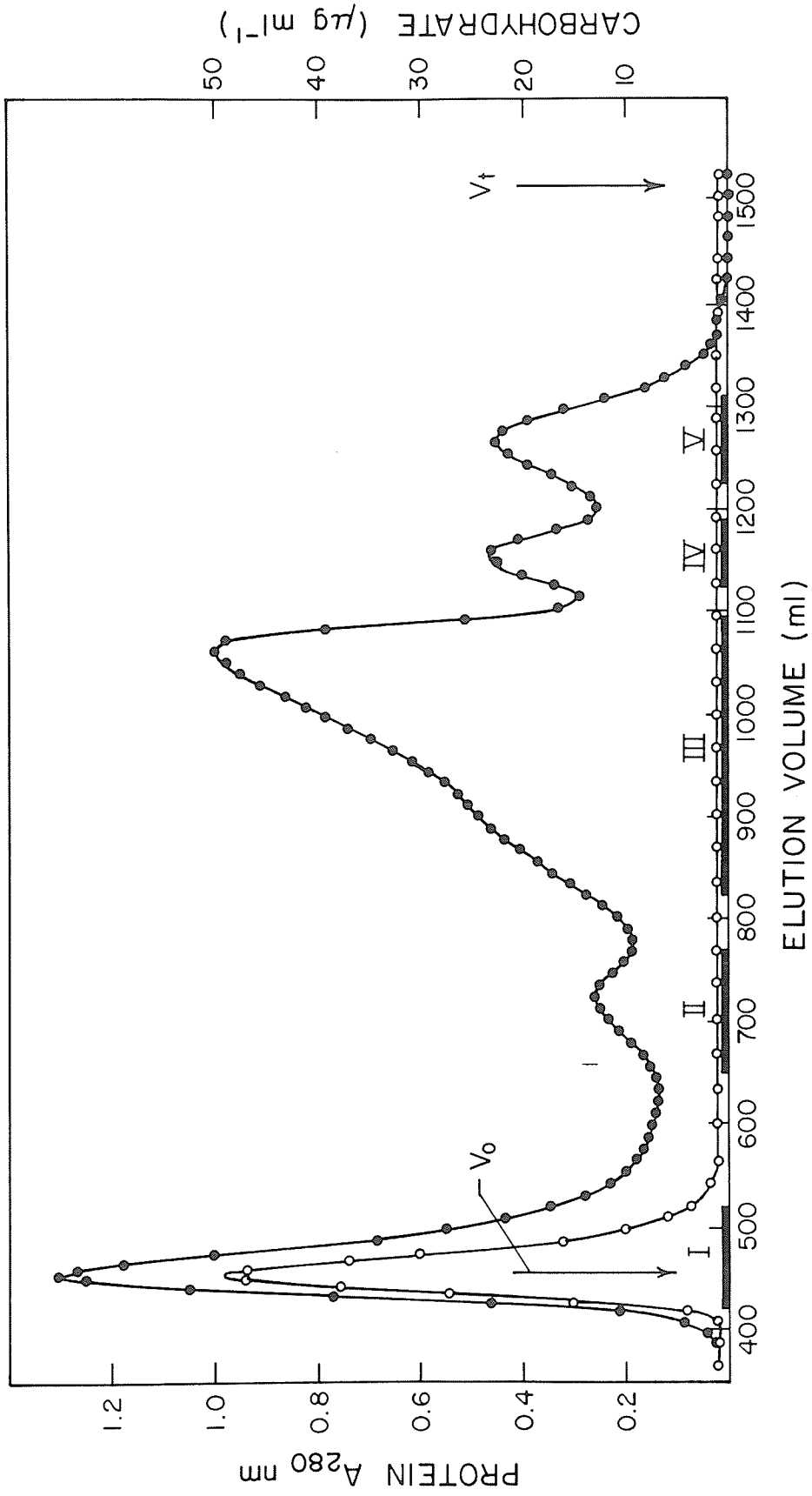
Peak III eluted as a broad assymetrical peak (825 to 1,100 ml), indicating that it was highly heterogenous in nature (by gel filtration chromatography). In addition, there appeared to be a "shoulder" in the leading edge of the peak which indicates the existence of an unresolved peak. Upon rechromatography, the shape of the elution profile of peak III was unchanged, however, the elution volume of the peak was significantly lower (950 ml compared with 1,060 ml). Another factor that can be invoked to explain the assymetry of peak III is the possibility of protein interaction with unprotonated carboxyl groups on the Sephadex matrix which would prevail under conditions of extremely low ionic strength.

Figure 4. Elution profile from gel filtration chromatography of alcohol-soluble gluten fraction on Sephadex G-200.

Conditions:

Column size	: 5.0 x 100 cm
Bed height	: 80 cm
Solvent	: acetic acid (0.1 N) + urea (3 M)
Flow rate	: 28.0 ml hr^{-1}
Fraction size	: 9.0 ml
Sample volume applied	: 32.0 ml
Sample concentration	: 20 mg ml^{-1}

Carbohydrate: o———o
Protein : ●———●


PROTEIN A_{280} nm
CARBOHYDRATE ($\mu g\ ml^{-1}$)
ELUTION VOLUME (ml)
V_o
V_t
I
II
III
IV
V

Rechromatography of peak III in a solvent containing 20 mM KCl (I = 0.02) gave a symmetrical peak at a lower elution volume (850 ml) (see Figure 27 in Appendix I). This result indicates that the assymetrical peak is due to ionic protein-protein interactions or interactions between protein and charged carboxyl groups on the Sephadex matrix.

Peak III proteins were also subjected to ultracentrifugation in solvents containing acetic acid and urea, and acetic acid, urea, and KCl to check for protein-protein interaction at low ionic strength. Without KCl in the solvent, the proteins again showed a skewed Schlieren pattern. Addition of KCl (20 mM) to the solvent resulted in a symmetrical profile and a doubling of the sedimentation coefficient. This is taken as a strong indication of protein-protein interactions being present in peak III proteins under conditions of low ionic strength. (The effect of 20 mM KCl on the ultracentrifugation and gel filtration profiles is described in detail in Appendix 1.) Thus, addition of a small amount of salt to the eluant resulted in peak III proteins eluting as a symmetrical protein peak. Subsequent studies using SDS-electrophoresis showed these proteins to be similar in molecular weight as they migrated as a single band (Figure 7).

The results of the examination of peak III proteins by gel filtration chromatography, under conditions of low ionic strength, suggest that estimations of molecular weight by this technique must be interpreted with considerable caution. The earlier elution of peak III on rechromatography suggests also that the possible presence of other peaks in the initial profile is an experimental artifact.

Peak IV (1,125 to 1,190 ml) and peak V (1,225 to 1,310 ml) were the last two protein peaks to be eluted. Although these peaks are clearly

distinct, there is a degree of overlapping. Both peaks are of low molecular weight and, therefore, a Sephadex matrix of a lower fractionation range such as G-50 or G-25 would provide a more satisfactory separation of these peaks.

The column fractions corresponding to the five protein peaks obtained by gel filtration were pooled as indicated by solid horizontal bars on the elution profile in Figure 4. The pooled samples were exhaustively dialyzed against frequent changes of distilled water to remove the acetic acid and urea, frozen, and freeze dried. Similarly, the column fractions between the peaks were pooled, dialyzed, and then frozen and freeze dried. This procedure gave estimates of the weight of protein in the peak fractions and total protein recovery from the column.

The relative proportion of peaks (by weight), peak elution volumes, K_{AV} values, and estimated molecular weights are given in Table 3. Peak I contained 21.7% of the eluted material. The elution volume of this peak (430 ml) equals the void volume of the column, thus giving a K_{AV} value of zero. Accordingly, the molecular weight of peak I is greater than 200,000 daltons, the exclusion limit of the Sephadex matrix. Peak II, which comprised 8.6% of the eluted protein, had an elution volume of 720 ml corresponding to a K_{AV} value of 0.27 and a molecular weight of 75,000 daltons. Peak III was the major protein peak, containing over half of the recovered material (56.4%). Its elution volume was 1,060 ml, the K_{AV} was 0.58, and the molecular weight 27,500 daltons. Peaks IV and V were relatively minor peaks, together accounting for only 13.3% of the recovered material. Their molecular weights were estimated to be 21,000 and 15,000 daltons, respectively. The weight recoveries of these proteins should be regarded as estimates since they tend to dialyze

TABLE 3. Peak weights, elution volumes, K_{AV} values and molecular weights of peaks obtained from gel filtration chromatography of alcohol-soluble proteins on Sephadex G-200.

	Peak weight[a] (%)	V_E (ml)	K_{AV}	MW[b] (daltons)
Peak I	21.7	430	0	>200,000
Peak II	8.6	720	0.27	75,000
Peak III	56.4	1060	0.58	27,500
Peak IV	5.0	1160	0.67	21,000
Peak V	8.3	1255	0.76	15,500
Between peaks[c]	7.1			

[a] Peak weight as a percentage of total weight of peaks recovered.

[b] Molecular weights estimated from Figure 3.

[c] Percentage of total weight recovered; total recovery = 88%.

through the dialysis tubing with extended time. Thus, it was impossible to achieve a condition of total removal of nitrogen containing substances in the solvent such as urea and minimum loss of protein during dialysis. The overall recovery by weight of material on the column was estimated at 88 to 90%.

The carbohydrate present in the alcohol-soluble fraction chromatographed as a single homogeneous peak, corresponding in elution volume to the high molecular weight protein in peak I. No carbohydrate was detected in any of the other protein peaks. The overall recovery of carbohydrate on the column (by weight) was estimated to be 82 to 85%. This indicates that the possibility of carbohydrate eluting from the Sephadex matrix itself into the protein fractions during gel filtration was not significant. Prior to use, the Sephadex column was washed thoroughly for a number of weeks until the carbohydrate content of the eluant was less than 2 μg ml^{-1}.

The alcohol-soluble peak I contained both carbohydrate and protein co-eluting at low elution volume (i.e. high molecular weight). This peak, for the purposes of further study, was termed the "alcohol-soluble fraction I", as indicated in the summary fractionation procedure given in Figure 1.

3. Polyacrylamide Gel Electrophoresis (PAGE) of Alcohol-Soluble Proteins

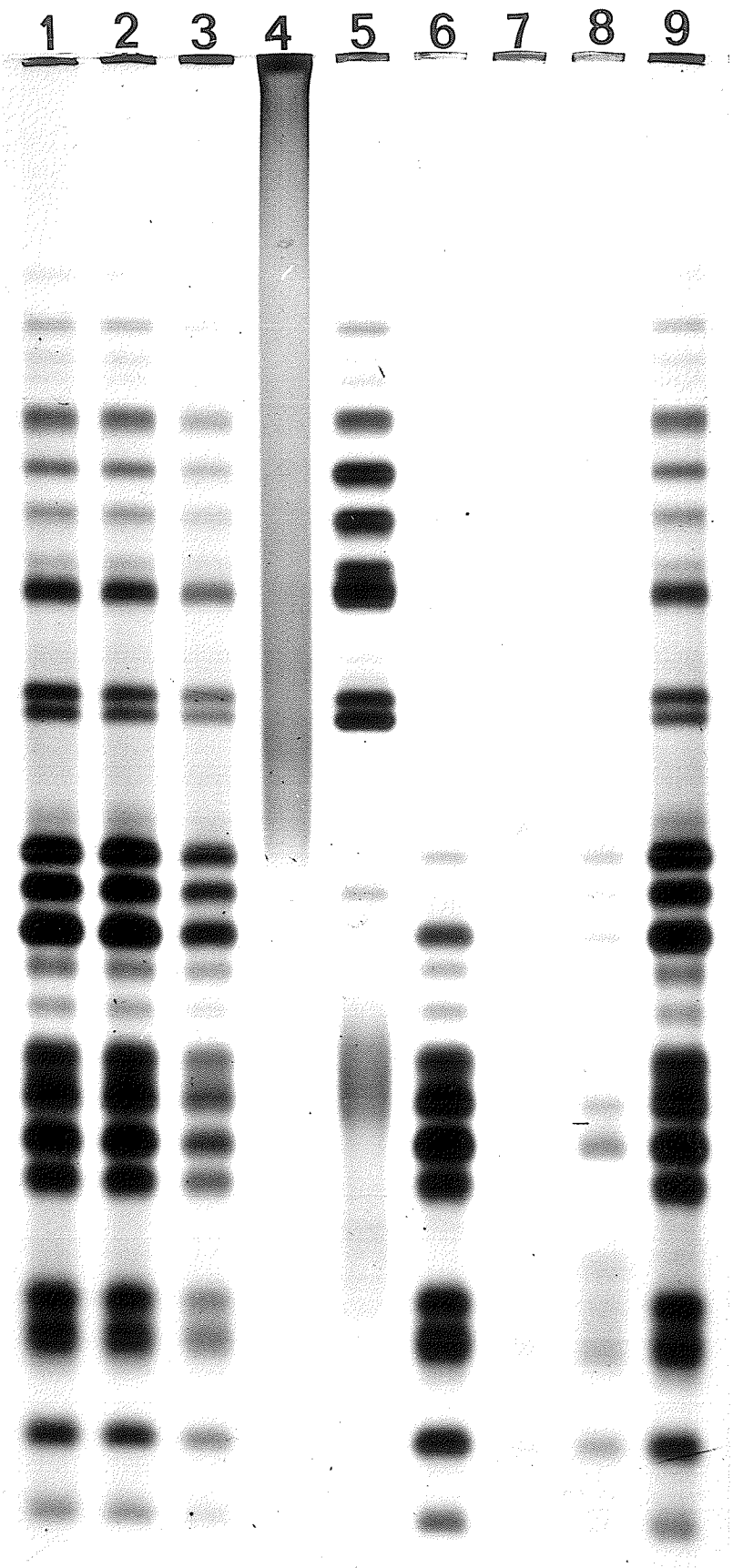
Freeze dried preparations of the alcohol-soluble gluten proteins and the peaks obtained by gel filtration chromatography on Sephadex G-200 were subjected to PAGE using aluminum lactate buffer at pH 3.1. The electrophoretic procedure followed was that of Bushuk and Zillman (1978) developed for the electrophoresis of gliadin protein. The electrophoregrams are shown in Figure 5.

Figure 5. Polyacrylamide gel electrophoresis of alcohol-soluble proteins and proteins in peaks obtained from gel filtration of the alcohol-soluble fraction on Sephadex G-200.

Pattern	Sample
1	70% ethanol extract of Napayo flour.
2	70% ethanol extract of defatted Napayo flour.
3	Freeze dried alcohol-soluble fractions obtained from dissolution of a wet gluten preparation.
4	Peak I from gel filtration of alcohol-soluble fraction.
5	Peak II from gel filtration of alcohol-soluble fraction.
6	Peak III from gel filtration of alcohol-soluble fraction.
7	Peak IV from gel filtration of alcohol-soluble fraction.
8	Peak V from gel filtration of alcohol-soluble fraction.
9	70% ethanol extract of Napayo flour.

Conditions:

Polyacrylamide gel: 6%
Buffer : aluminum lactate pH 3.1
Protein stain : Coomassie blue


1
2
3
4
5
6
7
8
9

To allow a comparison of the gliadin bands obtained from fractions prepared from the gluten ball, a standard 70% ethanol extract of undefatted Napayo flour was subjected to PAGE on the same gel. The electrophoregram of the flour extract is shown as pattern 1 in Figure 5. The electrophoregram of an analogous extract of defatted flour is shown as pattern 2 in this figure. The similarity of these two electrophoregrams (both in number of bands and band intensity) demonstrates that the defatting step did not alter the gliadin electrophoregram.

The electrophoregram of the freeze dried sample of the alcohol-soluble fraction obtained from the dissolution of the gluten ball is shown as pattern 3. All of the gliadin bands present in the extract of Napayo flour (patterns 1 and 2) are also present in the electrophoregram of the alcohol-soluble fraction of the gluten ball. Patterns 3, 4, 5, 6, 7, and 8 may be compared for relative band intensity as the same amount of protein was loaded from extraction of freeze dried preparations. These patterns, however, cannot be compared to patterns 1, 2, and 9 as far as band intensity is concerned, as patterns 1, 2, and 9 were the result of direct 70% ethanol extraction of flour. It is clear, however, that all of the gliadin components present in the flour form an integral part of the washed gluten. Because these components are present in the alcohol-soluble fraction derived from the gluten ball, it may be concluded that these components can be dissociated by extraction of the wetted gluten matrix with dissociating agents such as urea and cetyltrimethylammonium-bromide.

The electrophoregram of the protein in the alcohol-soluble fraction I (peak I) is shown as pattern 4. The proteins in this fraction remained at the slot and no distinct bands were observed in the trace amount of

protein that entered the gel. The gel, in this pattern, showed some deformation in the slot area (see pattern 4), indicating that the protein exerted a pressure on the gel matrix when the current was applied during electrophoresis. The general "smearing" or "streaking" observed in the pattern of peak I is typical of high molecular weight gluten proteins. The electrophoregram of peak II proteins (pattern 5) consists primarily of gliadin bands of lower mobility. There was only trace amounts of protein remaining at the slot position. The high mobility bands were absent in this electrophoregram.

Peak III proteins (pattern 6) comprise gliadins of generally higher mobility. The electrophoregrams of peaks II and III (patterns 5 and 6, respectively) are essentially equivalent to the electrophoregram of the total gliadin extract.

The electrophoregrams of the proteins in peaks IV and V are shown as patterns 7 and 8, respectively. These two fractions contain trace amounts (bands of low intensity) of the high mobility gliadin bands. There was essentially no protein at the point of application in these electrophoregrams. The same amount of protein was loaded onto the gels in these patterns as patterns 3, 4, 5, and 6. It was, therefore, concluded that peaks IV and V contained lower molecular weight albumins and globulins which, because of their charge, possessed greater mobility in the gel and had migrated off the end of the gel during electrophoresis.

4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Gluten Protein Fractions

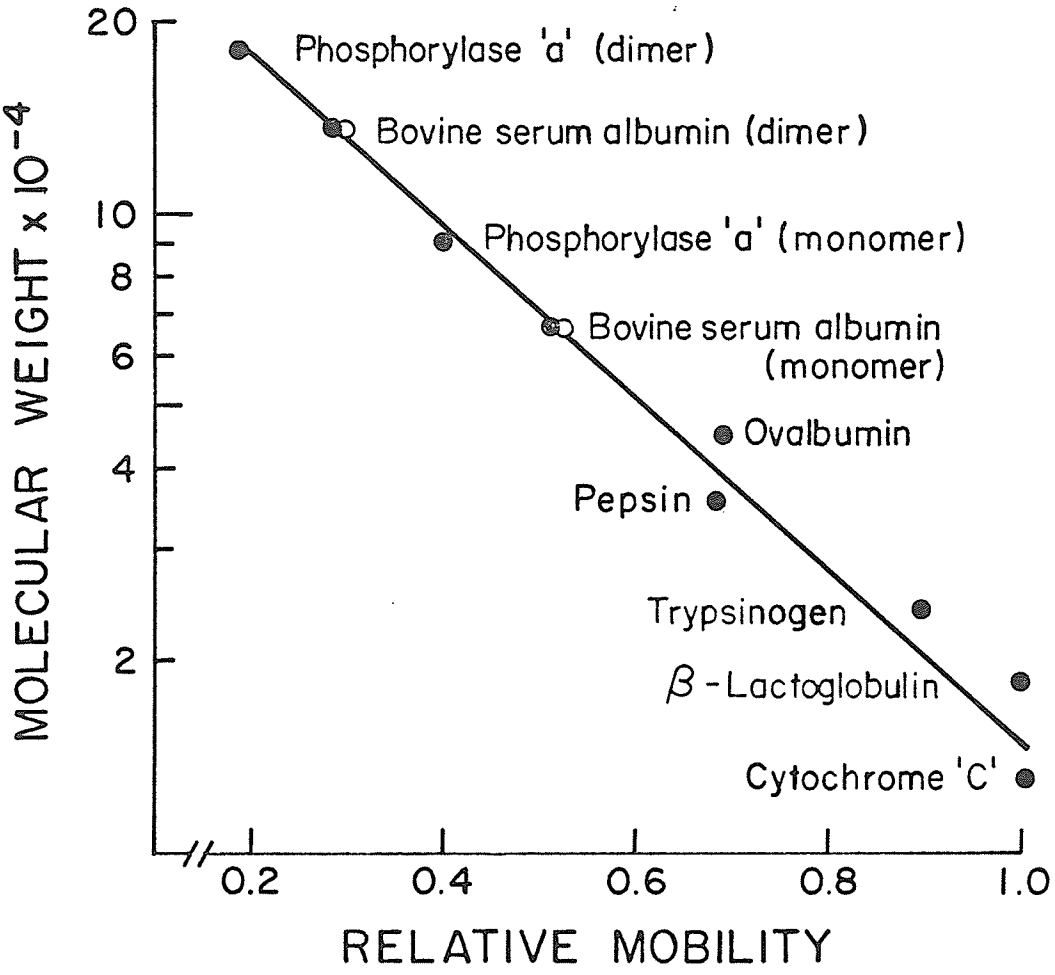
a. Determination of molecular weight. The molecular weights of gluten proteins were determined by SDS-PAGE according to the method of Weber and Osborn (1969). A calibration curve relating the log of the

Figure 6. Plot of the log of molecular weight of proteins of known molecular weight versus relative mobility on SDS-PAGE (molecular weight calibration curve).

o - Bovine serum albumin (cross-linked sample)
● - Bovine serum albumin (standard sample)

Conditions:

Polyacrylamide gel: 5%
Buffer : Tris-borate (pH 8.4), 0.1% SDS
Protein stain : Coomassie blue


MOLECULAR WEIGHT x 10^{-4}
20
10
8
6
4
2
Phosphorylase 'a' (dimer)
Bovine serum albumin (dimer)
Phosphorylase 'a' (monomer)
Bovine serum albumin (monomer)
Ovalbumin
Pepsin
Trypsinogen
β-Lactoglobulin
Cytochrome 'C'
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

molecular weight of proteins with known molecular weights to their relative mobility in the SDS gel is shown in Figure 6. The 5% polyacrylamide gel allows a reasonable estimate of the protein molecular weights in the range of 180,000 to 25,000 daltons. Below 25,000 daltons the separation is not sharp and, therefore, the estimates of molecular weights are less accurate. A gel of higher acrylamide concentration would be required to improve the resolution in the low region of molecular weight. There appeared to be no real advantage in using the expensive "cross-linked" bovine serum albumin marker subunits over crude samples of the protein (denoted by open circles in Figure 6).

The determination of molecular weights of proteins by the SDS-PAGE technique has led to some criticisms in the literature. These have usually centred around the assumptions made on the behaviour of proteins themselves in SDS systems (Wallach and Winzler 1974 and references cited therein). Some problems that are encountered with the theory of the technique are listed (Wallach and Winzler 1974).

i) It is generally assumed that 1.4 g of SDS is bound per gram of protein. In fact, this can range from 0.2 g SDS per gram protein for pepsin to 1.7 g SDS per gram protein for aldolase. In addition, the rate of SDS binding by proteins may vary considerably.

ii) SDS and disulfide reducers do not necessarily reduce all oligomeric proteins into their subunits.

iii) SDS does not necessarily effect complete lipid-protein separation.

iv) SDS does not necessarily unfold all proteins or reduce them to the same shape.

v) Aggregation may occur in SDS.

Any of the above situations can seriously affect the estimation of molecular weight of proteins by the SDS-PAGE technique. Wallach and Winzler (1974) state:

> "To summarize, we consider SDS-PAGE a useful, facile method, but one which would be far less attractive if some better alternative were available".

Nevertheless, the SDS-PAGE technique has proved extremely useful in the study of wheat gluten proteins. It has allowed workers to characterize the subunit patterns of the polypeptides present in wheat proteins and has been useful in determining the molecular weight ranges of the proteins in the gluten complex. The area of SDS binding to gluten proteins certainly requires further research. It is evident that large differences in amino acid composition occur between glutenin subunits (see later results) and, therefore, it is not unreasonable to expect differences in the binding of SDS to specific glutenin subunits.

b. SDS-PAGE of unreduced proteins in the alcohol-soluble and alcohol-insoluble fractions. The SDS-PAGE patterns of the unreduced proteins of the alcohol-soluble fraction, the five peak fractions obtained from gel filtration of the alcohol-soluble fraction on Sephadex G-200, and the alcohol-insoluble fraction are shown in Figure 7. Bovine serum albumin was also subjected to SDS-PAGE (pattern 1) as a reference protein.

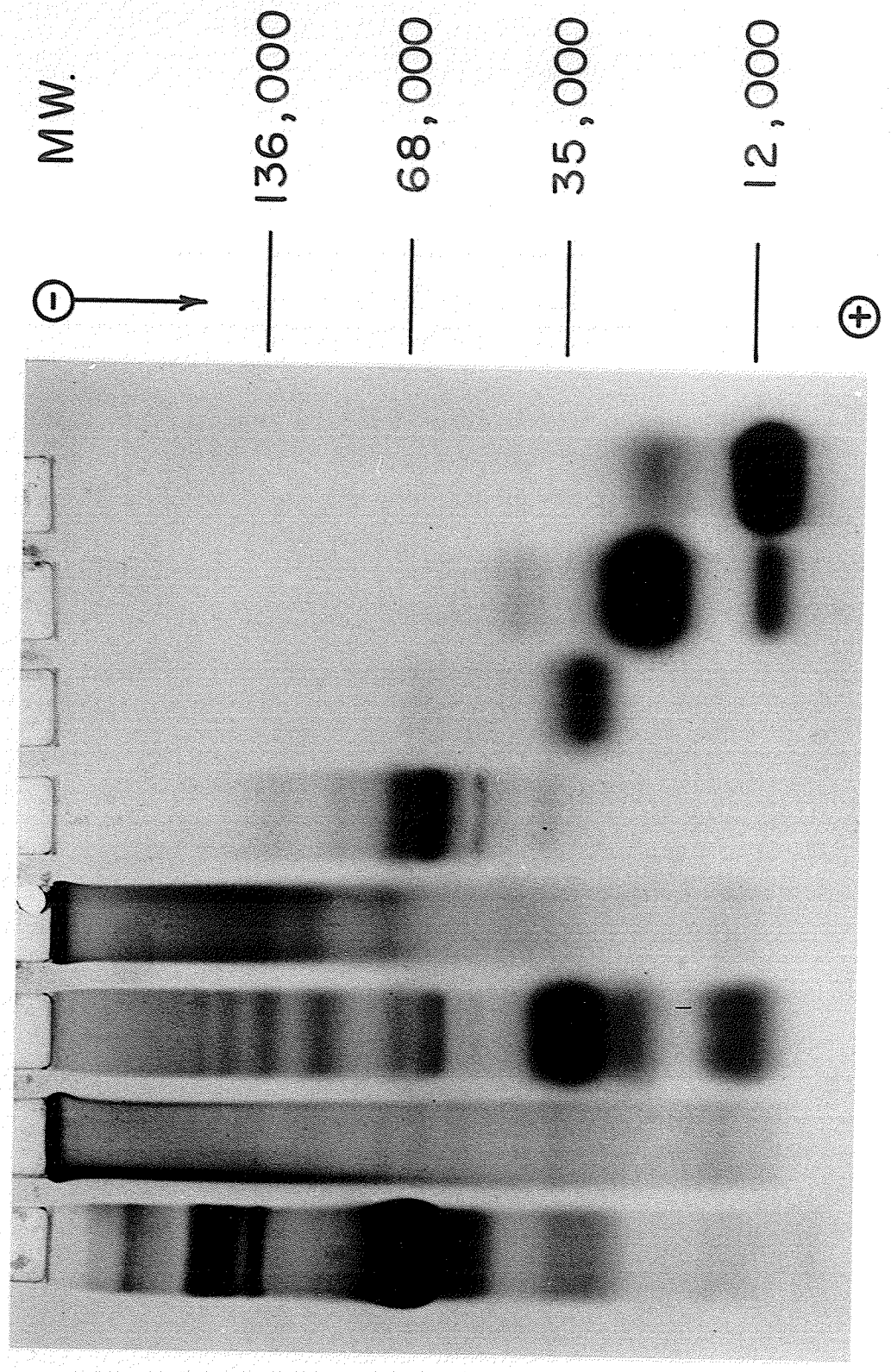
Most of the proteins in the alcohol-insoluble fraction (pattern 2) did not enter the gel. These proteins comprise large molecules (high molecular weight) and are referred to as glutenin II according to the model of glutenin structure recently proposed by Khan and Bushuk (1979a). This fraction contains trace amounts of protein components that enter the

Figure 7. SDS-PAGE patterns of unreduced proteins in the alcohol-insoluble and alcohol-soluble fractions.

Pattern	Sample
1	Bovine serum albumin
2	Proteins from alcohol-insoluble fraction
3	Proteins from alcohol-soluble fraction
4	Alcohol-soluble, peak I
5	Alcohol-soluble, peak II
6	Alcohol-soluble, peak III
7	Alcohol-soluble, peak IV
8	Alcohol-soluble, peak V

Conditions:

Polyacrylamide gel: 5%
Buffer : Tris-borate (pH 8.4), 0.1% SDS
Protein stain : Coomassie blue


MW.
136,000
68,000
35,000
12,000
⊖
⊕
1
2
3
4
5
6
7
8

gel on SDS-PAGE of unreduced glutenin; these components have been termed glutenin I subunits in the Khan and Bushuk model of glutenin structure. These subunits are believed to be held in the functional glutenin complex by strong non-covalent forces (Khan and Bushuk 1979a, b).

In contrast, most of the protein in the alcohol-soluble fraction enters the gel on SDS-PAGE (pattern 3). A large number of components are observed in this fraction with molecular weights ranging from 200,000 to 12,000 daltons. The alcohol-soluble fraction I (peak I) from gel filtration chromatography (pattern 4), although soluble in 70% (v/v) aqueous ethanol, is similar to the alcohol-insoluble fraction on SDS-PAGE. Most of the protein remains at the slot position and does not enter the gel. This fraction also contains some protein components that do enter the gel and whilst they cover a wide range of molecular weights, they are not easily discernible because the bands are extremely faint. This high molecular weight, alcohol-soluble fraction I also exhibits a "streaking" effect on the electrophoretic pattern as does the alcohol-insoluble pattern, even though the dissociating agent SDS is present.

The peak II fraction (pattern 5) contains several protein components ranging in molecular weight from 136,000 to 35,000 daltons. These proteins are soluble in 70% aqueous ethanol and comprise the gliadin bands of low mobility (see pattern 5 in Figure 5). The fact that Peak II proteins occur as a number of bands on SDS-PAGE indicates that the separation obtained with these proteins on gliadin electrophoresis (Figure 5) is due to molecular weight and possibly charge differences.

The SDS-PAGE patterns obtained for peaks IV and V (patterns 7 and 8) show that each comprises one major component. Fraction IV also contains two additional minor components. The component of higher molecular

weight may be an aggregated form of the major protein band of the peak. The lower molecular weight component appears to be the same as the major component of peak V. Peak V contains a trace amount of a component of the same mobility as the major component of peak IV. As mentioned previously, a Sephadex matrix with a lower fractionation range than G-200 (e.g. G-50) would be more effective for the separation of these low molecular weight peaks.

c. SDS-PAGE of reduced alcohol-insoluble and alcohol-soluble fractions. The SDS-PAGE patterns of the reduced proteins of the alcohol-insoluble and the alcohol-soluble fractions are shown in Figure 8. The patterns of the reduced proteins of the peak fractions (I to V) obtained from the gel filtration of the alcohol-soluble fraction are also included in this Figure. For this experiment, β-mercaptoethanol was added to the protein samples to reduce disulfide bonds.

The alcohol-insoluble fraction (glutenin) gives a pattern (1) that contains approximately 17 bands (subunits). These results are in agreement with those obtained by other workers for various glutenin preparations (Bietz and Wall 1972; Ewart 1972a; Hamauzu et al. 1972). Bietz and Wall (1972) demonstrated that glutenin is a complex mixture of approximately 15 different subunits ranging in molecular weight from 133,000 to 11,500 daltons. Different investigations have reported slightly different numbers of polypeptide subunits.

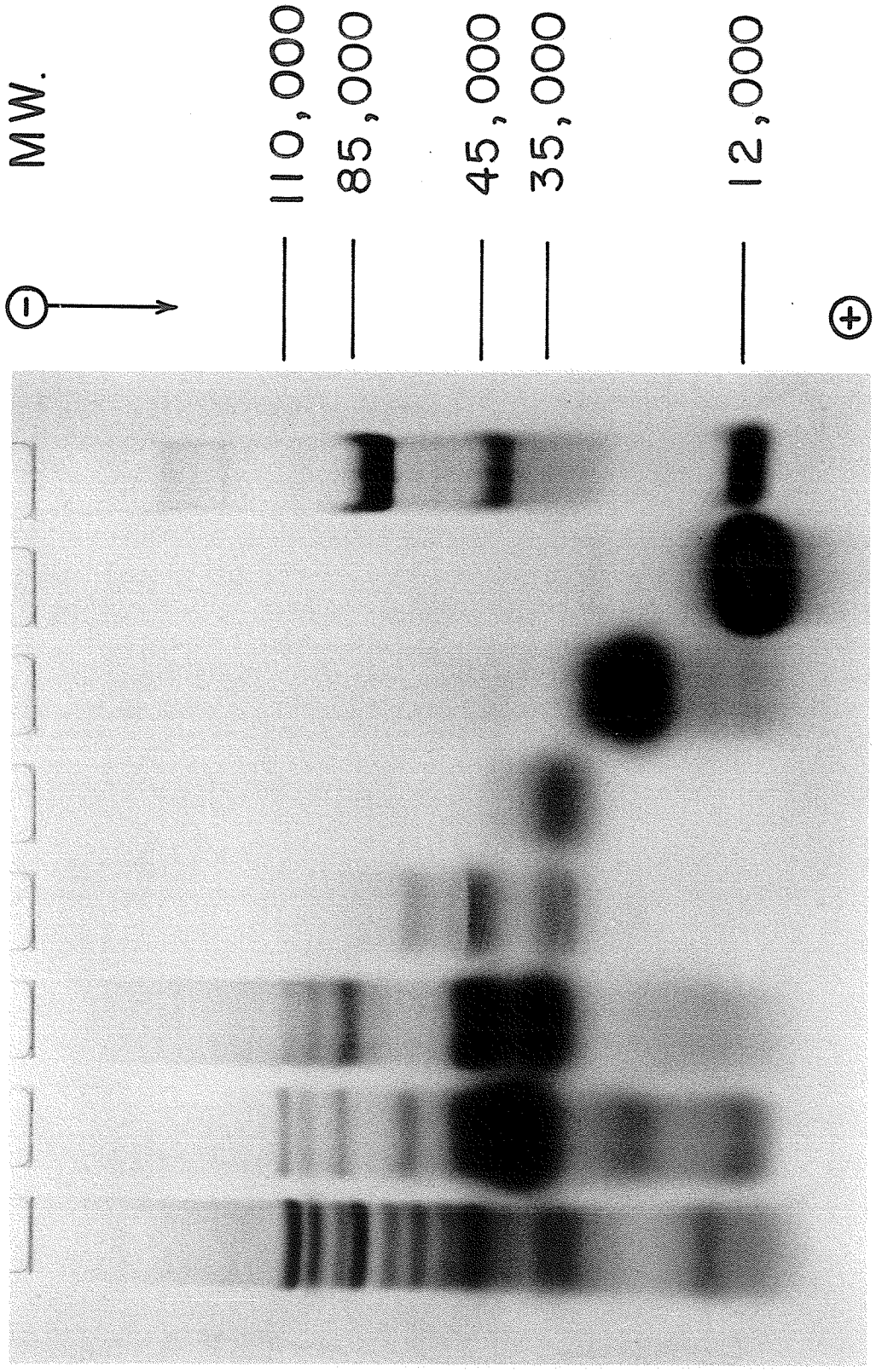
The estimates of the subunit molecular weights by SDS-PAGE have differed somewhat also, particularly those of the largest subunits. Orth and Bushuk (1973b) reported a value of 152,000 daltons for the highest molecular weight subunit, whereas Hamauzu et al. (1972) found a molecular weight of 104,000 daltons for the highest molecular weight

Figure 8. SDS-PAGE patterns of reduced proteins in the alcohol-insoluble and alcohol-soluble fractions.

Pattern	Sample
1	Protein from alcohol-insoluble fraction
2	Protein from alcohol-soluble fractions
3	Alcohol-soluble, peak I
4	Alcohol-soluble, peak II
5	Alcohol-soluble, peak III
6	Alcohol-soluble, peak IV
7	Alcohol-soluble, peak V
8	Reference proteins

Conditions:

Polyacrylamide gel	: 5%
Buffer	: Tris-borate (pH 8.4), 0.1% SDS
Protein stain	: Coomassie blue


MW.
110,000
85,000
45,000
35,000
12,000
⊖
⊕
1 2 3 4 5 6 7 8

subunit. Khan and Bushuk (1977, 1979a) reported a molecular weight of 134,000 daltons for this subunit and Payne et al. (1979) reported a value of 145,000 daltons. Paredes-Lopez (1980) reported a molecular weight as low as 90,000 daltons for what appears to be the same glutenin subunit. Obviously, slight variations in experimental procedures or the preparative procedures used for the proteins can produce substantial differences in the electrophoretic mobility of the gluten proteins. Certainly, some of the variations may be due to the theoretical problems associated with determining molecular weights using the SDS-technique that were previously mentioned. In this study, the molecular weight of the largest subunit of glutenin is 110,000 daltons, which is in the range of values obtained by other workers.

When subjected to SDS-PAGE in 0.125 M Tris-borate buffer at pH 8.4, the alcohol-insoluble (glutenin) proteins possess a characteristic group of four protein bands (see pattern 1, Figure 8). In this study, these bands ranged in molecular weight from 110,000 for the highest molecular weight band to 74,000 daltons for the lower molecular weight, fourth band. This group of polypeptide subunits can be separated from the other glutenin subunits by gel filtration chromatography of the reduced-alkylated alcohol-insoluble fraction on Sephadex G-200 (see later results).

The pattern of the alcohol-soluble fraction (pattern 2) showed three high molecular weight components in addition to the lower molecular weight gliadin-like components. The presence of these high molecular weight components was surprising as this group of subunits is normally associated with the alcohol-insoluble fraction of gluten proteins and has not been previously reported to be soluble in 70% (v/v) aqueous

ethanol. The electrophoretic mobility of the first protein bands is slightly lower than that of the analogous band in the pattern of the alcohol-insoluble fraction.

In general, slight changes in electrophoretic mobility were observed for the same proteins after fractionation by gel filtration chromatography. In pattern 2, the intensely stained band is probably the same as the peak III protein band (pattern 5). As indicated in the gel filtration profile (Figure 4) and the weight recoveries from the column (Table 3), this peak fraction forms a major percentage of the proteins present in the alcohol-soluble fraction.

Peak III proteins (pattern 5) migrate as a single band with the same mobility before and after reduction. This result indicates that this protein either has no disulfide bonds or if it has, then reduction of the bonds does not alter SDS binding and consequently mobility on SDS-PAGE. Whilst these proteins are homogeneous in molecular weight, they are quite heterogenous in charge density (see pattern 6, Figure 5). Thus, the high electrophoretic mobility and separation of the gliadin components that comprise peak III proteins (see Figure 5) is primarily due to differences in charge density of the polypeptides.

Peak III proteins appear to be similar to the low molecular weight gliadin proteins described by Bietz and Wall (1980). These authors described this fraction as a group of homologous polypeptides with a molecular weight of 36,000 daltons, but of different amino acid composition (hence variable charge density). This gives rise to a large number of bands when the fraction is examined by PAGE (see Figure 5).

Peaks IV and V proteins (patterns 6 and 7, respectively, Figure 8) also migrate as single bands on SDS-PAGE with the same mobility before

and after reduction. Whilst these proteins are homogenous in molecular weight they are also quite heterogenous in charge density (see Figure 5).

The peak II proteins (pattern 4) represent the highest molecular weight group of gliadin proteins. This group of gliadins has been classified as w-gliadins in the original nomenclature of Jones et al. (1959). Bietz and Wall (1980) showed that this group of proteins have low levels of the amino acids methionine and cysteine. This observation was confirmed in the amino acid composition studies of this peak fraction (see later results, Table 7). On SDS-PAGE, these proteins migrate as a number of subunits, some of which are higher in molecular weight than the peak III subunit. This probably accounts for their lower electrophoretic mobility on PAGE (see pattern 5, Figure 5).

All of the protein of the alcohol-soluble fraction I (peak I) enters the gel on SDS-PAGE (pattern 3), after reduction with β-mercaptoethanol. The four high molecular weight subunits, normally associated with the alcohol-insoluble fraction, are clearly evident in this pattern. The third and fourth bands are not completely resolved, however, both are present. The major proteins present are the 45,000 and 35,000 molecular weight components. Subunits of this molecular weight, obtained by reduction of glutenin, have been termed the "35,000 and 45,000 molecular weight non-gliadin polypeptides" and comprise a large proportion of glutenin (Bietz and Wall 1980). From the intensity of these bands in pattern 3, it is obvious that they do comprise a major proportion of the alcohol-soluble fraction I proteins.

5. Gel Filtration Chromatography of Reduced-Alkylated Alcohol-Insoluble Fraction on Sephadex G-200

The alcohol-insoluble fraction (glutenin) is only slightly soluble

in a solvent containing 0.1 N acetic acid and 3 M urea. To allow further study of the components present by gel filtration chromatography, the solubility of this fraction was increased by chemical modification of the protein component. The disulfide bonds in the proteins of this fraction were cleaved by reduction with β-mercaptoethanol according to the method of Friedman et al. (1970) and the exposed sulfhydryl groups were alkylated with 4-vinylpyridine to prevent reoxidation or reaction with any unreduced disulfide groups. After this modification, the alcohol-insoluble fraction was soluble in a solvent of acetic acid and urea.

The gel filtration profile of the reduced-alkylated alcohol-insoluble fraction (230 mg in 19.2 ml solvent) on Sephadex G-200 is shown in Figure 9. Gel filtration chromatography was performed at a rather low concentration of sample due to the viscous nature of wheat gluten proteins. At high concentrations (see Figure 30, Appendix II), the flow characteristics of the sample through the Sephadex G-200 matrix are not uniform. This leads to artifacts in the gel filtration profile and loss of resolution of peaks, especially those that appear near the void volume of the column. These artifacts can be eliminated by performing the gel filtration chromatography at a number of different sample concentrations and selecting an optimum concentration that results in a profile with symmetrical peaks and maximum resolution (see Appendix II). The gel filtration profile of the reduced-alkylated alcohol-insoluble fraction (Figure 9) has three distinct peaks. The elution volumes, K_{AV} values, and estimates of molecular weights for these peaks is given in Table 4.

The first protein peak chromatographed as a symmetrical peak in the void volume of the column. This peak, therefore, possessed a K_{AV} value

Figure 9. Elution profile from gel filtration chromatography of reduced-alkylated alcohol-insoluble fraction on Sephadex G-200.

Conditions:

Column size	: 5.0 x 100 cm
Bed height	: 80 cm
Solvent	: acetic acid (0.1 N) + urea (3 M)
Flow rate	: 28.0 ml hr^{-1}
Fraction size	: 9.0 ml
Sample volume	: 19.2 ml
Sample concentration	: 12 mg ml^{-1}

Carbohydrate: o——o
Protein : ●——●

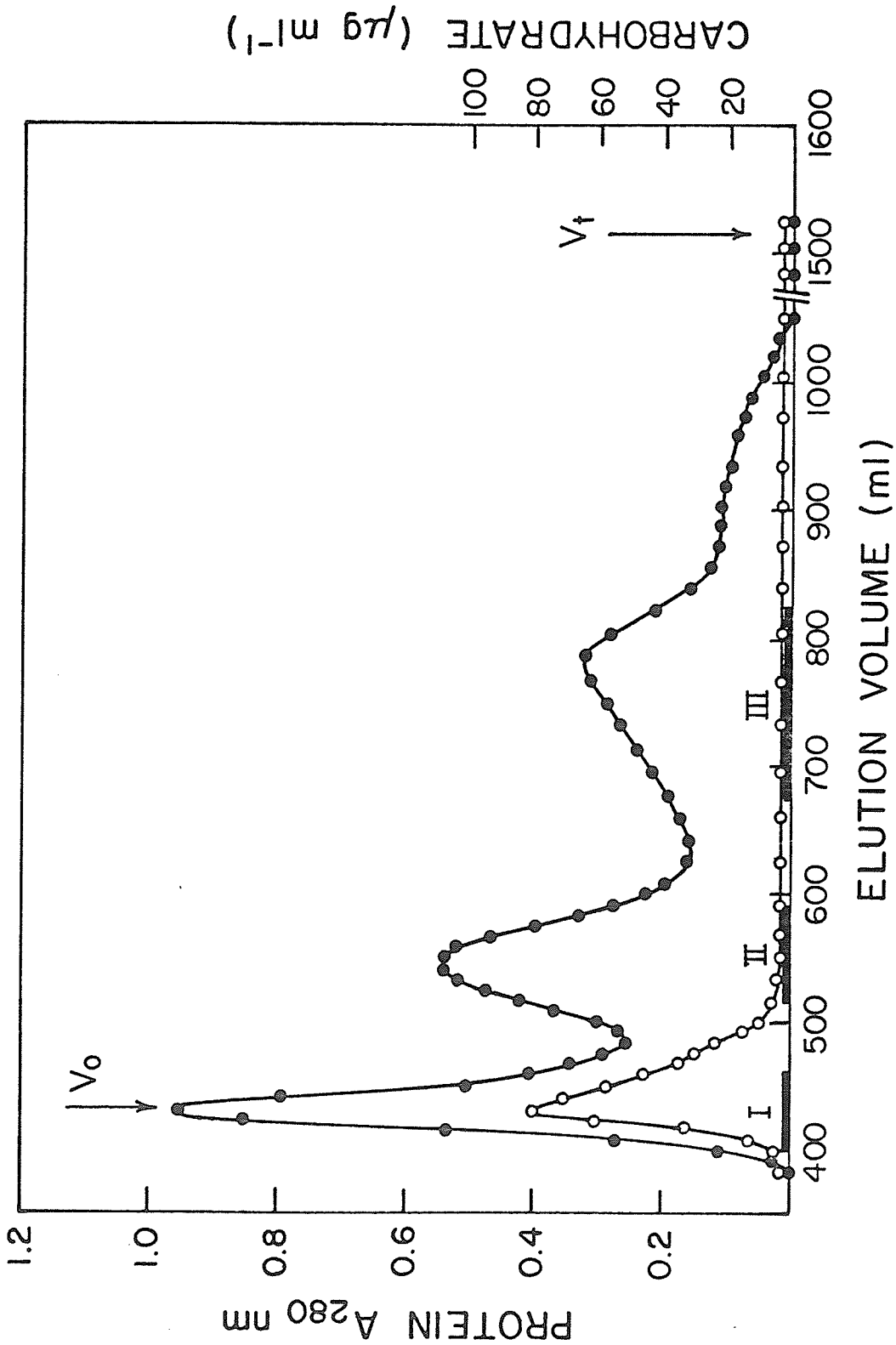

PROTEIN A280 nm
1.2
1.0
0.8
0.6
0.4
0.2
Vo
I
II
III
Vt
400
500
600
700
800
900
1000
1500
1600
ELUTION VOLUME (ml)
CARBOHYDRATE (μg ml⁻¹)
100
80
60
40
20

TABLE 4. Elution volumes, K_{AV} values, and molecular weights of protein fractions obtained by gel filtration chromatography of reduced-alkylated alcohol-insoluble fraction of gluten on Sephadex G-200.

Peak fractions	V_E (ml)	K_{AV}	MW[a] (daltons)
Peak I	430	0	>200,000
Peak II	545	0.11	125,000
Peak III	780	0.32	63,000

[a]Molecular weights estimated from Figure 3.

of zero and the estimated molecular weight was greater than 200,000 daltons. The second protein peak (peak II) also chromatographed as a single symmetrical peak at an elution volume of 545 ml corresponding to a K_{AV} value of 0.11 and a molecular weight of 125,000 daltons. The third protein peak (peak III) chromatographed as a fairly broad peak with an elution volume of 780 ml and a K_{AV} value of 0.32 equivalent to a molecular weight of 63,000 daltons. It is interesting to note that this peak showed a "leading edge" similar to that observed for peak III of the alcohol-soluble fraction (see Figure 4).

The weight recoveries of the peak fractions were not determined in this case due to difficulties in removing urea from the pooled fractions and losses of protein during the dialysis. The overall weight recoveries, after extensive dialysis, were usually in the range of 76 to 84%.

The gel filtration profile of the reduced-alkylated alcohol-insoluble fraction obtained in the present study is similar to the profiles obtained for reduced-alkylated glutenin preparations of Huebner and Wall (1974) and Khan and Bushuk (1978). The use of a large column and slower flowrates, as in the case of Figure 9, resulted in an improved resolution of peaks obtained on Sephadex G-200.

The fractions obtained by gel filtration of the reduced-alkylated alcohol-insoluble gluten protein were assayed for "total carbohydrate" by the phenol-sulfuric acid method of Dubois et al. (1956). A carbohydrate component eluted as a single peak with an elution volume corresponding to that of the peak I protein, i.e. the void volume of the Sephadex column. The peak fraction contained 80 μg ml^{-1} carbohydrate. The presence of carbohydrate in reduced-alkylated glutenin fractions was reported by Khan and Bushuk (1979b). In that study, values of carbohydrate were

determined on whole fractions that were pooled, dialyzed, and freeze dried using the less sensitive Anthrone method. It was reported that peak I contained 6.5%, peak II 1.0%, and peak III 0.3% carbohydrate. It is possible that some of this carbohydrate may be Sephadex fragments which had not been removed by the dialysis procedure. In the present study, carbohydrate was found only in the high molecular weight peak I and was not detected in the included protein peaks II and III. The reduced-alkylated alcohol-insoluble peak I was found to contain up to 27% carbohydrate by weight. The only other report of carbohydrate in glutenin fractions in the literature is that of Danno et al. (1978). In this report, a preparation of reduced-cyanoethylated glutenin was dissolved in 0.5% SDS and ethanol added to 70% v/v. The ethanol-insoluble fraction I was found to contain 30% carbohydrate by weight.

Thus, in two fractions derived from a gluten ball preparation, the alcohol-soluble fraction I and the reduced-alkylated alcohol-insoluble fraction, carbohydrate components were found to chromatograph on gel filtration chromatography as single peaks that eluted in the void volume of a Sephadex G-200 column.

6. Gel Filtration Chromatography of Reduced-Alkylated Alcohol-Soluble Fraction I

A comparison of the behaviour of the reduced-alkylated alcohol-insoluble fraction and the alcohol-soluble fraction I obtained by gel filtration chromatography (see Figure 4) was made. The disulfide bonds of the proteins of the alcohol-soluble fraction I were reduced and alkylated under the same conditions and by the same method as those in the alcohol-insoluble fraction described above. The freeze dried preparation of reduced-alkylated alcohol-soluble fraction I was dissolved in

a solvent containing acetic acid (0.1 N) and urea (3 M) and subjected to gel filtration chromatography under exactly the same conditions as the reduced-alkylated alcohol-insoluble fraction described in the previous section.

The gel filtration profile obtained is shown in Figure 10. This profile is quite similar to the profile of the reduced-alkylated alcohol-insoluble fraction shown in Figure 9. Three protein peaks were again obtained: peak I eluted in the void volume and peaks II and III were included in the column. The elution volumes, K_{AV} values, and molecular weights of the peaks are given in Table 5. From the data in Table 4 and Table 5, it can be seen that the similarity of the analogous peaks from the two reduced-alkylated fractions allow the peaks to be classified as similar proteins by gel filtration chromatography. The major difference between the two profiles (Figures 9 and 10) is the lesser proportion of peak II proteins in the alcohol-soluble fraction I.

As was observed for the alcohol-insoluble gluten fraction, carbohydrate again chromatographed as a single symmetrical peak with peak I proteins. The peak fraction contained approximately 80 μg ml^{-1} of carbohydrate. No carbohydrate was detected in the lower molecular weight peaks II and III.

7. SDS-PAGE of Proteins from Fractions (Peaks) Obtained by Gel Filtration Chromatography of Reduced-Alkylated Alcohol-Insoluble Fraction and Reduced-Alkylated Alcohol-Soluble Fraction I on Sephadex G-200

Column fractions representing the peaks from gel filtration of the reduced-alkylated alcohol-insoluble fraction (Figure 9) and reduced-alkylated alcohol-soluble fraction I (Figure 10) were pooled (indicated by horizontal bars on Figures 9 and 10), dialyzed, and the freeze dried samples examined by SDS-PAGE at pH 8.4. The patterns obtained are

Figure 10. Elution profile from gel filtration chromatography of reduced-alkylated alcohol-soluble fraction I on Sephadex G-200.

Conditions:

Column size	: 5.0 x 100 cm
Bed height	: 80 cm
Solvent	: acetic acid (0.1 N) + urea (3 M)
Flow rate	: 28 ml hr^{-1}
Sample volume	: 19.2 ml
Sample concentration	: 12.0 mg ml^{-1}

Carbohydrate: o———o
Protein : ●———●

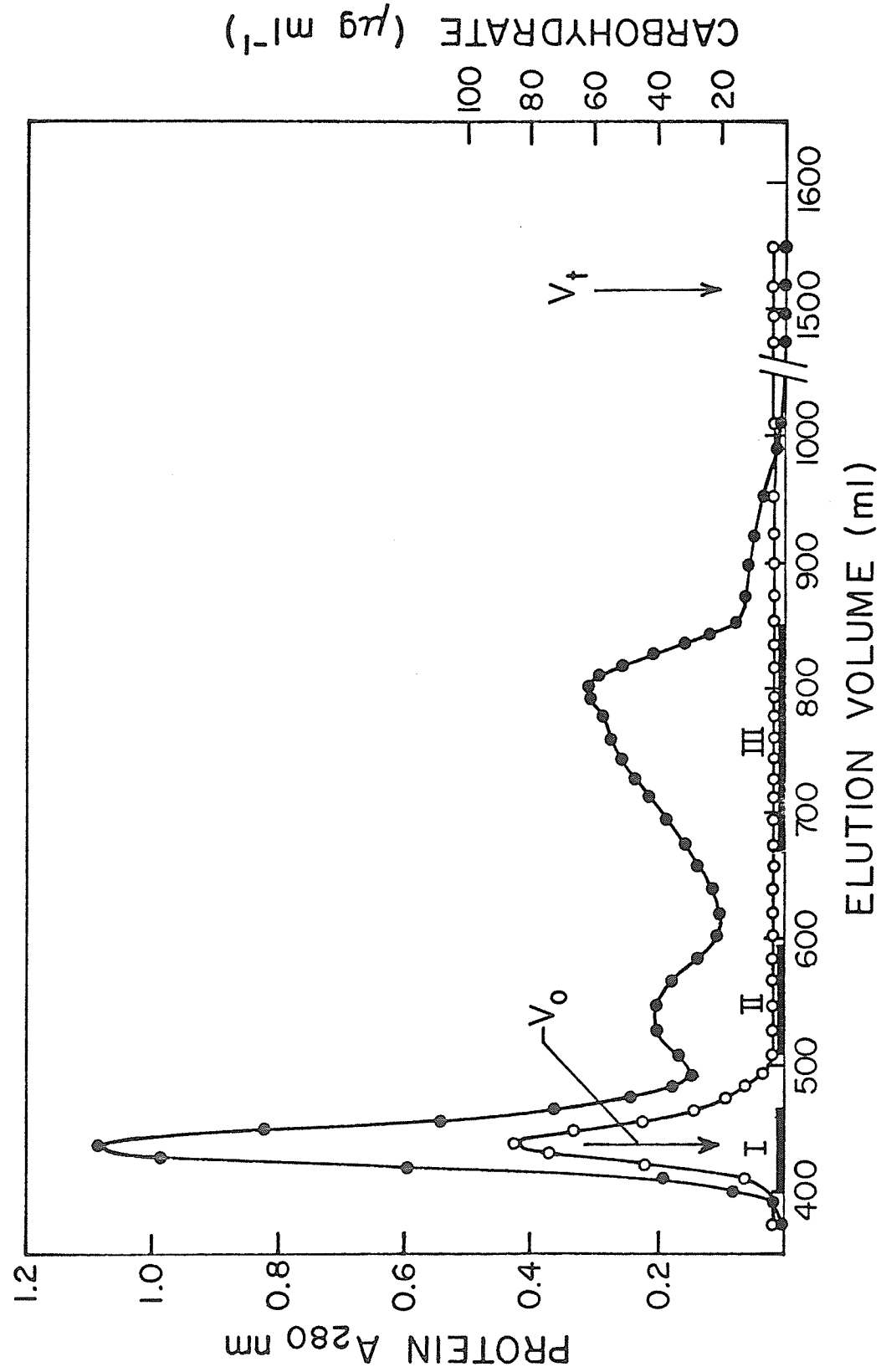

PROTEIN A280 nm
1.2
1.0
0.8
0.6
0.4
0.2
CARBOHYDRATE (μg ml⁻¹)
100
80
60
40
20
ELUTION VOLUME (ml)
400
500
600
700
800
900
1000
1500
1600
I
II
III
V0
Vt

TABLE 5. Elution volumes, K_{AV} values and molecular weights for protein fractions obtained from gel filtration chromatography of reduced-alkylated alcohol-soluble fraction I on Sephadex G-200.

Peak fractions	V_E (ml)	K_{AV}	MW[a] (daltons)
Peak I	430	0	> 200,000
Peak II	535	0.096	128,000
Peak III	800	0.34	59,000

[a]Molecular weights estimated from Figure 3.

shown in Figure 11. In this figure, pattern 1 represents the reduced-alkylated alcohol-insoluble fraction before gel filtration chromatography. Patterns 3, 4, and 5 correspond to peaks I, II, and III, respectively, obtained by gel filtration chromatography (see Figure 9). Pattern 1 is a typical electrophoregram of glutenin comprising approximately 17 subunits. The pattern of peak I proteins (pattern 2) lacks the four high molecular weight polypeptide subunits. This was somewhat surprising since this peak fraction represents the highest molecular weight fraction on the basis of elution volume on gel filtration. This fraction comprises subunits of lower molecular weight (68,000 to 12,000 daltons). Some protein material remained at the slot and a slight streaking was observed for this fraction. Obviously, these proteins have a high tendency to aggregate under conditions of gel filtration.

Peak II proteins (pattern 3) contains the four high molecular weight subunits observed by SDS-PAGE. Peak III fraction (pattern 4) contains a number of lower molecular weight subunits. The predominant subunits of molecular weight 45,000 and 35,000 daltons termed the "non-gliadin" proteins by Bietz and Wall (1980). The SDS-PAGE patterns obtained are consistent with the results obtained by Huebner and Wall (1974) and Khan and Bushuk (1979b) on gel filtration of reduced-alkylated glutenin preparations.

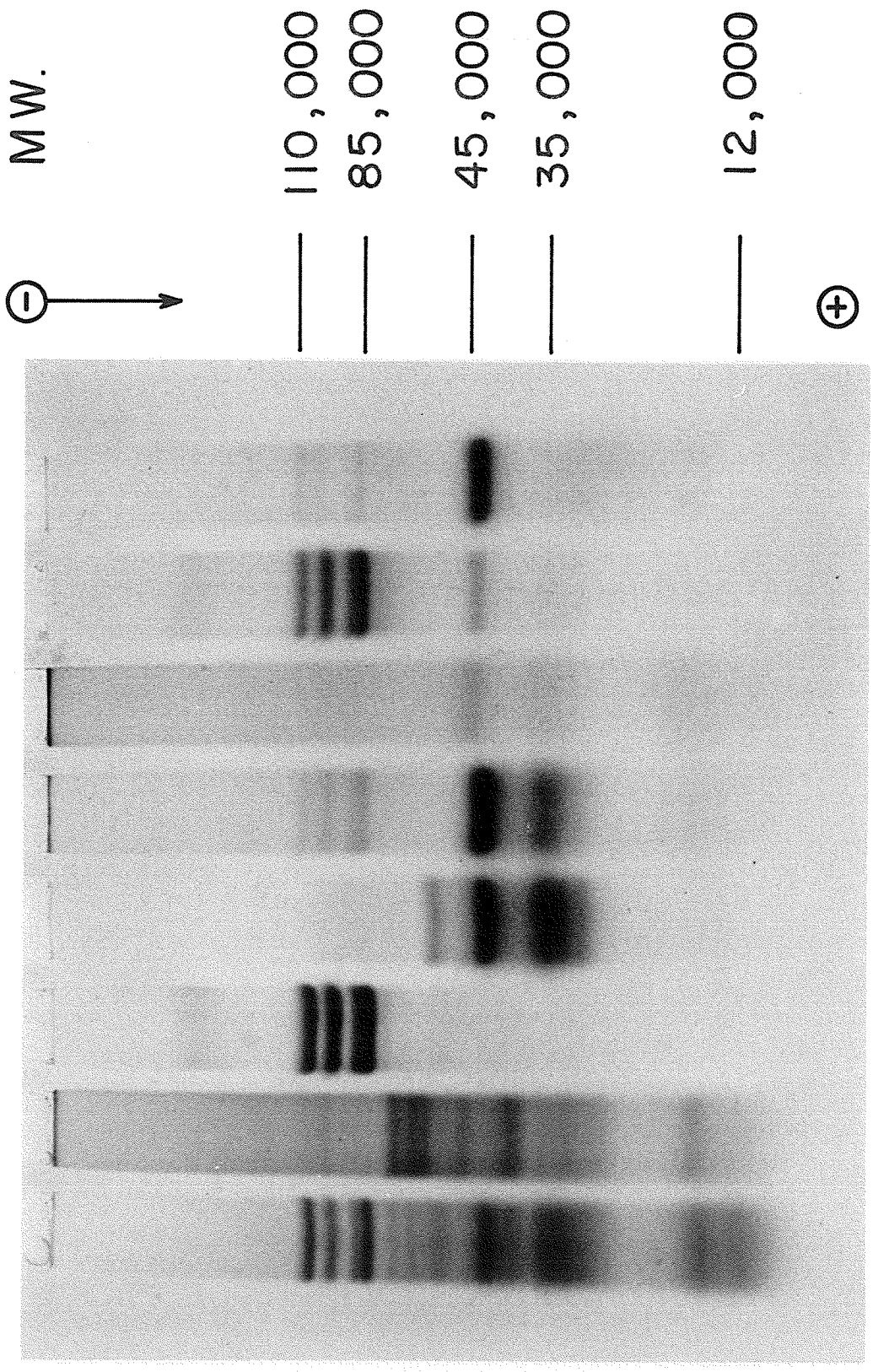
The analogous SDS-PAGE patterns obtained for the reduced-alkylated alcohol-soluble fraction I are shown as patterns 5, 6, 7, and 8 in Figure 11. The pattern for the total fraction before gel filtration chromatography (pattern 5) shows the presence of high molecular weight subunits although they are faint and other subunits, especially the 45,000 and 35,000 subunits. The amount of protein in the subunit patterns of these fractions appears to be less compared to the equivalent subunits

Figure 11. SDS-PAGE patterns of proteins from peaks obtained by gel filtration chromatography of reduced-alkylated alcohol-insoluble fraction and reduced-alkylated alcohol-soluble fraction I on Sephadex G-200.

Pattern	Sample
1	Reduced-alkylated (R-A) alcohol-insoluble proteins before gel filtration chromatography
2	R-A alcohol-insoluble peak I
3	R-A alcohol-insoluble peak II
4	R-A alcohol-insoluble peak III
5	R-A alcohol-soluble fraction I before gel filtration chromatography
6	R-A alcohol-soluble fraction I - peak I
7	R-A alcohol-soluble fraction I - peak II
8	R-A alcohol-soluble fraction I - peak III

Conditions:

Polyacrylamide gel: 5%
Buffer : Tris-borate (pH 8.4), 0.1% SDS
Protein stain : Coomassie blue


MW.
110,000
85,000
45,000
35,000
12,000
⊖
⊕
1
2
3
4
5
6
7
8

of the alcohol-insoluble fraction. The alcohol-soluble fraction I contains approximately 2.0% less protein by weight than the alcohol-insoluble fraction (Table 2). The high molecular weight subunits appear to be less in proportion to other subunits in this fraction. This result is consistent with the lower amount of peak II proteins in this fraction on gel filtration chromatography (see peak II in Figure 10 and Figure 9).

Peak I proteins (pattern 6) gave a pattern that is similar to that of peak I of the alcohol-insoluble fraction, however, the bands were much fainter. The high molecular weight polypeptides are again absent in this fraction (peak I). Some protein material remained at the slot position and again a slight streaking appeared in the electrophoregram. These proteins, in a similar fashion to the reduced-alkylated alcohol-insoluble peak I proteins, must aggregate under conditions of gel filtration chromatography.

The four high molecular weight subunits are present in the pattern of peak II proteins (pattern 7). A trace amount of the 45,000 dalton subunit is present in the pattern, however, this is probably the result of a small contamination of peak III proteins being present. Consequently, the high molecular weight bands from peak II show very faintly in the electrophoregram of the peak III proteins (pattern 8) as a result of this slight cross-contamination of fractions (peaks). The 45,000 "non-gliadin" subunit is clearly visible in the peak III protein pattern.

It was concluded that, in general, the SDS-PAGE patterns for the peaks obtained from gel filtration chromatography demonstrate a similarity between the two fractions. In fact, at this point it could be argued that the high molecular weight alcohol-soluble fraction I may simply

represent a contamination of the alcohol-soluble fraction by the alcohol-insoluble fraction. This is not the case, however, as the two fractions may be clearly differentiated on the basis of the monosaccharide composition of the carbohydrate component of the fractions. This will be reviewed later in the Results (see Section G; Tables 15 and 16; Figure 14).

A number of major conclusions can be seen from these experiments. The average molecular weights of the peak II proteins by gel filtration chromatography is 125,000 and 128,000 daltons for the alcohol-insoluble fraction and the alcohol-soluble fraction I, respectively. This figure is in the middle of the range of molecular weights reported for the highest molecular weight glutenin subunit (154,000 to 90,000) by SDS-PAGE mentioned previously. The molecular weight estimate for this group of polypeptides by gel filtration chromatography may be more useful as an average molecular weight value.

Modification of the proteins of both fractions by reduction and alkylation of the disulfide bonds present, alters the chromatographic profile of the protein considerably, however, it does not change the chromatographic profile of the carbohydrate components on Sephadex G-200. The reduction and alkylation of the protein components, allows a separation of the high molecular weight subunits by gel filtration chromatography. These subunits do not appear to have the tendency to aggregate under conditions of gel filtration in acetic acid-urea solvent and no streaking or material remains at the slot position on SDS-PAGE. Clearly, the carbohydrate component is not associated with this group of subunits. The question remains of how these proteins are associated into the gluten structure when their disulfide bonds are not disrupted. A number of

possibilities are evident. Firstly, these subunits may simply be joined together by interpolypeptide disulfide bonds and not to the other subunits in the glutenin pattern. Secondly, they may associate with the other groups of subunits by non-covalent forces and the change in structure (conformation) resulting from disulfide bond reduction may prevent the association (as in the model of Kasarda et al. 1976). Thirdly, these polypeptides may be linked directly by interpolypeptide bonds to the subunits of lower molecular weight. Finally, the role of these high molecular weight subunits in gluten structure can only be elucidated by intensive research in this area. Some comments, and further characteristics of these polypeptides based on amino acid composition studies, will be discussed in the following section.

On gel filtration of the two main gluten fractions, the carbohydrate component was found to occur exclusively with the high molecular weight protein fractions. The SDS-PAGE patterns for the high molecular weight peak (peak I) from gel filtration chromatography showed that the lower molecular weight subunits aggregate to form the proteins of this fraction. This underlines the strong aggregative tendency of these subunits, even in the presence of dissociating solvents. In addition, the presence of carbohydrate components exclusively in the aggregated protein fraction and the absence of carbohydrate in the other protein fractions provides strong circumstantial evidence of the carbohydrate component being involved in the aggregation mechanism.

Before further investigation of the associative properties of the protein and carbohydrate components in the gluten fractions was undertaken, further study on the individual protein and carbohydrate components themselves was carried out. Amino acid composition analyses of the

proteins were performed and hydrophobicities and charge potentials were calculated. Some details of secondary structure of A_2-gliadin based on the predictive scheme of Chou and Fasman (1974a, b) will be considered. The monosaccharide composition of the carbohydrate components were also determined. These are discussed in the following sections.

E. Amino Acid Composition Studies of the Proteins of Wheat Gluten Fractions

The amino acid composition was determined for the various gluten protein fractions prepared in this study. Comparisons of these fractions were made on the basis of the amino acid composition, average hydrophobicities, charge potentials, and charge frequency per residue. The wheat gluten proteins were also compared, on the basis of the above parameters, with proteins from other sources.

The values for the amino acid composition are given in grams amino acid per 100 g of protein. These values represent actual amino acid recoveries. That is, they have not been adjusted to grams amino acid recovered per 100 g of recovered amino acid basis, as is often done in these types of comparisons. Nitrogen recoveries were reasonably uniform and varied between 88 to 92%.

1. Calculation of Average Hydrophobicity ($\langle H \rangle_{ave}$) and Charge Potentials

Average hydrophobicity, positive and negative charge potential, charge ratios, and frequency of charged groups were calculated from the amino acid composition data. These parameters can be used to compare different proteins from the same source or proteins from other sources. A sample calculation, based on the amino acid composition data for the alcohol-insoluble protein given in Table 6, is as follows:

TABLE 6. Data for the calculation of protein hydrophobicity (HΦ total) and charge potentials.

Amino acid	% AA[a]	MW AA	Moles AA/ 100 kg protein	HΦ/ residue[b,c]	HΦ[c] total
Glutamic acid	35.04	147.13	238.16	0	0
Aspartic acid	4.31	133.10	32.38	0	0
Serine	5.12	105.09	48.72	0	0
Histidine	2.19	155.16	14.12	0	0
Glycine	5.34	75.07	71.13	0	0
Threonine	3.02	119.12	25.35	0.45	11.41
Arginine	9.35	174.20	24.97	0.75	18.73
Alanine	3.36	89.09	37.72	0.75	28.29
Cysteine	nd[d]	-	-	1.00	-
Methionine	1.77	149.21	11.86	1.30	15.42
Lysine	2.79	146.19	19.09	1.50	28.63
Valine	4.45	117.15	37.99	1.70	64.58
Leucine	7.24	131.17	65.20	2.40	132.47
Proline	10.76	115.13	93.46	2.60	242.99
Phenylalanine	4.33	165.19	26.21	2.65	69.46
Tyrosine	4.28	181.11	23.63	2.85	67.35
Isoleucine	3.40	131.17	25.92	2.95	76.47
Total			795.9		755.80

[a] From amino acid composition of alcohol-insoluble protein.

[b] Bigelow (1967).

[c] Kilocalories.

[d] nd = not determined.

i. Average hydrophobicity ($H\Phi_{ave}$)

$$= \frac{\text{Total } H\Phi}{\text{Total moles of amino acids/100 Kg protein}}$$

$$= \frac{755.8}{795.9}$$

$$= 0.949 \text{ Kcal mole}^{-1}$$

ii. Amide groups:

$$= \frac{\text{Weight of } NH_3 \text{ (g)}}{17} \times 10^3$$

$$= \frac{3.71 \times 1000}{17}$$

$$= 218.2 \text{ moles} \quad 100 \text{ Kg protein}^{-1}$$

iii. Negative charge potential:

= Total moles of glutamic acid + aspartic acid

= 238.2 + 32.4

= 270.6 moles 100 Kg protein^{-1}

iv. Negative charge potential less amide groups:

= 270.6 - 218.2

= 52.4 moles 100 Kg protein^{-1}

v. Positive charge potential:

= Total moles of arginine + lysine + histidine

= 24.97 + 19.09 + 14.12

= 58.2 moles 100 Kg protein^{-1}

vi. Ratio of positive to negative charges:

$$= \frac{58.2}{52.4}$$

$$= 1.11$$

vii. Frequency of charged groups:

$$= \frac{\text{Negative charge potential less amide + positive charge potential}}{\text{Total moles of amino acids/100 Kg protein}}$$

$$= \frac{52.4 + 58.2}{795.9}$$

$$= 0.19 \text{ charged groups residue}^{-1}$$

2. Hydrophobic Theory

The hydrophobicity values for the amino acid residues, as shown in column 4 of Table 6 were derived by Bigelow (1967) from the transfer free energies (ΔF_t) of amino acid sidechains as determined by Tanford (1962). The transfer free energy is defined as the free energy required to transfer one mole of amino acid from an aqueous solution to an ethanolic solution under standard conditions. The ΔF_t values were calculated from the ΔF_t of the corresponding amino acids by the following formula:

$$\Delta F_t = - RT \ln \frac{N_{EtOH}}{N_{HOH}}$$

where N_{EtOH} is the solubility of an amino acid in ethanol.

N_{HOH} is the solubility of an amino acid in water.

R = Gas constant.

T = Temperature ^{o}K.

The ΔF_t for sidechains of particular amino acids was determined by subtracting the ΔF_t of glycine from the ΔF_t of the particular amino acid. For example, the ΔF_t of glycine when subtracted from that of phenylalanine gives the ΔF_t for the benzyl sidechain. Tanford (1962) estimated that for any amino acid, the ΔF_t was close to the sum of the values of its original structural components.

The method as described used ethanol to simulate the hydrophobic environment of the interior of a protein molecule. Other organic solvents gave similar values to ethanol, however, the ΔF_t values for cysteine, cystine, histidine, and glutamic and aspartic acids were not derived by Tanford. It is difficult to assess the hydrophobicities for charged side-chains, but the values are probably small or negative. Negative values of hydrophobicity mean that the side chain has a higher affinity for water than ethanol and will tend to occur on the outer shell

of the protein molecule in an aqueous environment. Bigelow (1967) reasoned that while this may contribute to the stability of the protein, it is not the type of stabilization caused by the association of hydrophobic groups and assigned hydrophobicity of 0 Kcal to such groups. Cysteine was arbitrarily assigned a hydrophobicity value of 1.0 Kcal. The average hydrophobicity ($H\mathbb{Q}_{ave}$) of a protein, when multiplied by the number of residues in the molecule equals the total hydrophobicity. This is a measure of the stabilization that a molecule would achieve if all of the "non-polar" residues were buried inside the molecule. However, the average hydrophobicity term ($H\mathbb{Q}_{ave}$) is a convenient measure of a non-polarity (Bigelow 1967).

3. Amino Acid Composition of Napayo Flour Protein, Gluten Proteins, Alcohol-Insoluble Protein, Alcohol-Soluble Protein, and Gel Filtration Peak Fractions

The amino acid compositions of the above fractions are given in Table 7. The average hydrophobicities and charge potentials for the corresponding amino acid compositions have been calculated and are given in Table 8.

The amino acid compositions of the flour and gluten proteins are typical for wheat endosperm proteins in that they show high contents of glutamic acid, proline, and other non-polar amino acids (Kasarda et al. 1971; Wu and Dimler 1963a, b). Most of the glutamic and aspartic acids in wheat proteins occur in the amide form (Holme and Briggs 1959). This is confirmed by the amide group values and consequently the low negative charge potentials for the gluten proteins in Table 8. The high contents of glutamine and asparagine are believed to confer high hydrogen bonding potential in gluten protein, which is considered important in gluten structure. A number of studies have demonstrated the importance of

TABLE 7. Amino acid composition of Napayo flour protein, gluten, alcohol-insoluble proteins, alcohol-soluble proteins, and peak fractions obtained from gel filtration chromatography of alcohol-soluble proteins on Sephadex G-200.[a]

Amino acid	Flour protein (defatted)	Gluten	Alcohol-insoluble fraction (glutenin)	Alcohol-soluble fraction (gliadin)	Alcohol-soluble fraction				
					Peak[b] I	Peak[b] II	Peak[b] III	Peak[b] IV	Peak[b] V
Lysine	2.09	1.72	2.79	0.88	0.73	0.50	0.51	1.78	1.85
Histidine	2.13	2.01	2.19	1.98	1.68	1.49	2.00	4.04	1.31
Arginine	3.24	3.34	4.35	2.61	2.82	1.19	2.00	3.58	5.81
Ammonia	4.19	4.23	3.71	4.72	4.83	5.48	4.85	5.12	7.07
Aspartic acid	4.06	3.35	4.31	2.73	1.80	1.22	2.84	3.85	5.32
Threonine	2.73	2.61	3.02	2.11	2.70	1.53	1.71	4.48	3.83
Serine	4.41	4.79	5.12	4.58	5.64	4.12	4.18	5.33	3.74
Glutamic acid	39.27	40.61	35.04	43.32	43.24	51.12	44.10	29.74	14.42
Proline	12.91	13.57	10.76	15.31	14.01	20.42	15.80	9.10	8.05
Glycine	3.49	3.35	5.34	1.96	2.68	0.97	1.56	2.57	3.46
Alanine	2.93	2.40	3.36	2.12	1.34	1.03	2.17	3.45	3.30
Valine	4.42	4.01	4.45	3.94	4.18	1.56	4.15	5.65	5.43
Methionine	1.63	1.62	1.77	1.56	1.72	0.68	1.67	2.93	2.39
Isoleucine	3.81	3.83	3.40	4.04	3.70	3.27	4.62	4.68	2.85
Leucine	7.28	7.27	7.24	7.23	7.89	5.73	7.52	6.26	6.74
Tyrosine	2.95	3.36	4.28	2.58	1.93	1.47	2.72	1.90	4.12
Phenylalanine	5.60	5.51	4.33	6.01	5.58	11.15	6.34	1.18	1.77

[a]Grams amino acid per 100 grams protein; cysteine and tryptophan not determined.

[b]Peaks obtained from gel filtration chromatography of alcohol-soluble fraction on Sephadex G-200; see Figure 4.

TABLE 8. Average hydrophobicities and charge potentials of alcohol-insoluble and alcohol-soluble fractions of wheat gluten.

	Alcohol-insoluble fraction	Alcohol-soluble fraction	Alcohol-soluble fractions[a]		
			Peak I	Peak II	Peak III
Average hydrophobicity (Kilocalories residue^{-1})	0.949	1.064	1.016	1.136	1.100
Amide groups[b] (moles 100 Kg^{-1})	218.2	277.7	284.0	322.4	285.3
Negative potential (moles 100 Kg^{-1})	270.5	314.9	307.4	357.2	321.1
Negative potential less amide groups (moles 100 Kg^{-1})	52.3	37.2	23.3	34.8	35.8
Positive potential (moles 100 Kg^{-1})	58.2	33.8	32.0	19.9	27.86
Positive to negative charge ratio	1.11	0.90	1.37	0.57	0.78
Frequency of charged groups (groups residue^{-1})	0.19	0.09	0.08	0.07	0.08

[a]Fractions obtained from gel filtration chromatography of the alcohol-soluble fraction on Sephadex G-200; see Figure 4.

[b]Estimated from ammonia content in amino acid analysis.

hydrogen bonding in relation to dough properties. For example, acetylation of the amide groups in gluten proteins destroys the cohesiveness of dough (Barney et al. 1965). Another peculiar characteristic of wheat endosperm proteins is the high content of non-polar or hydrophobic amino acids.

Basic differences in the amino acid composition of the alcohol-insoluble (glutenin) proteins and the alcohol-soluble gliadin are evident from Table 7. The alcohol-soluble fraction contains more glutamic acid and proline than the alcohol-insoluble fraction. However, the latter fraction contains a higher amount of glycine, an amino acid often found in structural proteins such as collagen. The alcohol-insoluble fraction also contains higher levels of the basic amino acids lysine, histidine, and arginine. This is reflected in the higher positive charge potential of this fraction (58.2 moles) compared with the alcohol-soluble fraction (33.8 moles). The alcohol-insoluble fraction possesses a larger overall charge potential. This is reflected in the higher frequency of charged groups per residue for this fraction (0.19), compared with that for the alcohol-soluble fraction (0.09). The alcohol-soluble fraction contains a higher proportion of hydrophobic amino acids than the alcohol-insoluble fraction. The calculated average hydrophobicity of the alcohol-soluble fraction was 1.064 Kcal and 0.949 Kcal for the alcohol-insoluble fraction. Considering the low charge frequency per residue and the high value of hydrophobicity, it is not surprising that the prolamine fraction is soluble in an ethanolic solvent.

The low charge frequencies and the potential for hydrophobic bonding provide a theoretical basis for the low solubilities and the known aggregation tendency of wheat proteins. Dough structure is destroyed

by a number of solvents (e.g. hexane) which disrupt hydrophobic bonds (Ponte et al. 1967; Pomeranz et al. 1966). It has been demonstrated recently that sodium stearate solutions solubilize wheat proteins (Kobrehel and Bushuk 1977). Average hydrophobicity values have been estimated as 1.109 and 1.016 Kcal per residue for gliadin and glutenin, respectively (Woychik et al. 1961). A similar difference was obtained for gliadin and glutenin in the present study (Table 8). These values are considered high enough to stabilize gluten complexes by hydrophobic bonding (Pomeranz 1971). In fact, some authors conclude that hydrophobic bonding in proteins may be the most important single factor in non-covalent interactions in aqueous solutions where the strengths of electrostatic, charge transfer, and hydrogen bonds are reduced by the charge solvating and hydrogen-bonding ability of water (Jencks 1969). Hydrophobic bonding has been succinctly defined as

> "an interaction of molecules with each other which is stronger than the interaction of the separate molecules with water and which cannot be accounted for by covalent, electrostatic, hydrogen bond, or charge transfer forces" (Jencks 1969).

The amino acid compositions of the proteins in the fractions obtained by gel filtration of the alcohol-soluble fraction are also given in Table 7. The composition of peak I is quite similar to the original alcohol-soluble fraction. Peak I is an interesting fraction (termed alcohol-soluble fraction I), as it contains a significant amount of carbohydrate. It also has a high apparent molecular weight as it is excluded on gel filtration on Sephadex G-200 and will not enter a 6% acrylamide gel (Figure 5) or a 5% gel even in the presence of sodium

dodecyl sulfate. Peak I contains a high amount of glutamic acid and proline; similar to the unfractionated material. This peak contains a higher amount of glycine (2.68 g) than does the original alcohol-soluble fraction (1.96 g), peak II (0.97 g) or peak III (1.56 g). The charge potential of peak I proteins, given in Table 8, is low and this is reflected in the low frequency of charged groups per residue (0.08). In fact, this parameter has a low value for all of the alcohol-soluble fractions. Peak I fraction contains a slightly lower proportion of hydrophobic amino acids than either the original fraction, peak II, and peak III. This results in peak I proteins possessing a lower average hydrophobicity (1.016 Kcal residue^{-1}) than the other fractions and peaks.

The most notable characteristic of the amino acid composition of peak II proteins are the high contents of glutamic acid (51.12 g) and proline (20.42 g) and relatively low contents of glycine, valine, and the basic amino acids lysine, histidine, and arginine. The high degree of amidization of the glutamic acid and aspartic acid in this fraction is typical of the gliadin proteins (Ewart 1967). This composition leads to a low overall charge potential and a low frequency of charge groups per residue. This fraction contains a high proportion of hydrophobic amino acids, most notably phenylalanine. Its average hydrophobicity is the highest of the alcohol-soluble fractions.

The amino acid composition of peak III proteins more closely resembles that of the original alcohol-soluble fraction. Peak III proteins are gliadins of similar molecular weight by SDS-PAGE (see pattern 6, Figure 7), however, the components differ in charge density, illustrated by the PAGE results in Figure 5 (pattern 6). The contents of glutamic acid and proline are similar to those of peak I and the total

alcohol-soluble fraction. The level of the basic amino acids is low; the positive potential of this fraction is slightly lower than that of peak I and the alcohol-soluble fraction. Its charge frequency per residue is similar to the other alcohol-soluble fractions. A high proportion of hydrophobic amino acids has resulted in a fairly high value of average hydrophobicity of 1.100 Kcal residue^{-1}.

Peak IV and V fractions are low molecular weight proteins and their amino acid compositions (Table 7) resemble those of albumin (water soluble) and globulin (salt soluble) proteins. They contain lower proportion of glutamic acid (and glutamine), proline, hydrophobic amino acids, and contain a higher proportion of basic amino acids. The values for hydrophobicity and charge potentials were not calculated for these two peaks.

4. Amino Acid Composition of Reduced-Alkylated Alcohol-Insoluble Proteins and Reduced-Alkylated Alcohol-Soluble Fraction I and Peak Fractions Obtained from Gel Filtration Chromatography on Sephadex G-200

The amino acid compositions of the alcohol-insoluble gluten proteins and the alcohol-soluble fraction I (from gel filtration) gluten proteins after reduction and alkylation are given in Table 9. The compositions of the peak fractions obtained by gel filtration chromatography of these two fractions are also documented in Table 9. These fractions (I, II, and III) correspond to peaks I, II, and III in the gel filtration profiles shown in Figures 9 and 10.

From these gel filtration profiles and the SDS-PAGE patterns of the peak fractions, it appears that the total alcohol-insoluble fraction and the alcohol-soluble fraction I proteins are identical. The amino acid compositions (Table 9) indicate that they are similar, although there

TABLE 9. Amino acid composition of reduced-alkylated alcohol-insoluble proteins, reduced-alkylated alcohol-soluble fraction I proteins, and peaks obtained from gel filtration chromatography on Sephadex G-200.[a]

Amino acid	Alcohol-insoluble fraction				Alcohol-soluble fraction I			
	Reduced -alkylated[b]	Peak[c] I	Peak[c] II	Peak[c] III	Reduced -alkylated[b]	Peak[d] I	Peak[d] II	Peak[d] III
Lysine	2.70	5.61	1.16	1.34	0.75	1.72	0.94	0.65
Histidine	2.01	2.54	1.16	2.20	1.61	1.37	1.28	1.68
Arginine	6.10	7.35	4.29	6.54	5.39	4.41	3.54	4.79
Ammonia	3.99	2.17	4.26	4.19	4.47	4.22	4.71	4.60
Aspartic acid	4.07	9.67	1.21	2.48	1.69	3.37	0.99	1.31
Threonine	3.05	4.28	3.02	2.63	2.60	2.91	2.66	2.42
Serine	4.97	5.34	5.42	5.39	5.54	5.10	5.45	5.63
Glutamic acid	31.37	17.11	42.35	39.99	41.08	35.90	43.51	42.57
Proline	9.53	6.21	10.52	12.34	12.42	11.74	12.62	13.42
Glycine	4.85	5.28	10.67	2.66	3.03	3.92	7.55	3.24
Alanine	3.24	5.97	2.21	2.50	1.73	2.74	1.79	1.49
Valine	4.33	6.58	1.85	4.49	3.84	3.94	2.25	3.29
Methionine	1.65	2.56	0.44	1.88	1.58	1.53	0.78	1.35
Isoleucine	3.20	4.50	1.05	3.89	3.31	3.43	1.85	2.89
Leucine	6.79	9.51	4.46	7.49	7.26	7.95	5.42	7.13
Tyrosine	4.37	4.57	7.80	2.34	2.52	2.58	5.55	2.83
Phenylalanine	4.63	5.42	0.72	6.01	4.66	4.21	2.24	4.38

[a]Grams amino acid per 100 grams protein; cysteine and tryptophan not determined.

[b]Sulfhydryl groups in reduced proteins alkylated with 4-vinylpyridine.

[c]Peaks obtained from gel filtration chromatography of reduced-alkylated alcohol-insoluble fraction on Sephadex G-200; see Figure 9.

[d]Peaks obtained from gel filtration chromatography of reduced-alkylated alcohol-soluble fraction I on Sephadex G-200; see Figure 10.

does appear to be some significant differences. For a more detailed comparison of these fractions and peaks, the average hydrophobicities and charge potentials were calculated from the corresponding amino acid compositions, and are given in Table 10.

The alcohol-insoluble and the alcohol-soluble fraction I proteins possess high contents of glutamic acid and proline. However, the alcohol-soluble fraction I contains a greater proportion of these amino acids than does the alcohol-insoluble fraction. The alcohol-insoluble fraction, however, contains greater proportions of the basic amino acids lysine, histidine, and arginine. This is reflected in the high positive potential observed for this fraction (66.4 moles 100 Kg^{-1}) compared with the alcohol-soluble fraction I proteins (46.5 moles 100 Kg^{-1}). Both fractions contain a similar proportion of hydrophobic amino acids and this is confirmed in the average hydrophobicity values of 0.975 and 0.974 Kcal per residue for this parameter.

Peak I proteins of the alcohol-insoluble fraction show a marked reduction in glutamic acid; 17.11 g compared to 31.37 g in the original fraction. However, an increase in aspartic acid content was noted for peak I proteins. Increased contents of the basic amino acids histidine, arginine, and especially lysine was also observed. This resulted in the fraction having a high positive potential (96.9 moles 100 Kg^{-1}). This characteristic has prompted some authors to claim that these proteins resemble albumin (water soluble) and globulin (salt soluble) proteins in amino acid composition (Huebner et al. 1974). The higher frequency of charged groups per residue (0.20) for these proteins supports this view. In general, the peak I proteins contained a higher proportion of hydrophobic amino acids, especially leucine (9.51 g) and isoleucine

TABLE 10. Hydrophobicities and charge potentials of reduced-alkylated alcohol-insoluble protein and reduced-alkylated alcohol-soluble fraction I, and of peaks obtained from gel filtration chromatography on Sephadex G-200.[a]

	Alcohol-insoluble fraction				Alcohol-soluble fraction I			
	Reduced-alkylated	Peak I	Peak II	Peak III	Reduced-alkylated	Peak I	Peak II	Peak III
Average hydrophobicity (Kilocalories residue^{-1})	0.975	1.057	0.728	1.010	0.974	0.990	0.829	0.964
Amide groups[b] (moles 100 Kg^{-1})	234.7	127.6	250.6	246.5	262.9	248.2	277.1	270.6
Negative potential (moles 100 Kg^{-1})	243.8	188.9	296.9	290.4	292.0	269.3	303.2	299.2
Negative potential less amide groups (moles 100 Kg^{-1})	9.1	61.3	46.3	44.2	29.1	21.1	26.1	28.6
Positive potential (moles 100 Kg^{-1})	66.4	96.9	40.0	60.9	46.5	45.9	35.0	42.8
Positive to negative charge ratio	7.30	1.58	0.86	1.34	1.60	2.18	1.34	1.50
Frequency of charged groups (groups residue^{-1})	0.10	0.20	0.12	0.14	0.10	0.09	0.08	0.10

[a]Values correspond to the amino acid composition of fractions and peaks in Table 9.

[b]Estimated from ammonia content in amino acid analysis.

(4.5 g) than the original or the other two peak fractions. This was reflected in a higher value for average hydrophobicity (1.057 Kcal residue^{-1}).

Peak I proteins of the alcohol-soluble fraction I also contained a lesser proportion of glutamic acid, although the difference is not as great as that between the alcohol-insoluble peak I and the original alcohol-insoluble fraction. A concomitant increase in aspartic acid content was also observed in the alcohol-soluble fraction I (peak I). The high level of proline (11.74 g) was maintained in peak I compared to the total fraction (12.4 g). In contrast to the alcohol-insoluble peak I, no general increase in the levels of basic amino acids was observed on gel filtration of the alcohol-soluble fraction I. This resulted in a low positive charge potential (45.9 moles 100 Kg^{-1}) for this fraction, compared to peak I proteins of the alcohol-insoluble fraction (96.9 moles 100 Kg^{-1}). In addition, the high degree of amidization of glutamate and aspartate in this peak is reflected in lowered overall charge potentials and a low charge frequency per residue (0.09) compared to the alcohol-insoluble peak I (0.20). A small increase in the average hydrophobicity of this peak (0.990 Kcal) over the original fraction (0.974 Kcal) indicates an increase in the proportion of hydrophobic amino acids present. The increased hydrophobicity and the very low charge potentials may, in part, explain why these proteins are soluble in ethanolic solvent. The increased average hydrophobicity of the peak I proteins exhibited in both the alcohol-insoluble fraction and the alcohol-soluble fraction I suggests that hydrophobic bonding is probably involved in the aggregation of these proteins.

The amino acid compositions of the peak II proteins of both the

alcohol-insoluble and alcohol-soluble fraction I are similar. These proteins contain large proportions of glutamic acid and proline. A large increase, compared with original fractions and other peak fractions, in the glycine content of the peak II proteins is also notable. Peak II proteins also contain a lower proportion of basic amino acids, resulting in the lowest positive charge potentials of all the fractions studied. Peak II proteins have the lowest frequency of charged groups per residue (0.12 for the alcohol-insoluble fraction; 0.08 for the alcohol-soluble fraction I). The proportion of hydrophobic amino acids is lower in peak II proteins which results in these proteins having low values of average hydrophobicity. The average hydrophobicities of the peak II proteins of the alcohol-insoluble fraction and the alcohol-soluble fraction I are 0.728 and 0.829 Kcal residue^{-1}, respectively. This means that the chance of a hydrophobic residue occurring at the surface of a molecule of this protein is much less than the proteins of peak III or peak I fractions. Accordingly, it is speculated that the role of hydrophobic bonding in the structure of peak II proteins is less significant. Peak II proteins are interesting in that they consist of the four high molecular weight components as determined by SDS-PAGE. This is clearly illustrated in the patterns obtained with the reduced-alkylated proteins in Figure 11. The gel-filtration profiles (Figures 9 and 10) showed that there is no carbohydrate component associated with the peak II proteins. Given the high contents of glycine and proline in this fraction, it is reasonable to postulate that its secondary structure may contain β-sheets. Proline and glycine have a high propensity for reverse turns and thus would provide the necessary structural requirements of β-sheets and β-meanders (Schulz and Schirmer 1979). The glutamine residues would provide the high hydrogen bonding

potential required for stabilization of such structures. Glycine is generally found in higher levels in structural proteins. Whilst proline is known as a strong "helix breaker" (Lewis et al. 1970), tyrosine, which occurs in increased levels, is a known "helix-former" (Finkelstein and Ptitsyn 1976). Thus, there also exists the possibility of the presence of some α-helicity in peak II proteins. Of course, further evidence using techniques such as optical rotary dispersion, peptide sequence analysis, and perhaps X-ray crystallography would be required to confirm the presence of these structures.

The amino acid composition of the peak III proteins (Table 9) of the alcohol-insoluble fraction and the alcohol-soluble fraction I are similar. These proteins contain high levels of glutamic acid and proline as do the peak II proteins. The outstanding characteristic of these proteins is the very low glycine content. Compared with the peak II fraction, peak III proteins contain a higher proportion of hydrophobic amino acids. The average hydrophobicity of the peak III proteins for the alcohol-insoluble fraction and the alcohol-soluble fraction I is 1.010 and 0.964 Kcal residue^{-1}, respectively (Table 10). These proteins also possess very low charge potentials and low frequencies of charged groups per residue.

5. Comparison of Average Hydrophobicities and Charge Frequencies of Wheat Gluten Fractions and Proteins From Other Sources

Proteins from diverse sources, together with the average hydrophobicity and charge frequency values calculated from their respective amino acid compositions, are listed in Table 11. These values may be compared with those of the wheat protein fractions determined in this study in Tables 8 and 10. The values of average hydrophobicity calcu-

TABLE 11. Values of average hydrophobicities ($\mathbb{H}_{ave}$) and charge frequencies per residue for some proteins.

Protein	$H\mathbb{Q}_{ave}$ (Kcal residue^{-1})	Charge frequency (groups residue^{-1})
Fibroin (B. Mori)[a]	.480	0.03
(Tussah)[a]	.600	0.08
α-soluble collagen[a]	.940	0.16
Edestin (Hemp)[a]	.950	0.23
Wild rice[b]	.970	0.275
Pumpkin seed globulin[a]	.980	0.28
Glutenin (wheat)[c]	1.016	nd
Cultivated rice[b]	1.026	0.247
Wool (merino)[a]	1.030	0.16
Hemagglutin (soya)[a]	1.070	0.31
α-amylase (B. subtilis)[a]	1.070	0.25
Elastin (bovine aorta)[a]	1.080	0.045
Gliadin (wheat)[c]	1.109	nd
Leghaemoglobin (soya)[a]	1.150	0.25
Zein (maize)[a]	1.310	0.028

[a]Bigelow (1967).
[b]Watts (1980).
[c]Woychik et al. (1961).

lated for the protein fractions in this study may be slightly underestimated as tryptophan was not included in the calculation. The average hydrophobicities of proteins cover a wide range of values, however, most proteins fall within the range of 1.000 to 1.100 Kcal residue^{-1} (Bigelow 1967).

The alcohol-soluble proteins (gliadins) in this study possess high average hydrophobicities compared to most other proteins (e.g. peak fractions II and II; Table 8 gave values of 1.100 and 1.136 Kcal residue^{-1}, respectively). The peak II proteins (high molecular weight glutenin subunits) in Table 10 had a very low average hydrophobicity value (0.728 and 0.829 Kcal residue^{-1}) compared with other proteins. These values are similar to the low values of fibrous type proteins (e.g. edestin, 0.950 Kcal residue^{-1}). The charge frequencies (groups residue^{-1}) are also very low for wheat gluten proteins, similar to the values for the structural proteins fibroin (0.03 and 0.08), wool (0.16), and elastin (0.045). Most other proteins have charge frequencies of 0.2 and 0.35 groups per residue.

Bigelow (1967) pointed out that charge frequency and hydrophobicity are likely to be the two structural features that have the greatest influence on the solubility of a protein. Proteins with unusual solubility properties tend to occur with low values for one or both of these parameters. The low solubility of gluten proteins is certainly consistent with their low values of charge frequency.

6. Amino Acid Composition and the Presence of Carbohydrate

Peak I fraction of the reduced-alkylated alcohol-insoluble fraction and the reduced-alkylated alcohol-soluble fraction I both contain a significant carbohydrate component (Figures 9 and 10). The amino acid

composition of these fractions were examined closely for any enrichment of amino acid residues that are known to occur in covalent linkages between carbohydrate and protein as in glycoproteins. No abnormally high values were observed for the proportion of serine or threonine. However, the two peak I fractions contained a greater proportion of aspartic acid than the original preparations. The amide of aspartic acid is a common amino acid linked to N-acetyl glucosamine in the "high mannose" type of glycoproteins found in mammalian tissues. This class of glycoproteins is not widely distributed in plants, however.

One of the difficulties in assessing the amino acid data in this light, is the fact that the peak I proteins are quite heterogenous, as shown by the protein patterns obtained by SDS-PAGE. Therefore, given the low carbohydrate contents, it may be difficult to see any significant increases in select amino acids due to partial purification of a possible glycoprotein. The possibility of the existence of these linkages will be discussed in detail in the later part of this study.

Another important consideration relative to the presence of carbohydrate in the peak I proteins is the possible effect of this component on the hydrophobicity of the protein itself. These proteins have relatively high hydrophobicity (Tables 10 and 11) and it has been postulated that hydrophobic bonds may be involved in the aggregation of the proteins to give the peak I fractions. The average hydrophobicity value is a theoretical value reflecting the chance that a hydrophobic group may be located on the surface of a protein molecule. However, it is reasonable to assume that the tertiary protein structure itself can alter the frequency of hydrophobic residues on the surface of a molecule. Differences of this nature are used as a separation technique for many

proteins using a hydrophobic column matrix (Hofstee 1973). Caldwell (1979) effected a fractionation of gliadin components by hydrophobic interaction chromatography using octyl-Sepharose 4B as the matrix. The hydrophobic affinity of the gliadin proteins was quite high as aqueous ethanol was required to effect desorption of the protein from the matrix. However, a fraction consisting of about 25% of the applied protein was not bound to the matrix and eluted at the void volume of the column. When examined on PAGE, this fraction did not enter the gel. The protein fraction was dismissed by the author, in that it contained no gliadin bands and was "presumed to be low molecular weight glutenin or irreversibly aggregated gliadins". This fraction appears to correspond to the peak I fraction of the alcohol-soluble fraction of gluten in this study. This fraction is of high molecular weight (see gel filtration profile, Figure 4) and does not enter the gel on PAGE (Figure 5). Its average hydrophobicity is only 50 cal residue^{-1} less than that of the total gliadin fraction. Therefore, it is quite possible that the carbohydrate component associated with this fraction may influence the hydrophobicity of the surface of the molecule or cause a change in the tertiary structure of the protein itself.

Thus extreme care must be taken by researchers in the assessment of hydrophobicities of the gluten proteins and extending these to molecular (secondary and tertiary) structural level.

F. Secondary Structure of Wheat Gluten Proteins

Very little information is available on the types of secondary structures that wheat gluten proteins may possess. Wu et al. (1967), using optical rotary dispersion techniques, showed that glutenin proteins had only a small proportion of α-helical structure (10 to 15%) in

3 M urea. Other types of secondary structure could not be identified from the types of measurements carried out by Wu et al. (Kasarda et al. 1976).

Information on the secondary structures of gluten proteins has not been forthcoming for two main reasons. Firstly, the high insolubility properties of gluten proteins requires the use of strong dissociating solvents, which in all probability solubilize the proteins by changing the secondary (and other levels of) structure of the protein. Wheat gluten proteins, when solubilized, often form aggregates with molecular weights in the millions, even in the presence of dissociating solvents. The insolubility problem is akin to that of membrane proteins where Wasserman (1974) stated that the major problem is that;

> "no medium has yet been found to satisfy all three criteria; quantitative extraction of most proteins, reasonable stability in the medium, and a dispersion of molecules rather than aggregates".

Secondly, wheat gluten proteins are extremely heterogeneous. This heterogeneity makes protein purification very difficult, especially if extensive purification is required as for sequence analysis. The general lack of data on the primary structure of gluten proteins can be directly attributed to the poor solubility and the inherent heterogeneity of these proteins.

Consequently, no successful attempts to crystallize wheat gluten proteins have been reported. Therefore, there is no information on the structure of the proteins by X-ray crystallography. From 1970 to 1980, a number of theoretical methods were developed and tested for the estimation of secondary structure of proteins. These methods were

initially based on amino acid composition, however, now they are almost totally based on amino acid sequences. There have been no reports in the literature of the application of these methods to the estimation of secondary structure in wheat gluten proteins. Accordingly, the available predictive methods will be examined and one of the methods will be applied to a known N-terminal sequence of A_2-gliadin protein. This part of the study was undertaken so as to provide some information on the possible secondary structures of gluten proteins. The information obtained on the association of carbohydrate with gluten protein may, therefore, be interpreted more reasonably by taking information on secondary structure of the proteins into account.

1. Prediction of Secondary Protein Structures from Amino Acid Composition and Amino Acid Sequence

a. Alpha-helix and β-sheet structures. Many schemes for the prediction of protein structure have been proposed in recent years. These all require a semi-empirical evaluation of the involvement or association of certain amino acids with secondary structures. A large number of crystalline proteins have been examined with the goal of determining conformational preferences for the various amino acids (Lewis et al. 1971; Chou and Fasman 1974a, b; Crawford et al. 1973; Beghin and Dirkx 1975). In the present study, the method of Chou and Fasman (1974a, b) for quantifying protein conformational preferences will be examined.

The data given for each amino acid residue in Table 12 was derived from the analysis of the crystal structures of 29 proteins. The values were derived as follows. The fractional occurrence (f) of each residue in a given conformational structure, for example α-helix, (f_α) is determined. This parameter is defined as:

TABLE 12. Conformational parameters and classification of amino acid residues as formers, breakers or indifferent for α-helical and β-sheet regions.[a]

Helical residues	P_α[b]	Class[c]	β-sheet residues	P_β[b]	Class[c]	β-turn residues	P_T[b]
Glutamic acid	1.51	H	Valine	1.70	H	Asparagine	1.56
Methionine	1.45	H	Isoleucine	1.60	H	Glycine	1.56
Alanine	1.42	H	Tyrosine	1.47	H	Proline	1.52
Leucine	1.21	H	Phenylalanine	1.38	h	Aspartic acid	1.46
Lysine	1.16	h	Tryptophan	1.37	h	Serine	1.43
Phenylalanine	1.13	h	Leucine	1.30	h	Cysteine	1.19
Glutamine	1.11	h	Cysteine	1.19	h	Tyrosine	1.14
Tryptophan	1.08	h	Threonine	1.19	h	Lysine	1.01
Isoleucine	1.08	h	Glutamine	1.10	h	Glutamine	0.98
Valine	1.06	h	Methionine	1.05	h	Threonine	0.96
Aspartic acid	1.01	I	Arginine	0.93	i	Tryptophan	0.96
Histidine	1.00	I	Asparagine	0.89	i	Arginine	0.95
Arginine	0.98	i	Histidine	0.87	i	Histidine	0.95
Threonine	0.83	i	Alanine	0.83	i	Glutamic acid	0.74
Serine	0.77	i	Serine	0.75	b	Alanine	0.66
Cysteine	0.70	i	Glycine	0.75	b	Methionine	0.60
Tyrosine	0.69	b	Lysine	0.74	b	Phenylalanine	0.60
Asparagine	0.67	b	Proline	0.55	B	Leucine	0.59
Proline	0.57	B	Aspartic acid	0.54	B	Valine	0.50
Glycine	0.57	B	Glutamic acid	0.37	B	Isoleucine	0.47

[a]Data from Chou and Fasman (1978a, b).

[b]Conformational parameters: for definitions see text.

[c]Classification: H = strong fromer; h = former; I = weak former; i = indifferent; b = breaker; B = strong breaker.

$$f_{\alpha} = \frac{n_{\alpha}}{n}$$

where n = total number of residues of a given kind in the 15 proteins

n_{α} = the number of these residues that are located in the α-helical sections.

From f_{α}, a conformational parameter (P_{α}) can be determined for each residue. P_{α} is defined as:

$$P_{\alpha} = \frac{f_{\alpha}}{f_{\alpha\,ave}}$$

where $f_{\alpha\,ave}$ is the average value of f_{α} (that is, the sum of the f_{α} values divided by 20, the number of different residues).

Hence, when $P_{\alpha} = 1.0$ for a residue, then that residue adopts the α-helical conformation at the same frequency as the average residue in the polypeptide chain. When $P_{\alpha} > 1.0$, there is a greater than average chance for the residue to adopt the α-helical conformation. A value of $P_{\alpha} < 1.0$ means a less than average frequency of occurrence of α-helical conformation for that residue.

The concept that the end of helixes for example, are occupied on the average, by residues with "helix breaking" tendencies, and that these residues act to prevent further helix growth may be introduced. In this case, the average conformational parameter ($P_{\alpha\,ave}$) of this cluster of residues is less than 1.0 ($P_{\alpha\,ave} < 1.00$). Conversely, the centres of helixes are occupied by residues of greater than average helix forming tendency ($P_{\alpha\,ave} > 1.0$).

Similar analyses have shown that residues with $P_{\beta\,ave}$ values below unity tend to occur at the boundaries of β-sheet regions and those with relatively high $P_{\beta\,ave}$ values occur within the inner sheet region.

To aid in developing procedures for predicting secondary structures,

each residue may be classified according to its α-helix or β-sheet "forming" or "breaking" tendency based on the conformational parameters given in Table 12. Each residue may be further classified as a strong helix former, helix former, helix breaker, indifferent conformational preference, strong β-sheet former, β-sheet former, or β-sheet breaker. This then allows for the prediction of secondary structures upon examination of amino acid composition and primary structure. Various rules have been developed in applying this procedure (Chou and Fasman (1978a, b). For example, a cluster of three β-formers (H or h) out of five residues along the protein sequence will nucleate a β-sheet. The β-sheet is extended in both directions until β-tetrapeptide breakers with $P_{\beta\,ave} < 1.0$ are reached. Any segment with $P_{\beta\,ave} \geq 1.05$, as well as $P_{\beta\,ave} > P_{\alpha\,ave}$ is predicted as a β-sheet (Chou and Fasman 1974b).

Considering the conformational parameters for the amino acid residues given in Table 12 and the amino acid composition of wheat gluten proteins, it is not surprising that the secondary and tertiary structures have remained an enigma to the cereal chemist. The cereal chemists have generally regarded that the high levels of proline in wheat endosperm protein disrupt secondary protein structure (Ewart 1967). However, from Table 12 it can be seen that glycine is a strong helix breaker, whilst asparagine and tyrosine would be moderate helix breakers. Thus, the levels of these amino acids in most wheat protein fractions certainly explains the low degree of α-helicity observed in the optical rotary dispersion studies of gliadin and glutenin by Wu and Cluskey (1965). Proline and glycine, however, are found in high proportion in structural proteins such as collagen. Collagen consists of a tightly wound triple helix consisting of interchain hydrogen bonds. The proline

rings are oriented towards the outside and glycine residues, because of their small size and the steric constraints involved, occur every third residue in a repeating sequence on the inside.

Glutamic acid, alanine, methionine, and leucine are regarded as strong helix formers. These amino acids are in relatively low proportion in wheat gluten proteins. The low proportion of glutamic acid in gluten proteins is due to the high degree of amidization of the amino acid. Glutamine is classified as only a weak α-helix former (see Table 12). The alcohol-insoluble and the alcohol-soluble fractions (see Table 7) contain similar amounts of strong helix breakers (i.e. glycine + proline). However, the alcohol-soluble peak II proteins contain 21.3 grams of strong helix breaker and slightly less of the strong helix formers, leucine, methionine, and alanine. Alpha-helical configurations could not be predicted for these gliadin proteins.

The conformational parameters (P_β) for residues to occur in β-sheet structure are also given in Table 12. Valine, isoleucine, and tyrosine are strong β-sheet formers, whereas the other hydrophobic amino acids, phenylalanine, tryptophan, tyrosine, leucine, and methionine are regarded as β-sheet formers. Proline, aspartic acid, and glutamic acid are regarded as strong β-sheet breakers. The high proportion of hydrophobic amino acids in wheat gluten proteins and the relatively high values of average hydrophobicity may well make β-sheets a possibility in the secondary structure of these proteins. Two other pieces of information are relevant here. Firstly, polypeptides in a β-sheet conformation have generally been found to be insoluble, a well known characteristic of wheat proteins. In fact, the sparsity of information on β-formers in proteins has been attributed to this factor

(Chou and Fasman 1978a). Secondly, with increasing structural data from X-ray crystallography studies on proteins, it has become more apparent that β-sheets play as essential a role as α-helices in the spatial architecture of proteins (Chou and Fasman 1978a). The hydrogen-bonding residues involved in β-sheets have now been shown to outnumber helices in ribonuclease (Richards and Wychoff 1971), α-chymotrypsin (Birktoft and Blow 1972), elastase (Shotton and Watson 1970), concanavalin A (Reeke et al. 1975), and the immunoglobulins (Poljak 1975). Thus, the high levels of glutamine conferring the large hydrogen bonding potentials in gluten proteins would be suitable for the stabilization of β-sheet secondary structure. Finally, charged residues are frequently found at the helix boundaries of proteins. However, they are conspicuously absent at the β-sheet boundary regions. In addition, charged residues are not favoured in the central β region of β-sheets. The low charge potential exhibited by wheat gluten proteins would, therefore, suit a β-sheet secondary structure.

b. Beta-turns as secondary structures. In structural chemistry of proteins, β-turns are also referred to as β-bends hairpin loops, reverse turns, and 3_{10} bends. A β-turn structure consists of four consecutive amino acid residues in a protein where the polypeptide chain folds back on itself by nearly 180°. Venkatachalam (1968), whilst searching for favourable conformations of three consecutive peptide units (C^{i} to C^{i+3}) found three arrangements with a characteristic hydrogen bond between O_i and N_{i+3}. The author termed these arrangements reverse turns or β-bends I, II, and III. Lewis et al. (1973) defined reverse turns as those quartets of amino acid residues i, $i+1$, $i+2$, and $i+3$, in which the distance between the C_α-atoms in positions

i and $i + 3$ is less than 7 Å and the chain is not in α-helical conformation.

The fractional occurrence of amino acid residues in the structures and the values of the conformational parameter (P_t) have been calculated on a similar basis to the α-helix and β-sheet parameters. The conformational parameters of Chou and Fasman (1978a) based on a data base for 29 proteins was given in Table 12. These values allow for the accurate prediction of chain reversal and tertiary folding in proteins. Residues with the highest potential for β-turns, considering all four positions, are asparagine, glycine, proline, aspartic acid, and serine ($P_t > 1.4$). It is interesting to note that the α-helix breaking residues (proline, glycine, and asparagine) with P_α values less than 0.70 are the strong β-turn formers with $P_t > 1.5$.

Researchers have also considered the option that there may be positional preferences for certain amino acid residues in the four positions that constitute a β-turn. Lewis *et al.* (1971) derived frequencies for each type of residue at each position in the turn, i, $i + 1$, $i + 2$, and $i + 3$. These frequencies were defined as the "propensity of the residue type to occur at the particular position". These frequencies were updated as the X-ray crystallography data base was expanded (Crawford *et al.* 1973; Chou and Fasman 1974b). Chou and Fasman (1978a, b) studied the positional preferences of amino acid residues based on 408 β-turns. The frequency values for a particular amino acid occurring at a particular position i or $i + 1$ or $i + 2$, or $i + 3$ in the tetrapeptide are given in Table 13.

Proline is a prominent amino acid in the β-turn secondary structure, occurring almost exclusively at position $i + 1$. Glycine occurs

TABLE 13. Fractional occurrence (bend frequency) of amino acid residues in the four positions i, $i + 1$, $i + 2$ and $i + 3$ of the β turn.[a]

Residues at position i	f_i[b]	Residues at position $i + 1$	f_{i+1}[b]	Residues at position $i + 2$	f_{i+2}[b]	Residues at position $i + 3$	f_{i+3}[b]
Asparagine	0.161	Proline	0.301	Asparagine	0.191	Tryptophan	0.167
Cysteine	0.149	Serine	0.139	Glycine	0.190	Glycine	0.152
Aspartic acid	0.147	Lysine	0.115	Aspartic acid	0.179	Cysteine	0.128
Histidine	0.140	Aspartic acid	0.110	Serine	0.125	Tyrosine	0.125
Serine	0.120	Threonine	0.108	Cysteine	0.117	Serine	0.106
Proline	0.102	Arginine	0.106	Tyrosine	0.114	Glutamine	0.098
Glycine	0.102	Glutamine	0.098	Arginine	0.099	Lysine	0.095
Threonine	0.086	Glycine	0.085	Histidine	0.093	Asparagine	0.091
Tyrosine	0.082	Asparagine	0.083	Glutamine	0.077	Arginine	0.085
Tryptophan	0.077	Methionine	0.082	Lysine	0.072	Aspartic acid	0.081
Glutamine	0.074	Alanine	0.076	Threonine	0.065	Threonine	0.079
Arginine	0.070	Tyrosine	0.065	Phenylalanine	0.065	Leucine	0.070
Methionine	0.068	Glutamic acid	0.060	Tryptophan	0.064	Proline	0.068
Valine	0.062	Cysteine	0.053	Glutamine	0.037	Phenylalanine	0.065
Leucine	0.061	Valine	0.048	Leucine	0.036	Glutamic acid	0.064
Alanine	0.060	Histidine	0.047	Alanine	0.035	Alanine	0.058
Phenylalanine	0.059	Phenylalanine	0.041	Proline	0.034	Isoleucine	0.056
Glutamine	0.056	Isoleucine	0.034	Valine	0.028	Methionine	0.055
Lysine	0.055	Leucine	0.025	Methionine	0.014	Histidine	0.054
Isoleucine	0.043	Tryptophan	0.013	Isoleucine	0.013	Valine	0.053

[a]From the data of Chou and Fasman (1978a, b).
[b]Fractional occurrence - for definition see text.

frequently at position $\underline{i}$ + 2 and $\underline{i}$ + 3, but not at $\underline{i}$ or $\underline{i}$ + 1. Tryptophan is predominant at position $\underline{i}$ + 3 but not at $\underline{i}$ + 1, $\underline{i}$, or $\underline{i}$ + 2. Cysteine and histidine occur frequently at $\underline{i}$, but not at $\underline{i}$ + 1. The positional preference of amino acid residues in β-turns is generally attributed to sterochemical considerations that make certain residues more energetically stable at specific positions in the β-turn (Chou and Fasman 1978b). Beta-turns do contain a reasonable frequency of charged or hydrophillic amino acids. It is not surprising, therefore, that in a survey of the location of β-turns in proteins they were found to be concentrated at the protein surface (Kuntz 1972). Conversely, most hydrophobic, β-sheet forming amino acid residues, for example valine, isoleucine, and leucine are not found in the β-turn structure. Chou and Fasman (1978a) note that whilst these residues show the lowest bend potential, they are often found in regions just beyond the β-turns. An environmental analysis of β-turn neighbouring residues shows that reverse chain folding is stabilized by anti-parallel β-sheet regions (Chou and Fasman 1977).

Considering the amino acid composition of wheat gluten proteins, the high levels of proline and glycine in some fractions and the low levels of the charged amino acids, it would appear that these proteins would be good candidates for β-turn secondary structures. The high levels of the hydrophobic amino acids would also provide the required β- sheet secondary structure for the stabilization of the chain reversal regions. In fact, the apparent predisposition of the gluten proteins, due to their amino acid compositions, for forming β-sheet and β- turn secondary structures may explain some of the observed properties of these proteins. For example, this type of structure is generally

regarded to confer insolubility to proteins. Conformational changes have been observed in preparations of A-gliadin under different conditions. These include reversible changes from compactly folded structures to random coils and fibrillar aggregates. The reversibility of these conformational states indicates that the aggregation mechanism involves only secondary forces (Kasarda et al. 1974a). The compactly folded conformation would certainly require β-turn regions in the secondary structure of the protein. In all probability, these regions are absolutely necessary in the folding and unfolding mechanism itself.

2. Prediction of the Secondary Structure of A_2 Gliadin from Amino Acid Composition and Amino Acid Sequence

a. Prediction of α-helix and β-sheet structure. In order to test some of the hypotheses presented in the two previous sections, the possible secondary structures of A_2 gliadin were examined using the semi-empirical methods of Chou and Fasman (1978a,b). Using the conformational parameters, based on 29 proteins, Chou and Fasman formulated the following empirical rules to elucidate the secondary structural regions of proteins.

i. A cluster of four helical residues (either H or h residues) out of six residues along the protein sequence will nucleate a helix, with weak helical forming residues (I_α) counting as 0.5 h . The helical segment is extended in both directions until α-tetrapeptide breakers with $P_{\alpha ave} < 1.00$ are reached (e.g. b_4, b_3i, b_2i_2, b_3h). Proline cannot occur in the inner helix or at the C-terminal end. Proline, aspartic acid, glutamic acid, and histidine,

lysine, arginine are incorporated, respectively at the N- and C-terminal helical ends. Any segment with P_{α} ave $\geq$ 1.03, as well as P_{α} ave > P_{β} ave is predicted as helical.

ii. A cluster of three β-formers (H or h) out of five residues along the protein sequence will nucleate a β-sheet. The β-sheet is extended in both directions until β-tetrapeptide breakers with P_{β} ave < 1.00 are reached. Any segment with P_{β} ave $\geq$ 1.05 as well as P_{β} ave > P_{α} ave is predicted as β-sheet.

iii. When regions in proteins contain both α- and β-forming residues, the overlapping region is helical if P_{α} ave > P_{β} ave, or β-sheet if P_{β} ave > P_{α} ave. The helix and β-sheet boundary frequency tables are also used to delineate whether the region is α or β (Chou and Fasman 1978b).

The prediction methods have been shown to be very accurate in determining secondary structure. Using the conformational parameters calculated from 15 data base proteins, Chou and Fasman (1974b) demonstrated a high success rate for predicting the structures of trypsin inhibitor, thermolysin, lamprey hemoglobin, and concanavilin A. A number of predictive methods were compared on adenylate kinase, where investigators were invited to submit their predictions based on a known amino acid sequence, prior to any knowledge of the X-ray crystallography results (Schulz et al. 1974). The overall helical predictions were in good agreement with the experimental data. The Chou and Fasman method correctly

localized nine of 10 helices. Three of the five central β-stranded parallel β-pleated sheet structures were correctly identified and seven of the 10 were predicted to within $\pm$ 1 residue. Seven of the 10 β-bend regions were also localized.

Figure 12 shows the primary amino acid sequence of A_2-gliadin, taken through 25 residues from the N-terminal end (Kasarda et al. 1974b). Using the method of Chou and Fasman (1974b), the amino acids were classified according to their α-helix and β-sheet forming or breaking tendencies, the conformational parameters listed, and the average conformational parameters calculated for groups of five amino acid residues. In order to compare the calculated $P_{\alpha\,ave}$ and $P_{\beta\,ave}$ values in Figure 12, the $P_{\alpha\,ave}$ and $P_{\beta\,ave}$ values obtained in the 80 helical and 64 β regions of the 15 proteins studied by Chou and Fasman (1974a) are listed.

$P_{\alpha\,ave}$ = 1.08 for all helical segments.

$P_{\beta\,ave}$ = 1.11 for all β segments.

$P_{\alpha\,ave\ N}$ = 1.04 for residues at N-terminal of helix.

$P_{\alpha\,ave\ I}$ = 1.13 for residues at the internal helix.

$P_{\alpha\,ave\ C}$ = 1.09 for residues at C-terminal of helix.

$P_{\beta\,ave\ N}$ = 1.17 for residues at N-terminal boundary region.

$P_{\beta\,ave\ I}$ = 1.17 for residues at central sheet region.

$P_{\beta\,ave\ C}$ = 1.11 for residues at C-terminal boundary.

$P_{\beta\,ave\ nC}$ = 0.94 for residues at C-terminal non β-region.

Considering the first five N-terminal residues of A_2-gliadin in Figure 12, an α-helix structure can be clearly dismissed as the $P_{\alpha\,ave}$

Figure 12. N-terminal 25 residue sequence of A_2-gliadin with average conformational parameters for prediction of α-helix and β-sheet structure for five pentapeptides.

Class:

H = strong former
h = weak former
i = indifferent
b = breaker
B = strong breaker

P_{α} - Conformational parameter for α-helix structure

$P_{\alpha ave}$ - Average conformational parameter for α-helix structure

P_{β} - Conformational parameter for β-sheet structure

$P_{\beta ave}$ - Average conformational parameter for β-sheet structure

Residue number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	H_2N	VAL	- ARG	- VAL	- PRO	- VAL	- PRO	- GLN	- LEU	- GLN	- PRO	- GLN	- ASN	- PRO	- SER	- GLN
Class		h	i	h	B	h	B	h	H	h	B	h	b	B	i	h
P_α		1.06	0.98	1.06	0.57	1.06	0.57	1.11	1.21	1.11	0.57	1.11	0.67	0.57	0.77	1.11
$P_{\alpha ave}$				0.95					0.92					0.85		
Class		H	i	H	B	H	B	h	h	h	B	h	i	B	b	h
P_β		1.70	0.93	1.70	0.55	1.70	0.55	1.10	1.30	1.10	0.55	1.10	0.89	0.55	0.75	1.10
$P_{\beta ave}$				1.32					0.92					0.87		

Residue number	16	17	18	19	20	21	22	23	24	25
	GLN	- GLN	- PRO	- GLN	- GLU	- GLN	- VAL	- PRO	- LEU	- VAL -
Class	h	h	B	h	H	h	h	B	H	h
P_α	1.11	1.11	0.57	1.11	1.51	1.11	1.06	0.57	1.21	1.06
$P_{\alpha ave}$			1.08					1.00		
Class	h	h	B	h	B	h	H	B	h	H
P_β	1.10	1.10	0.55	1.10	0.37	1.10	1.70	0.55	1.30	1.70
$P_{\beta ave}$			0.84					1.27		

= 0.95 (i.e. $P_{\alpha\,ave} < 1.03$, the required value) and $P_{\alpha\,ave} < P_{\beta\,ave}$. Also, proline, the sixth residue, is in the C-terminal position of the segment and also occurs in the middle of the segment at position 3. However, three of the five residues are strong β-sheet formers giving a very high average β-conformational parameter value of 1.32. Thus, it can be confidently predicted that this segment forms a β-sheet structure.

It is more difficult to assign a secondary structure to the second segment (residues 6 to 10). Two strong breakers for helices and sheets are located at position 6 and 10. For this segment $P_{\alpha\,ave} = 0.92 = P_{\beta\,ave}$. Alpha-helix can be virtually ruled out because of the proline residue at the C-terminal side of the helix initiator leucine at the central position 8. Considering that the first five residues are in β-sheet conformation and that a considerable drop in $P_{\beta\,ave\ nC}$ values occurs at the β-sheet boundary (see above $P_{\beta\,ave\ nC} = 0.94$), then it is reasonable to assign a β-sheet boundary region to these residues.

The third segment (residues 11 to 15) is unique. The conformational parameters are too low for both α-helix and β-sheet formation ($P_{\alpha\,ave}$ = 0.85, $P_{\beta\,ave} = 0.87$). Closer inspection of this segment shows a typical sequence for a chain reversal. Asparagine, proline, serine, and glutamine occur in the four most favourable positions: $\underline{i}$, $\underline{i} + 1$, $\underline{i} + 2$, and $\underline{i} + 3$ for a β-turn structure (refer to Table 13). The possibility of this segment forming a β-turn structure will be considered in some detail in the following section.

The fifth segment (residues 21 to 25) contains four residues out of five that are β-formers, two of which are strong β-formers. The $P_{\beta\,ave}$ value of 1.27 is greater than the $P_{\alpha\,ave}$ value (1.0) for this segment. The possibility of α-helix structure is eliminated as proline (position

23) cannot occur in a central position of the helix. Thus, this segment must be in a β-conformation. This assignment may aid in determining the conformation of the preceding fourth segment.

The fourth segment (residues 16 to 20) contains four helix formers out of the five residues with proline (the strong helix breaker) located centrally at position 18. Proline also occurs as the third C-terminal residue from the strong helix initiator, glutamic acid located at position 20. With proline located at these positions, α-helical structure must be dismissed even though the value of $P_{\alpha\,ave}$ is 1.08 for this segment. From a β-structure point of view, this segment consists of two strong breakers and three β-formers. From the prediction rules, it is possible that a β-sheet could be nucleated in this segment. The $P_{\beta\,ave}$ of this segment provides a problem as it is extremely low for sheet structure (0.84). Possibly the segment could be classified as an extreme boundary region (N-terminal non β-region) leading into the strong β-sheet forming residues of the fifth segment. This assignment would accommodate the low $P_{\beta\,ave}$ for the segment.

b. Prediction of β-turn structure from amino acid sequence. Chou and Fasman (1978a, b) have also developed a method for the prediction of β-turns in secondary structure based on the fractional occurrence (f) of amino acid residues at the four β-turn positions $\underline{i}$, $\underline{i}$ + 1, $\underline{i}$ + 2, and $\underline{i}$ + 3. The fractional occurrence (f) is defined as:

$$f = \frac{n_i}{n_{total}}$$

where n_i = number of particular residues that occur at position $\underline{i}$ in the 408 β-turns from the 29 proteins studied.

n_{total} = total number of the particular residues that occur in the 29 proteins studied.

The fractional occurrence values for all the amino acids at the four positions of the β-turn is given in Table 13. As discussed previously, certain amino acids have strong positional preferences in the β-turn structure.

The probability (p_t) of a β-turn occurring at residue $\underline{i}$ is calculated from:

$$p_t = f_{\underline{i}} \times f_{\underline{i}+1} \times f_{\underline{i}+2} \times f_{\underline{i}+3}$$

The average probability ($p_{t\ ave}$) of β-turn occurrence, based on analysis of 408 β-turns was found to be 0.55×10^{-4} (Chou and Fasman 1977). For a β-turn to be predicted, the method requires that the following three criteria are fulfilled by a given tetrapeptide:

i. p_t value $> 0.75 \times 10^{-4}$ (i.e. $1.5 \times p_{t\ ave}$).

ii. $P_{t\ ave} > 1.00$ ($P_{t\ ave}$ = average conformational turn parameter for the tetrapeptide).

iii. $P_{\alpha\ ave} < P_{t\ ave} > P_{\beta\ ave}$.

Using this method, Chou and Fasman (1977) correctly predicted 70% of the bend and non-bend residues in 29 proteins and 78% of the β-turns were located correctly within $\pm$ two amino acid residues.

The amino acid sequence of A_2-gliadin shown in Figure 12 was examined for the presence of potential β-turn regions. The following procedure was adopted. The six proline residues in the sequence (at positions 4, 6, 10, 13, 18, and 23) were placed in position $\underline{i} + 1$. The fractional occurrence of proline in a β-turn in this position is very high (0.301 from Table 13). This high value means that proline almost exclusively occupies this position in a β-turn. By using this procedure, the possibility of locating a β-turn ($p_t > 0.75 \times 10^{-4}$) is maximized as the calculated p_t is also maximized. For the six tetrapeptides in

the sequence with proline at position $\underline{i}$ + 1, the probability of turn occurrence (p_t), the average conformational turn parameter ($P_{t\ ave}$), $P_{\alpha ave}$, and $P_{\beta ave}$ were calculated. The values obtained are given in Table 14. The first tetrapeptide (positions 3, 4, 5, and 6 in the sequence) cannot be involved in a β-turn secondary structure. The p_t value is well below the $p_{t\ ave}$ of 0.55×10^{-4} and the value 0.75×10^{-4} required for β-turn prediction. The value of $P_{t\ ave}$ is also less than the value of $P_{\beta ave}$ for the tetrapeptide. This conclusion is consistent with the previous prediction that these residues form a β-sheet structure. For the second tetrapeptide (positions 5, 6, 7, and 8 in the sequence), none of the required criteria is fulfilled for prediction of a β-turn structure.

As the examination passes through the predicted β-sheet structure, the third tetrapeptide (at positions 9, 10, 11, and 12 in the sequence) becomes an interesting point. The p_t value is 0.75×10^{-4}, a borderline value for β-turn prediction. The $P_{t\ ave}$ is $>$ 1.00 and $P_{\alpha ave} < P_{t\ ave} > P_{\beta ave}$. Thus, the basic conditions are fulfilled for the prediction of a β-turn structure for these residues. However, before assigning a definite β-turn structure to this tetrapeptide, the next tetrapeptide must be examined.

The fourth tetrapeptide comprises residues at positions 12, 13, 14, and 15 in the sequence. Asparagine occupies position $\underline{i}$ in the tetrapeptide and from Table 13 the f value for asparagine to occur in this position is 0.161, the highest of any amino acid. Similarly, proline at $\underline{i}$ + 1 occurs most frequently at this position in a β-turn. Serine occupies position $\underline{i}$ + 2 and also has a high f value (0.125) for occurring in that position. Glutamine in position $\underline{i}$ + 3 has a moderately

TABLE 14. Prediction of β-turn structure in A_2 gliadin[a] by the method of Chou and Fasman (1978a).

Tetrapeptide	P_{tave}[b]	$P_{\alpha ave}$[b]	$P_{\beta ave}$[b]	P_t[c]	β-turn
3, 4, 5, 6[d]	1.01	0.85	1.13	0.36×10^{-4}	NO
5, 6, 7, 8[d]	0.90	0.99	1.16	0.48×10^{-4}	NO
9, 10, 11, 12[d]	1.26	0.86	0.91	0.75×10^{-4}	(?) NO
12, 13, 14, 15[d]	1.37	0.78	0.82	5.96×10^{-4}	YES
17, 18, 19, 20[d]	1.06	1.08	0.78	0.52×10^{-4}	NO
22, 23, 24, 25[d]	0.78	0.98	1.31	0.42×10^{-4}	NO
8, 9, 10, 11 [e]	1.02	1.00	1.01	0.20×10^{-4}	NO

[a] Sequence determined by Kasarda *et al.* (1974b).

[b] Average conformational parameter (β-turn, α-helix, β-sheet) for tetrapeptide obtained from the conformational parameter of amino acid residues in Table 12.

[c] Probability of β-turn occurrence at residue i. $p_t = f_i \times f_{i+1} \times f_{i+2} \times f_{i+3}$; bend frequencies calculated from data in Table 13.

[d] Amino acids in tetrapeptide from sequence data (Figure 12) in positions i, $i + 1$, $i + 2$, $i + 3$; proline located at position $i + 1$.

[e] As above, except proline located at position $i + 2$.

high f value (0.098) for occurring in this position in a β-turn. The p_t value for the tetrapeptide is 5.96×10^{-4} which is eight times the required value of 0.75×10^{-4} for β-turn prediction. The second criterion for prediction is also fulfilled as $P_{t\ ave} = 1.37$ is > 1.00. The third criterion is also fulfilled as $P_{\alpha\ ave} < P_{t\ ave} > P_{\beta\ ave}$. Thus, this tetrapeptide can be confidently predicted as β-turn secondary structure. Again, this is consistent with the very low α-helix and β-sheet parameters for the segment comprising residues 11 through 15 (see previous section).

Because the third and the fourth segment overlap by one amino acid residue, the third tetrapeptide must be reconsidered. The $\underline{i} + 3$ residue (asparagine) of the third tetrapeptide is the $\underline{i}$th residue of the fourth tetrapeptide (i.e. overlapping occurs). By definition, a β-turn consists of four consecutive amino acid residues. Therefore, having clearly assigned the (fourth) tetrapeptide (residues 12, 13, 14, and 15) as the β-turn, then the third segment (9, 10, 11, and 12) cannot be assigned this structure. To examine this further, the third segment was moved back by one amino acid residue towards the N-terminal end to accommodate the β-turn of the fourth tetrapeptide. The new tetrapeptide comprises residues 8, 9, 10, and 11 in the sequence, proline being now in position $\underline{i} + 2$ in the peptide. From Table 15 it can be seen that the tetrapeptide cannot have a β-turn structure ($p_t = 0.20 \times 10^{-4}$).

The fifth tetrapeptide (residues 17, 18, 19, and 20) and the sixth tetrapeptide (residues 22, 23, 24, and 25) have low p_t values (0.52×10^{-4} and 0.42×10^{-4}, respectively) and cannot be predicted as having a β-turn secondary structure.

Thus, one β-turn structure is clearly predictable in the first N-

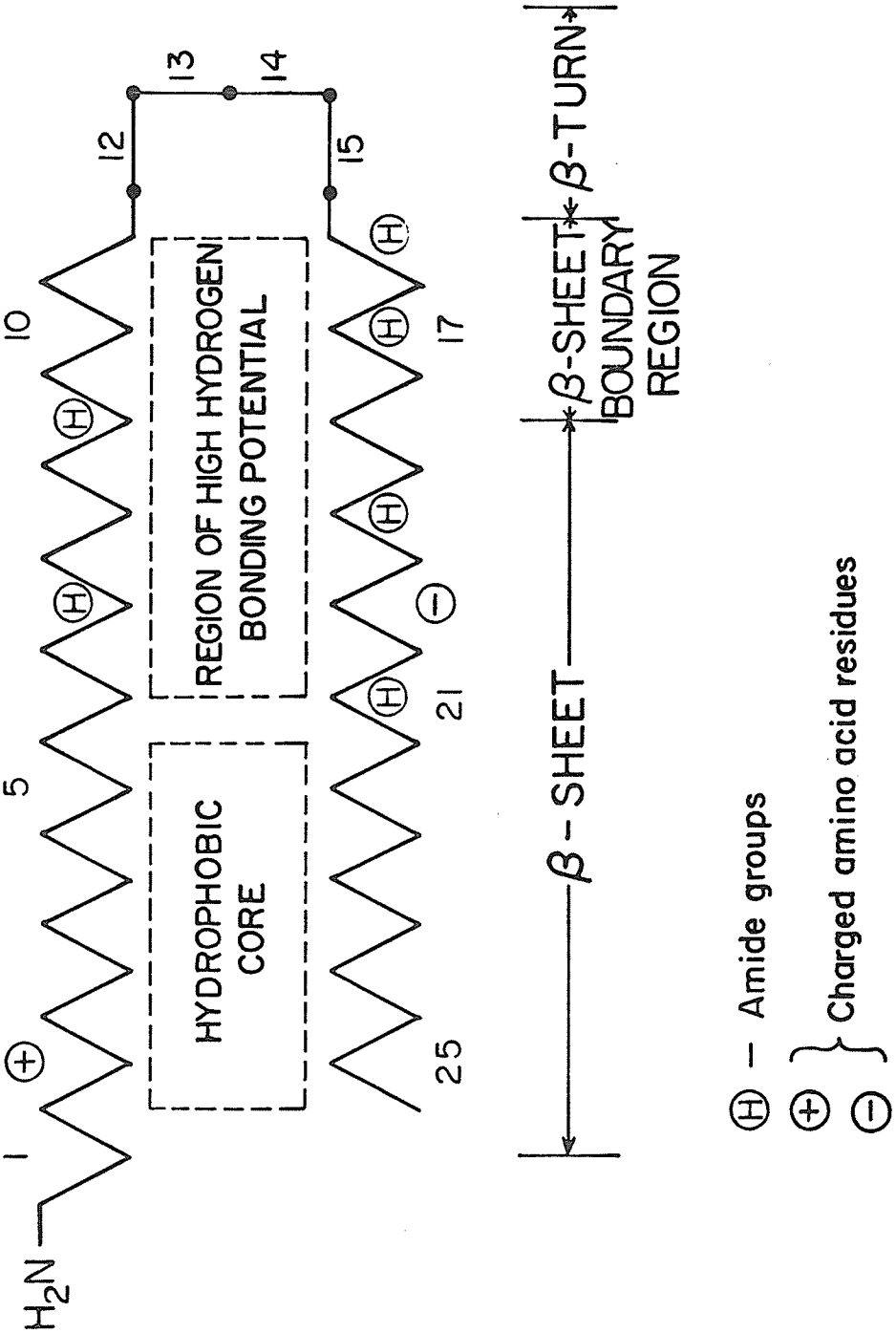
terminal 25 residues of the amino acid sequence of A_2-gliadin. Moreover, the β-turn tetrapeptide occurs between two regions of predicted β-sheet structure - a common location for chain reversals in proteins with known β-sheet secondary structure.

c. Secondary structure of A_2-gliadin. On the basis of the considerations in the previous sections, a secondary structure for the 25 N-terminal residues of A_2-gliadin can now be predicted as follows.

Residue numbers from N-terminal of the polypeptide	Assigned secondary structure
1 to 8	β-sheet.
9 to 11	β-sheet C-terminal boundary region.
12 to 15	β-turn.
16 to 18	β-sheet N-terminal boundary region.
19 to 25	β-sheet.

The predicted conformational structure for A_2-gliadin is shown pictorially in Figure 13. Clearly, the sheeted structure is stabilized by hydrogen bonding; glutamine is present at positions 16, 17, 19, 21, and positions 7 and 9. Hydrogen bonding across the β-sheet structure is a common feature in the most of the structures studied. This inter-sheet H-bonding also stabilized the β-turn structure (residues 12 to 15). Another interesting feature of the model in Figure 13 is that residues 3 to 6 and residues 22 to 25 can be in close proximity in the three-dimensional structure. These eight residues consist of proline, valine, and leucine, which are very hydrophobic (for average residue hydro-

Figure 13. A schematic diagram of the predicted secondary structure for A_2-gliadin.


H2N
1
5
10
12
13
14
15
17
21
25
HYDROPHOBIC CORE
REGION OF HIGH HYDROGEN BONDING POTENTIAL
β-SHEET
β-SHEET BOUNDARY REGION
β-TURN
H — Amide groups
+ − Charged amino acid residues

phobicity see Table 6). Thus, it is reasonable to expect the formation of a "hydrophobic core" in this region of the molecule, especially if the ionic strength of the solvent is increased slightly; A_2-gliadin is known to form fibrillar aggregates as ionic strength is increased (Kasarda et al. 1967). This change in structure may well be brought about by the "clumping together" of hydrophobic residues in areas of the structure described. The schematic diagram of the conformation of A_2-gliadin shows that the structure has the potential to be quite flexible. The reversible aggregation to fibrils can be explained by the intermediate energy of the hydrophobic interactions and the hydrogen bonding. The compactly folded structure at low ionic strength is certainly due to the β-structure of the sheets and turns. Finally, the unfolded structure in 8 M urea is almost certainly due to the destabilization of the structure through the competition of the solvent for hydrogen bonds across the β-sheets.

The most satisfying aspect of the predicted secondary structure is that in the first N-terminal 25 residues, the β-sheets and chain reversals that would be suspected from the initial amino acid composition are both present, whilst no α-helical sections were predicted. As more amino acid sequence data becomes available, a greater number of structures for gluten proteins may be predicted. Ultimately, future X-ray crystallography studies may reveal that the often overlooked β-sheet and β-turn structures are dominant in wheat gluten proteins.

G. The Determination of Sugars in Protein Fractions Containing a Carbohydrate Component

Three gluten fractions in this study have been shown to contain a carbohydrate component. These fractions are the ethanol-soluble frac-

tion, the ethanol-soluble fraction I, and the ethanol-insoluble fraction. On gel filtration chromatography on Sephadex G-200, the carbohydrate component of all three fractions elutes in the void volume as a high molecular weight protein-carbohydrate complex (see Figures 4, 9, and 10). The modification of the protein component by reduction and alkylation of the disulfide bonds results in a change in the gel filtration profile for the protein. However, the gel filtration profile of carbohydrate component was unchanged; it still elutes with a protein component as the high molecular weight fraction I.

The carbohydrate component of the three fractions was qualitatively analyzed by paper chromatography. An example of some of the fractionation of sugars obtained by paper chromatography is shown in Figure 14. The solvent system consisting of a mixture of ethyl acetate, pyridine, and water in the ratio of 8:2:1 proved effective in the separation of pentoses and hexoses. This is also reflected in the R_G values for the neutral sugars given in Table 15.

Figure 14 and Table 16 give the monosaccharide composition of the three carbohydrate containing fractions. The major sugar in the ethanol-soluble fraction is galactose; arabinose and glucose are present in trace amounts. The alcohol-insoluble fraction, however, contains glucose as the major sugar with only a trace of galactose and arabinose. Xylose and mannose were not detected in any of the fractions by paper chromatography. Maltose and maltotriose were not detected, indicating complete hydrolysis of the sugars. The uronic acids (glucuronic acid and galacturonic acid) often associated with cell wall polysaccharides, were not detected in the digests of these fractions. The sensitivity of the $AgNO_3$ detection method is somewhat reduced for the uronic acids,

Figure 14. Paper chromatogram of sugars in digests of the alcohol-soluble fraction, alcohol-soluble fraction I and alcohol-insoluble fraction together with standard sugar mixtures.

Paper : Whatman No. 1
Solvent system : Ethyl acetate:pyridine:water (8:2:1)
Method : Downward flow
Run time : 36 hr
Sugar detection: Ag NO_3 method of Trevelyan *et al.* (1950)

Pattern	Sample
1	Standard sugar mixture (5%) a. galactose b. glucose c. mannose d. arabinose e. xylose
2	Alcohol-soluble fraction I digest
3	Alcohol-soluble fraction digest
4	Standard sugar mixture (as above)
5	Alcohol-insoluble fraction digest
6	Maltose with contaminating amounts of glucose and maltotriose
7	D-glucosamine

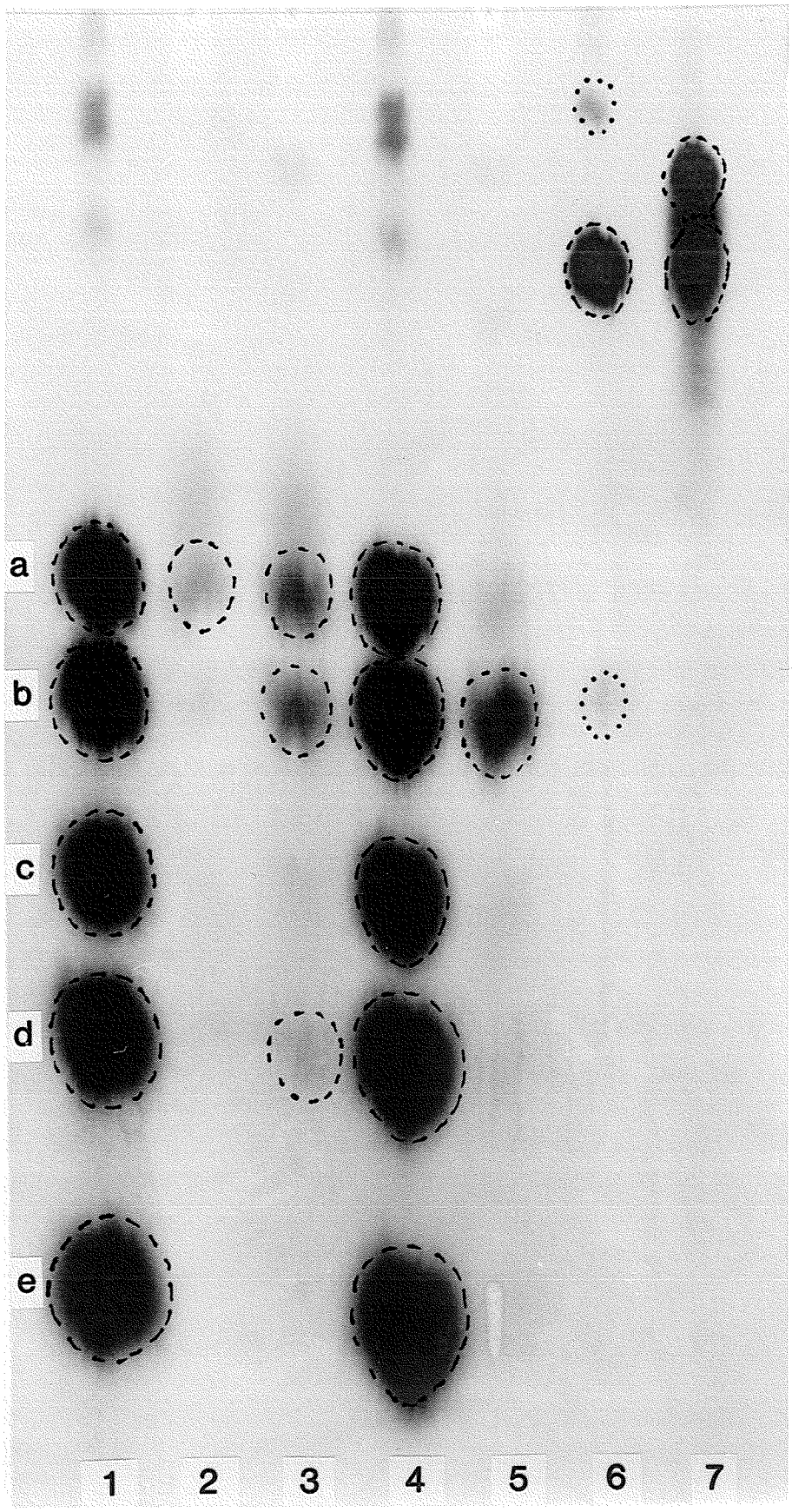

a
b
c
d
e
1
2
3
4
5
6
7

TABLE 15. Summary of the carbohydrate composition obtained from the paper chromatography[a] of the digests of the alcohol-soluble fraction, alcohol-soluble fraction I and alcohol-insoluble fraction.

	R_G	Alcohol-soluble fraction	Alcohol-soluble fraction I	Alcohol-insoluble fraction
Monosaccharides:				
Galactose	0.82	+ + +[b]	+ + +	+
Glucose	1.00	+ +	+	+ + + +
Mannose	1.28	0	0	0
Arabinose	1.50	+	+	+
Xylose	1.87	0	0	0
Glucosamine	(0.25) (0.39)	0	0	0
Maltose	0.38	0	0	0
Maltotriose	0.14	0	0	0
Uronic acids:				
Glucuronic acid	streak	0	0	0
Galacturonic acid	streak	0	0	0
Urea[c]	1.87	0	0	0

[a] Chromatography paper: Whatman No. 1.
Solvent system : ethyl acetate/pyridine/water, (8:2:1).
Method: downward flow.

[b] Sugar detection: Ag NO_3 method of Trevelyan et al. (1950).
+ + + + : high intensity spots.
+ : very low intensity spots.
0 : not detected.

[c] Urea detected as a white spot after development of paper chromatogram.

however, if cell wall polysaccharides were an important constituent of the carbohydrate component in these fractions then the levels would be expected to be above the detectable limits. Glucosamine was also not detected on paper chromatography of the digests. The fact that both glucosamine and mannose were not detected precludes the possibility of the presence of "high mannose" type glycoproteins. These glycoproteins do occur in plants (for example soybean agglutinin) and the linkage (N-glycosidic) is usually between asparagine and glucosamine with mannose occurring in the carbohydrate side chains.

Another interesting observation in the paper chromatography results of the fractions examined in this study is that if urea is present then it will interfere with the procedure. Urea must be removed completely from the preparations by appropriate dialysis before freeze drying and digestion of the sample. Urea chromatographs with xylose in the system described and upon the development of the chromatogram appears as a white spot which can mask the presence of xylose. Urea also interferes strongly in the assay for reducing sugar (see Figure 15).

The neutral sugar content of the carbohydrate component in the three carbohydrate-containing fractions was quantitatively estimated by gas-liquid chromatography of the respective alditol acetate derivatives of the sugars (see Table 16). The alcohol-soluble fraction I contains 70% galactose, 11% arabinose, and 11% glucose. Only small amounts of xylose (1.9%) and mannose (3.5%) were detected. In contrast, the alcohol-insoluble fraction contained 97.0% glucose and only small amounts of other neutral sugars. These results are in good agreement with the qualitative results by paper chromatography.

The total carbohydrate levels in the fractions as determined, by

Figure 15. Calibration curve for the assay of reducing sugar showing a plot of absorbance at 600 nm versus micrograms of glucose. Method of Nelson (1944) as modified by Roybt and Whelan (1968).

Solvents:

o——o : acetic acid (0.1 N)
●——● : acetic acid (0.1 N), ethanol 70% (v/v)
△——△ : acetic acid (0.1 N), urea (3 M)

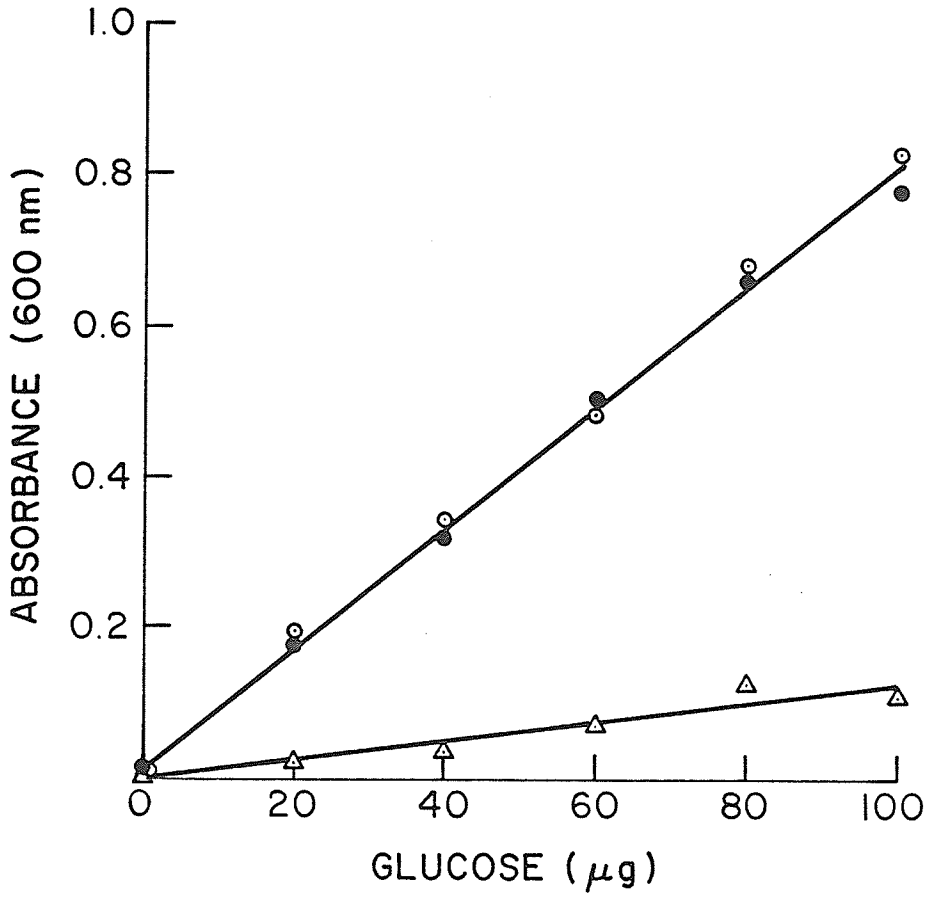

1.0
0.8
0.6
0.4
0.2
ABSORBANCE (600 nm)
0
20
40
60
80
100
GLUCOSE (μg)

TABLE 16. Carbohydrate content[a], reducing sugar content[b] and monosaccharide composition[c] of the alcohol-soluble and alcohol-insoluble fractions.

	Alcohol-soluble fraction	Alcohol-soluble fraction I	Alcohol-insoluble fraction
Carbohydrate (%)	0.6	2.9	17.0
Reducing sugar (%)[d]	21.0	8.0	10.8
Monosaccharide composition (%)[d]			
Galactose	55.5	70.3	0.7
Glucose	20.7	11.3	97.0
Mannose	6.2	3.5	0.4
Arabinose	16.1	11.1	1.2
Xylose	1.2	1.9	0.8

[a]Carbohydrate content determined as "total carbohydrate" by the phenol-sulfuric acid method of Dubois et al. (1956).

[b]Reducing sugar determined by the copper-reduction method of Nelson (1944) as modified by Roybt and Whelan (1968).

[c]Neutral sugars estimated as the alditol acetate derivatives by gas-liquid chromatography.

[d]Of total carbohydrate.

the phenol-sulfuric method, are also given in Table 16. The ethanol-soluble fraction contains 0.6% (w/w) carbohydrate. This was concentrated to 2.9% carbohydrate in the alcohol-soluble fraction I, which was obtained by gel filtration chromatography of the total alcohol-soluble fraction (refer to Figure 4). The alcohol-insoluble fraction contained 17.0% carbohydrate. Thus, carbohydrate is a significant component of this fraction.

The levels of reducing sugar present in the carbohydrate component was estimated by the copper-reduction method of Nelson as modified by Roybt and Whelan (1968). The standard curve using glucose as the standard sugar (in three different solvents) was shown in Figure 15. The protein blank used for this analysis (peak III proteins from Figure 4) gave no absorbance at 600 nm and caused no interference in the assay for reducing sugar. The ethanol-soluble fraction contained 21.0% reducing sugars, ethanol-soluble fraction I contained 8.0%, and the ethanol-insoluble fraction contained 10.8% reducing sugars (Table 16).

The proteins of the alcohol-soluble fraction I and the alcohol-insoluble fraction, when compared, show a large degree of similarity. Firstly, the gel filtration profiles of the reduced-alkylated proteins are similar, each giving three peaks (refer to Figures 9 and 10). The carbohydrate component elutes with the high molecular weight peak in each case. Examination of the protein subunits from these peaks, also reveals some similarities in the corresponding peaks of each fraction (see Figure 11). Only minor differences in amino acid composition were observed between the two fractions (previously discussed; see Table 7). Considering the close similarity of the proteins in these fractions, it would be assumed that the fractions are indeed the same and that the

presence of the ethanol-soluble fraction I in the ethanol-soluble fraction may simply be the result of "contamination" by the alcohol-insoluble fraction. The monosaccharide composition of the carbohydrate component in these fractions clearly demonstrates that the fractions are different. The difference in the solubility of these fractions may well depend on the carbohydrate component.

The experiments carried out and the data obtained so far in this study serve as a general background and provide some interesting insights into the potential contribution of both the carbohydrate and protein components in the fractions to gluten structure. The next major question that requires investigation is whether the carbohydrate component in the three fractions examined is associated with the protein by secondary interactions or is a part of a covalent structure. Experiments designed to answer this question are described in the following sections of this thesis.

H. The Association of Protein and Carbohydrate Components in the Alcohol-Soluble Fraction I

A number of experiments were carried out to determine the nature of the association between the protein and carbohydrate components in the alcohol-soluble fraction I. These experiments were specifically designed to provide information on whether the protein and carbohydrate components were covalently or non-covalently linked.

1. Molecular Weight of the Carbohydrate Component

The carbohydrate component of the alcohol-soluble fraction appears to be a high molecular weight component from the results of gel filtration. On gel filtration chromatography on Sephadex G-200, the carbohydrate component elutes with a protein component at the void volume of

the column. Partial modification of the protein structure via reduction of the disulfide bonds and alkylation of the resultant sulfhydryl groups of the protein moiety did not release the carbohydrate component. On gel filtration of the reduced-alkylated alcohol-soluble fraction I on Sephadex G-200, the carbohydrate again eluted as a single peak with a protein component in the void volume. These results indicate that if the carbohydrate is a separate entity, then its molecular weight by gel filtration chromatography is greater than 200,000 daltons.

Thus, the question that remains unanswered at this stage is whether the carbohydrate component is actually "associated" with the protein or is it simply of high molecular weight and, therefore, elutes co-incidently with the high molecular weight protein component? A possible answer to this question was sought through two different experimental approaches.

a. Estimation of reducing end-group content of the carbohydrate component. The reducing end-group content of the carbohydrate was determined using the copper-reduction method of Nelson (1944) as modified by Robyt and Whelan (1968). This analysis gave a value of 8% for the alcohol-soluble fraction I (see Table 16). The assay was carried out with a sample of alcohol-soluble fraction I dissolved in a solution of aqueous ethanol (70% v/v). It was shown in Figure 15 that this solvent does not interfere with the assay. A sample of peak III proteins, obtained from gel filtration chromatography of the ethanol-soluble fraction (see Figure 4), was also assayed to serve as a protein blank for the determination. This fraction was chosen because it was derived from the same gluten fraction as the ethanol-soluble fraction I and it did not contain any carbohydrate.

A value of 8.0% reducing sugar present in the carbohydrate compo-

nent indicates that the carbohydrate itself cannot be very high in molecular weight. The calculated average chain length (CL_{ave}) is approximately 12 glucose units which corresponds to a molecular weight of 2,160 daltons. Accordingly, it was concluded from this experiment that the molecular weight of the carbohydrate is at least several orders of magnitude lower than the value indicated from the gel filtration results. The carbohydrate component must, therefore, elute in the void volume of the Sephadex G-200 because it is "associated" with the protein to form a high molecular weight complex.

As the reducing sugar estimations were made in a relatively crude mixture of protein and carbohydrate, an alternative approach to the question posed above was made using proteolytic digestion of the protein component.

b. Enzymic cleavage of the protein component of alcohol-soluble fraction I by Proteinase K. Proteinase K was chosen to selectively cleave and disrupt the protein component of alcohol-soluble fraction I. This enzyme is derived from the fungus Tritirachium album (Ebeling et al. 1974) and is available in a highly purified form. It is an endoprotease that has been developed for the isolation of native, high molecular weight nucleic acids free of contaminating protein. In contrast to crude pronase preparations, no contaminating endonucleases are present in Proteinase K (McCormick et al. 1974). The enzyme exhibits approximately sixfold the proteolytic activity in comparison to pronase from Streptomyces griseus (Ebeling et al. 1974). Furthermore, highly purified Proteinase K does not contain any reported carbohydrase activities and is, therefore, particularly suitable for the purpose of this experiment.

A sample of the alcohol-soluble fraction I (12.8 mg) was incubated

with a solution of Proteinase K (1.0 ml) in a buffer containing Tris-HCl (20 mM), $CaCl_2$ (5 mM), pH 8.3 for 16 hr at 40° C. An enzyme protein to substrate protein ratio of approximately 1 to 30 was used. After 16 hr incubation, a further 0.2 ml enzyme solution was added and the incubation extended for 4 hr. The digest was then centrifuged (10,000 x g, 15 min, 20° C) and the supernatant assayed for carbohydrate. The remaining pellet was washed with Tris-HCl buffer and recentrifuged, and the supernatant again assayed for carbohydrate. The second pellet was then suspended in a solvent of acetic acid (0.1 N) and urea (3 M) by mixing vigourously on a Vortex mixer. The resulting suspension was assayed for carbohydrate and then centrifuged. The carbohydrate content of the supernatant (i.e. carbohydrate solubilized by the acetic/urea solvent) was determined.

The carbohydrate contents of the various preparations described above are given in Table 17. Hydrolysis of the protein component of the alcohol-soluble fraction I released 260 μg carbohydrate into the Tris-HCl buffer. This represents 72% of the total carbohydrate recovered. No further carbohydrate was removed by washing the pellet with Tris-HCl buffer. A total of 100 μg carbohydrate remained in the pellet, accounting for 28% of the recovered carbohydrate. An overall recovery of 97% of the carbohydrate was obtained in this experiment. A further 30 μg of carbohydrate was solubilized by the treatment of the pellet with acetic acid/urea solvent. Thus, a total of 290 μg of carbohydrate, representing 81% of recovered carbohydrate, was solubilized.

Thus, it may be concluded that treatment of the alcohol-soluble fraction I with Proteinase K released a major proportion of the carbohydrate component present in this fraction. This result indicates that

TABLE 17. Solubilization of carbohydrate[a] by the action of Proteinase K on the protein component of the alcohol-soluble fraction I.[b]

		Carbohydrate (μg)	Percentage of carbohydrate recovered
Carbohydrate solubilized by Proteinase K digestion	(A)	260	72
Buffer wash of pellet	(B)	0	0
Resuspended pellet in 0.1 N acetic acid + 3 M urea	(C)	100	28
Acetic acid-urea soluble carbohydrate in pellet	(D)	30	8
Total carbohydrate solubilized	(A + B + D)	290	80
Total carbohydrate recovered[c]	(A + B + C)	360	100

[a]Carbohydrate determined as "total carbohydrate" by the phenol-sulfuric acid method of Dubois et al. (1956).

[b]12.8 mg of alcohol-soluble fraction I containing 371 g of carbohydrate was subjected to digestion with Proteinase K.

[c]Overall recovery of carbohydrate: $\frac{360}{371} \times \frac{100}{1} = 97\%$.

the carbohydrate is strongly held in the gluten complex since it is released only after hydrolysis of the protein. Furthermore, it appears that the protein structure stabilizes the protein-carbohydrate complex.

c. Gel filtration chromatography of Proteinase K digest on Sephadex G-200. The alcohol-soluble fraction I, after proteolysis with Proteinase K, was chromatographed on a Sephadex G-200 column and the elution profile compared to that of the original sample. If the carbohydrate was of high molecular weight and not "associated" with the protein component, it would be expected to elute in the void volume of the column when the proteolytic digest was chromatographed. However, if the carbohydrate component was of low molecular weight, then it would elute at a higher elution volume corresponding to its molecular weight.

The elution profile of the ethanol-soluble fraction I on the Sephadex G-200 column before proteolytic hydrolysis is given in Figure 16A. Both the carbohydrate and protein components elute as a symmetrical peak at the void volume of the column. For this experiment, 10 mg of the fraction was dissolved in 2.0 ml of a solvent containing acetic acid (0.1 N) and urea (3 M). This sample concentration was chosen after a series of gel filtration experiments involving increasing sample concentrations. (These ancillary results are described in Appendix II and the elution profiles shown in Figure 29.) At increased sample concentration peak symmetry is lost and the flow characteristics of the column break down, producing some artifacts; for example "shoulders" and additional peaks in the profile. However, at a concentration of 5.0 mg ml^{-1}, the carbohydrate was easily detected in the void volume of the column as shown in Figure 16A. Accordingly, the carbohydrate would easily be detected if it eluted at the void volume of the column after

Figure 16. A. Elution profile from gel filtration chromatography of alcohol-soluble fraction I (before proteolytic hydrolysis) on Sephadex G-200.

Conditions:

Column size	: 2.0 x 100 cm
Bed height	: 90 cm
Solvent	: acetic acid (0.1 N) + urea (3 M)
Flow rate	: 8.0 ml hr^{-1}
Fraction size	: 3.5 ml
Sample volume	: 2.0 ml
Sample concentration	: 5 mg ml^{-1}

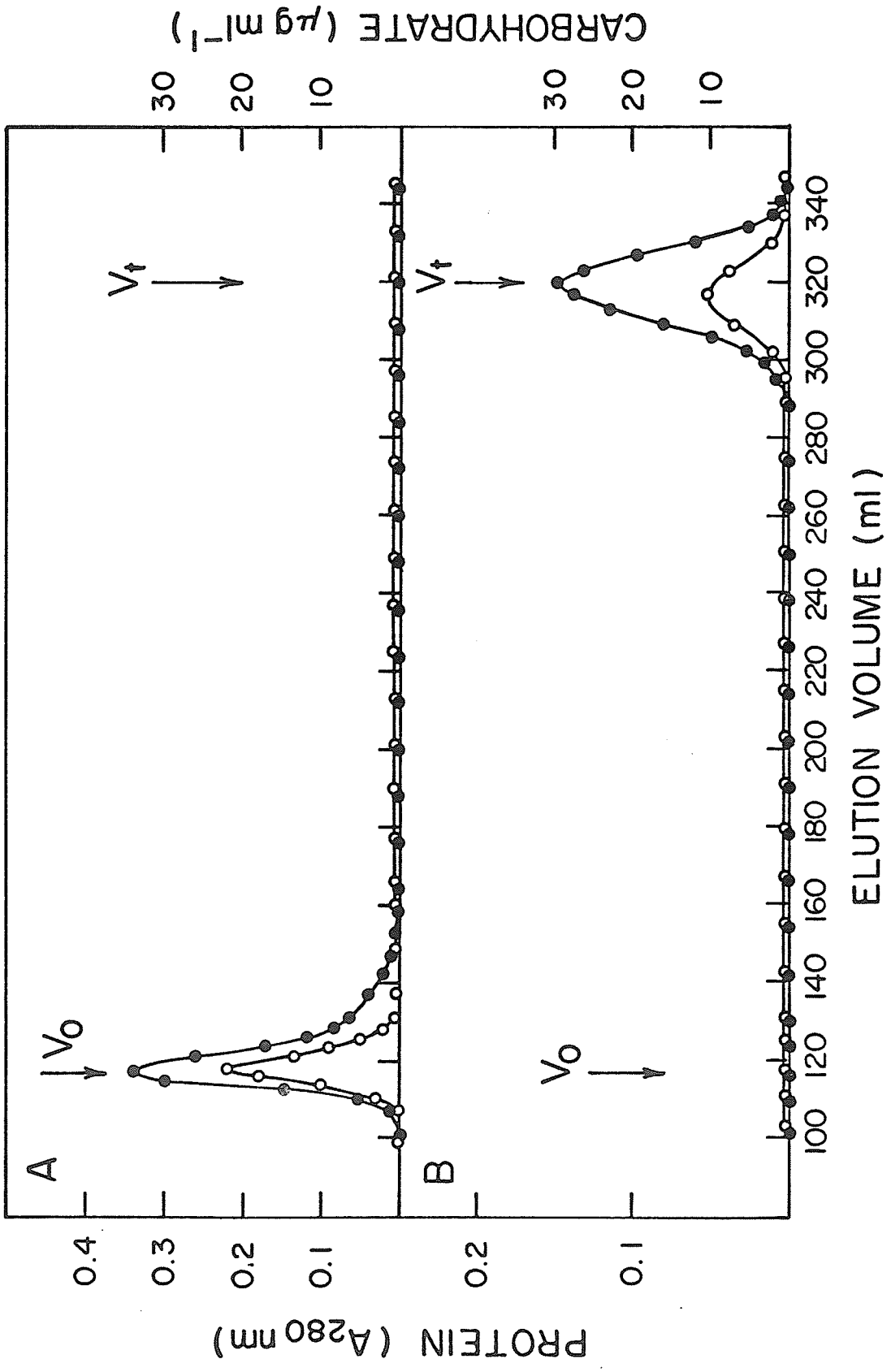
Carbohydrate: o———o
Protein : ●———●

B. Elution profile from gel filtration chromatography of Proteinase K digest of alcohol-soluble fraction I on Sephadex G-200.

Conditions:

Column size	: 2.0 x 100 cm
Bed height	: 90 cm
Solvent	: acetic acid (0.1 N) + urea (3 M)
Flow rate	: 8.0 ml hr^{-1}
Fraction size	: 3.5 ml
Sample volume	: 2.0 ml

Carbohydrate: o———o
Protein : ●———●


A
B
V_o
V_t
PROTEIN ($A_{280\,nm}$)
CARBOHYDRATE ($\mu g\ ml^{-1}$)
ELUTION VOLUME (ml)
0.4
0.3
0.2
0.1
30
20
10
100
120
140
160
180
200
220
240
260
280
300
320
340

the protein was removed by proteolysis.

The elution profile of the Proteinase K digest of the alcohol-soluble fraction I on gel filtration chromatography on Sephadex G-200 is shown in Figure 16B. No protein or carbohydrate eluted in the void volume of the column. Both the protein and the carbohydrate components eluted in the total volume (V_t) of the column (i.e. were of relatively low molecular weight). This result shows that the carbohydrate component of alcohol-soluble fraction I has a relatively low molecular weight. Accordingly, this component chromatographs on gel filtration as "apparently high molecular weight" due to its association with the protein component. This association appears to be quite strong, since it is not destroyed by dissociating solvents such as 3 M urea. This experiment also demonstrates that modification of the protein to low molecular weight peptides is required to release the carbohydrate component from the aggregated complex.

2. Electrophoresis of Alcohol-Soluble Fraction and Alcohol-Soluble Fraction I on Polyacrylamide Disc Gels

The alcohol-soluble fraction and the alcohol-soluble fraction I were subjected to electrophoresis on polyacrylamide disc gels. After electrophoresis, gels were stained for protein with Coomassie Blue R and for carbohydrate with periodic acid-schiff reagent. The purpose of these experiments was to determine whether the protein and carbohydrate components would separate during electrophoresis or migrate as a single component. The latter evidence has been cited as indicative of a covalent linkage between the carbohydrate and protein moieties. Most of the glycoproteins reported in the literature have been initially identified using this criterion.

Figure 17. A. PAGE of alcohol-soluble fraction stained for protein and carbohydrate.

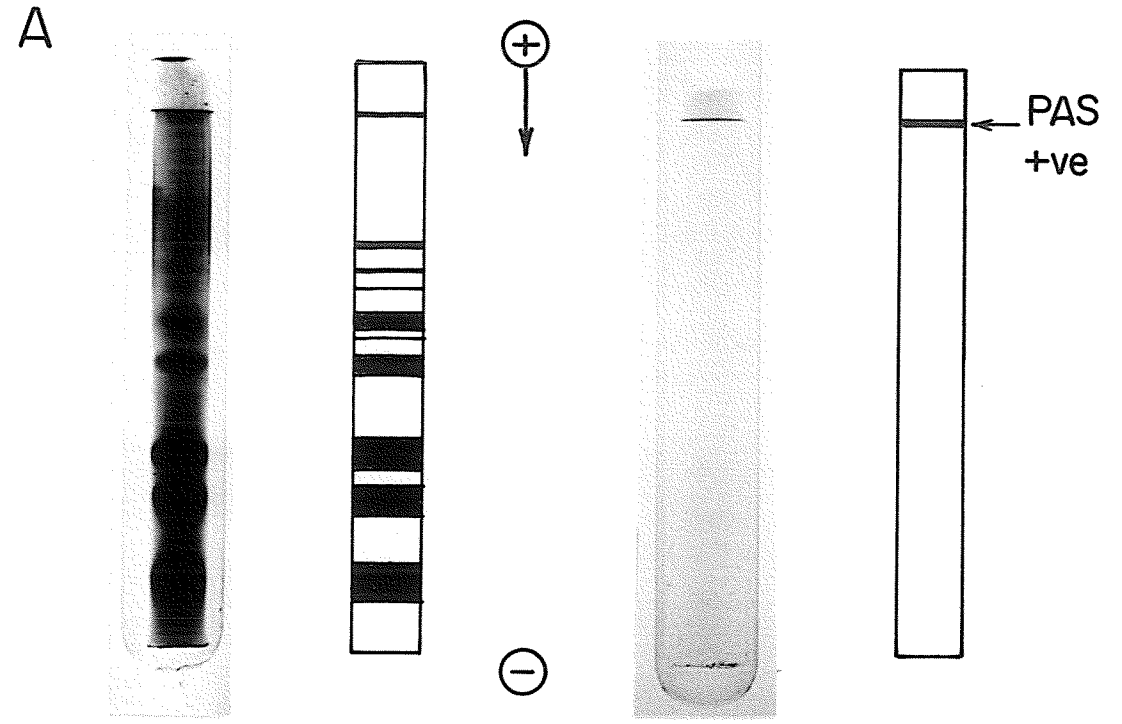
Conditions:

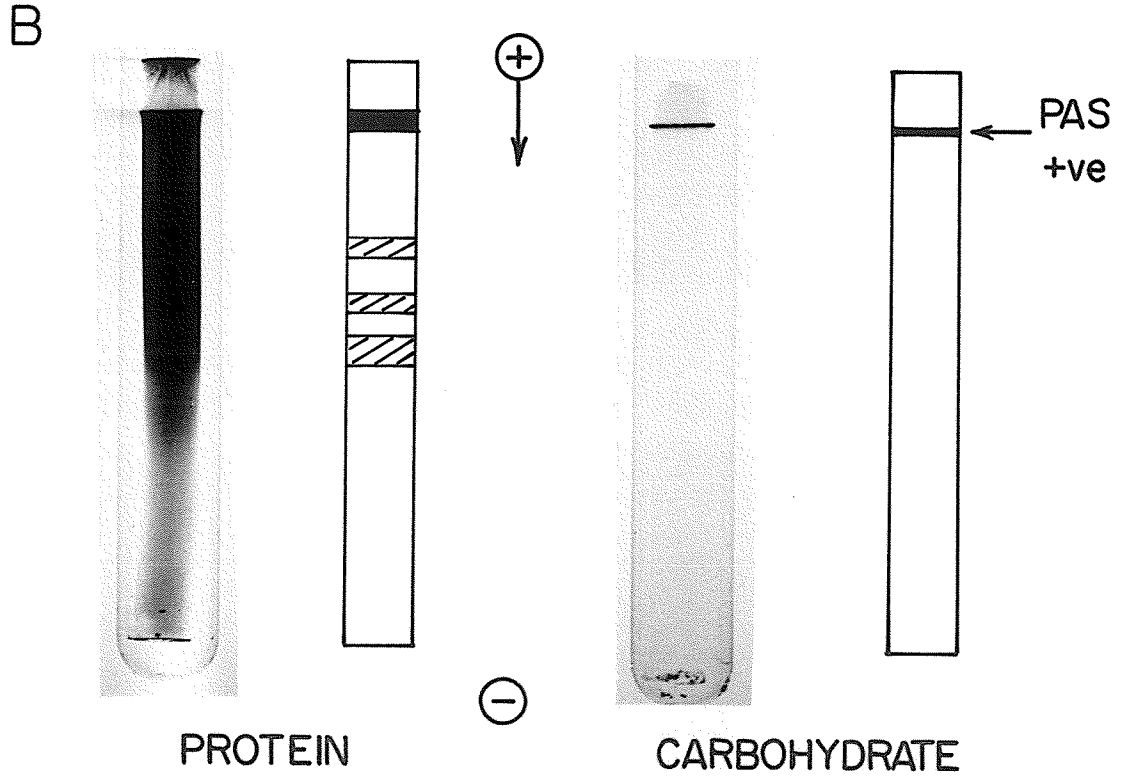
Polyacrylamide disc gel	4% acrylamide
Buffer	β-alanine pH 4.3
Protein stain	Coomassie blue
Carbohydrate stain	Periodic acid-schiff reagent

B. PAGE of alcohol-soluble fraction I stained for protein and carbohydrate.

Conditions:

as above


A
PAS
+ve
PROTEIN
CARBOHYDRATE


B
PAS
+ve
PROTEIN
CARBOHYDRATE

Samples of the alcohol-soluble fraction and alcohol-soluble fraction I were dissolved in β-alanine buffer (pH 4.3) and subjected to polyacrylamide disc gel electrophoresis. The gel system included a large-pore stacking gel and a running gel consisting of 4% polyacrylamide. The gels were prepared by the method of MacGregor and Meredith (1971), which is described in the Methods section. Disc gels had two advantages over slab gels for the purposes of this experiment. Firstly, gels that are very porous (i.e. low acrylamide concentration) are more easily handled and secondly, disc gels are more efficient for some staining procedures such as those required for the detection of carbohydrate. Acrylamide disc gels containing levels of less than 4.0% acrylamide possess very poor handling properties and cannot be used as running gels.

The electrophoretic pattern showing stains for both carbohydrate and protein for the alcohol-soluble fraction is given in Figure 17A. When stained for protein, the electrophoretic pattern is heterogeneous with many components entering the gel and some high molecular weight protein that did not enter the small-pore running gel. It is interesting to compare this electrophoretic pattern with that of the flatbed electrophoregram (Figure 5, pattern 3). The sample applied is the same in both cases. The comparison demonstrates the large increase in the resolution of protein components obtained using the flatbed apparatus (higher polyacrylamide concentration).

When stained for carbohydrate, a bright pink coloured band was observed, indicating a positive periodic acid-schiff reaction and the presence of carbohydrate. This band corresponded to the protein band that did not enter the polyacrylamide running gel. No other bands that

stained positively for carbohydrate were observed.

The electrophoretic patterns obtained for the alcohol-soluble fraction I are shown in Figure 17B. This fraction, isolated by gel filtration chromatography on Sephadex G-200 (refer to Figure 4), contains 2.9% carbohydrate compared with 0.6% carbohydrate in the total alcohol-soluble fraction (see Table 16). A general streaking was obtained when the disc gel was stained for protein. This was similar to the result obtained for this fraction on the flatbed apparatus (Figure 5, pattern 4). Some distinct faint bands that entered the gel were observed under these conditions and are indicated by the cross-hatched bands in Figure 17B. The major protein band stained an intense blue colour and did not enter the gel. It is estimated that this band contained over 90% of the protein in the sample applied to the gel. Some protein material did not enter the stacking gel.

When stained for carbohydrate, one major band was periodic-acid schiff positive (Figure 17B) and corresponded to the major protein band that did not enter the polyacrylamide gel. Another positive stain occurred for carbohydrate (not shown in Figure 17B), which corresponded to the protein band that did not enter the stacking gel, however, this was much fainter than the positive carbohydrate stain shown in Figure 17B. This particular stain is not shown in the electrophoretic pattern in Figure 17B, because this part of the gel was lost in handling due to its fragile nature (low acrylamide concentration). An increase in the intensity of the carbohydrate stain was observed in the alcohol-soluble fraction I, compared with that of the total alcohol-soluble fraction. This is consistent with the increased carbohydrate level in the former fraction.

It can be concluded that the carbohydrate component of the alcohol-soluble fraction I cannot be separated from the major portion of the protein component under the conditions of electrophoresis used in this study. The results obtained here are consistent with the hypothesis that the carbohydrate and protein in this fraction are linked by a covalent bond. However, the protein component is extremely complex (see for example SDS-PAGE results; Figures 8 and 11) and it is possible that the carbohydrate is held in the complex of aggregating polypeptides by strong noncovalent forces.

3. Electrophoresis of Alcohol-Soluble Fraction I on Agarose-Acrylamide Disc Gels

Because the co-incident protein and carbohydrate bands of the alcohol-soluble fraction I did not enter the 4.0% acrylamide gel on electrophoresis, an alternative matrix was investigated. Matrices containing various combinations of agarose and acrylamide have been used by others to form porous gels which allow molecules of molecular weights upwards of a million daltons to enter the gel. Gels containing a combination of agarose and acrylamide possess superior handling properties than low concentration polyacrylamide gels.

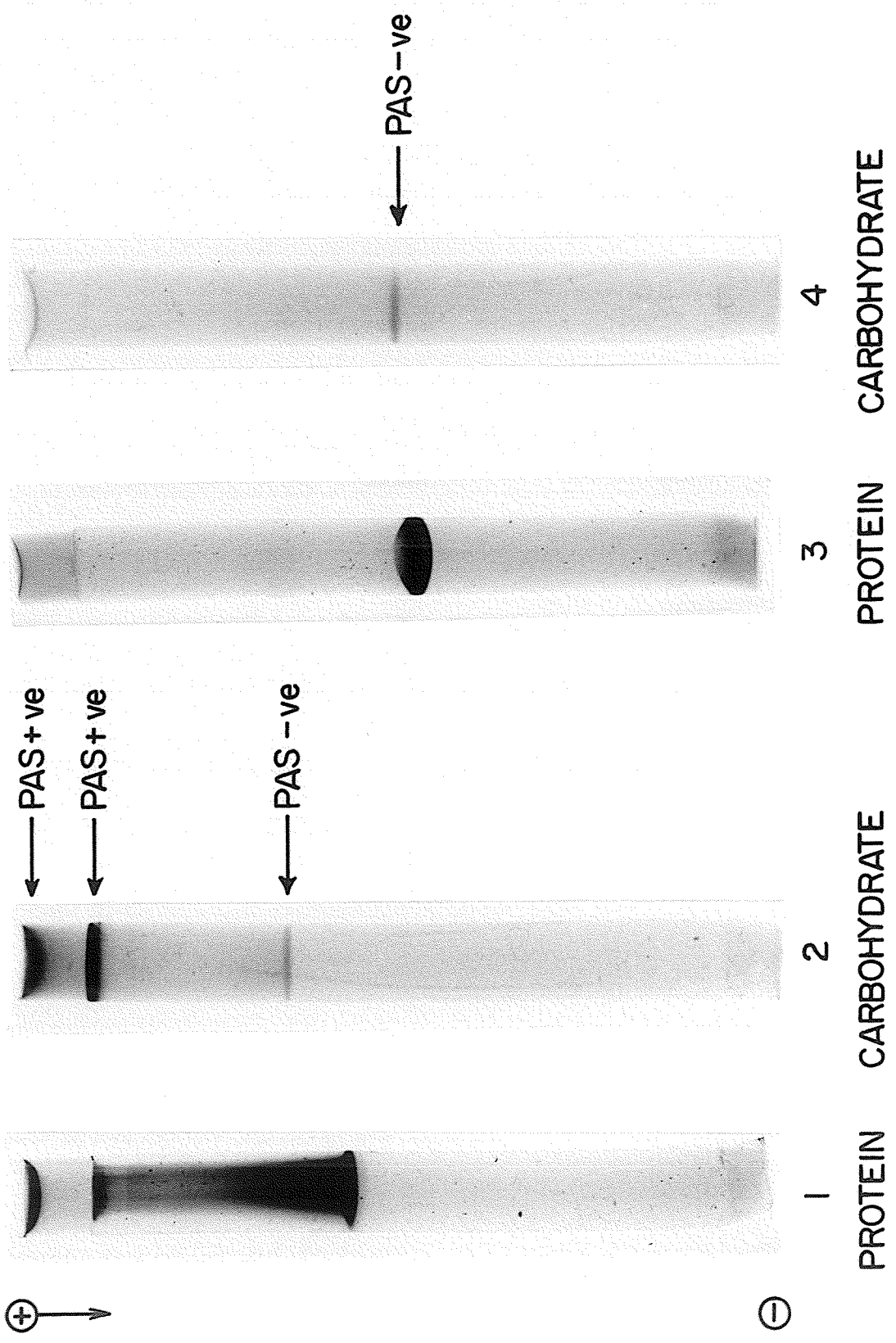
Agarose-acrylamide disc gels were prepared according to the method of Perret et al. (1979) as described in the Materials and Methods section. The gels were prepared giving a final concentration of 0.5% agarose and 2.0% acrylamide in β-alanine buffer (pH 4.3). The electrophoretic patterns obtained from the electrophoresis of the alcohol-soluble fraction I are shown in Figure 18 (patterns 1 and 2). Two gels were run simultaneously; one was stained for protein with Coomassie Blue dye and the other for carbohydrate with periodic acid-schiff reagent. For comparison, peak III fraction obtained from the gel filtration of

Figure 18. Electrophoresis of alcohol-soluble fraction I and alcohol-soluble peak III proteins in 2.0% acrylamide - 0.5% agarose disc gels.

Conditions:

Buffer : β-alanine pH 4.3
Protein stain : Coomassie blue
Carbohydrate stain: Periodic acid-schiff reagent

Pattern	Sample
1	Alcohol-soluble fraction I - stained for protein
2	Alcohol-soluble fraction I - stained for carbohydrate
3	Alcohol-soluble peak III proteins - stained for protein
4	Alcohol-soluble peak III proteins - stained for carbohydrate


PAS+ve
PAS+ve
PAS-ve
PAS-ve
⊕
⊖
1
2
3
4
PROTEIN
CARBOHYDRATE
PROTEIN
CARBOHYDRATE

the alcohol-soluble fraction (see Figure 4) were also subjected to electrophoresis under the same conditions. This fraction was chosen because it did not contain any carbohydrate.

The electrophoresis patterns that were obtained (Figure 18) indicate that the top portion of the gel cylinder acts as a stacking gel. It is presumed that in this zone the gel is of lower acrylamide concentration (i.e. higher porosity) due to the diffusion of surface water into the gel solution before polymerization was complete.

The protein pattern of alcohol-soluble fraction I (pattern 1) showed three bands. One did not enter the disc gel at all; the other entered the fortuitous stacking gel but not the main (running) gel; and the third major band migrated well into the gel. The latter band showed the most intense stain for protein, however, it did not stain for carbohydrate (pattern 2). The band which entered the stacking gel stained poorly for protein (pattern 1) but stained very intensely for carbohydrate (pattern 2) as did the band at the top of the gel. A band that stains poorly for protein and intensely for carbohydrate under these conditions is a typical reaction for glycoproteins where the linkage between the carbohydrate and protein moiety is covalent.

Peak III fraction gave a positive protein band but was negative when stained for carbohydrate (see patterns 3 and 4, respectively). The faint band in pattern 4 that appears on the black and white photograph was opaque (not pink) and was probably due to precipitation of the protein in the gel by periodic acid. The same reaction was noted for the major protein component of the alcohol-soluble fraction I which migrated well into the gel (pattern 2). These bands (compare patterns 1 and 2) did not migrate exactly the same distance in the two gels. This was

attributed to a slight difference between the two porous gels.

It was concluded from this experiment that a major portion of the protein in the alcohol-soluble fraction I was not associated with the carbohydrate component. This result is consistent with the information obtained in the gel filtration studies of the reduced-alkylated alcohol-soluble fraction I (see Figure 10). It will be recalled that on gel filtration of the reduced-alkylated fraction, the carbohydrate component eluted at the void volume of the column together with aggregating polypeptides, and was not associated with all of the proteins in the fraction under these conditions. In addition, the carbohydrate-protein complex does not enter the agarose-acrylamide running gel confirming that the complex is of extremely high molecular weight.

In summary, electrophoresis of the alcohol-soluble fraction I on agarose-acrylamide disc gels indicates that the carbohydrate present in the fraction is very strongly associated with a minor protein component. The possibility of a covalent linkage of these components cannot, therefore, be ruled out at this stage. Further experiments designed to elucidate the nature of the possible linkage are described in the next section.

I. Examination of the Alcohol-Soluble Fraction I for the Presence of a Covalent Linkage Between the Carbohydrate and Protein Moieties

1. Evidence for a Covalent Linkage of Carbohydrate and Protein Components in Wheat Gluten

Throughout this study a number of experimental results have provided strong indications that the carbohydrate and protein components in certain wheat gluten fractions may be covalently linked, forming either a glycoprotein or proteoglycan. These results are summarized

below and discussed in this context.

a. Gel filtration chromatography of the alcohol-soluble fraction on Sephadex G-200. In the initial fractionation of the alcohol-soluble fraction by gel filtration chromatography on Sephadex G-200 (see Figure 4), both the carbohydrate and the protein eluted as symmetrical peaks at the same elution volume.

b. Gel filtration chromatography of reduced-alkylated alcohol-soluble fraction I on Sephadex G-200. Partial modification of the structure of the protein component in the alcohol-soluble fraction I by reduction and alkylation of the disulfide bonds present did not release the carbohydrate component from the complex. On gel filtration chromatography of the reduced-alkylated alcohol-soluble fraction I on Sephadex G-200, the carbohydrate and protein components again eluted as symmetrical peaks at the same elution volume (see Figure 10).

c. Gel filtration chromatography of reduced-alkylated alcohol-insoluble fraction on Sephadex G-200. The protein and carbohydrate components of this fraction also eluted as symmetrical peaks at similar elution volumes when subjected to gel filtration chromatography on Sephadex G-200 (see Figure 9).

d. Gel filtration chromatography of the proteolytic digest of alcohol-soluble fraction I on Sephadex G-200. The fact that the carbohydrate and protein components eluted as co-incident peaks before proteolytic digestion means that either the two components are low molecular weight and strongly linked or that both the carbohydrate and the protein are of similar high molecular weight. Chromatography of the proteolytic

digest of the alcohol-soluble fraction I on Sephadex G-200 demonstrated that the latter was not the case. The carbohydrate in both fractions was of low molecular weight and, therefore, it appears in the high molecular weight fraction on gel filtration due to "association" with the protein. This experiment could not distinguish between covalent linkage or strong secondary association between the carbohydrate and protein.

e. Co-incident protein and carbohydrate bands on disc gel electrophoresis. The strongest evidence for a covalent linkage between the carbohydrate and protein components was provided by the disc gel electrophoresis results. The alcohol-soluble fraction and the alcohol-soluble fraction I showed co-incident stains for both protein and carbohydrate in the disc gels (see Figure 17). The bands that possess co-incident stains for protein and carbohydrate, however, did not enter the 4% acrylamide gel, indicating that they are of extremely high molecular weight. The possibility of these stains occurring because both components are simply high molecular weight is unlikely. However, to confirm this a sample of alcohol-soluble fraction I was subjected to electrophoresis on a much more porous 0.5% agarose-2.0% acrylamide gel system. This allowed the major portion of the protein to migrate well into the gel. This major protein was not associated with carbohydrate (see Figure 18; patterns 1 and 2). However, a slower migrating band entered the stacking gel, staining for both protein and carbohydrate. The carbohydrate stain was extremely intense and the stain for protein much less intense. This differential in staining intensity is typical of glycoproteins.

f. Monosaccharide composition of the carbohydrate component of gluten fractions. The major sugars present in the alcohol-soluble fraction I are galactose (70.3%), arabinose (11.1%), and glucose (11.3%) (see Table 16). Galactose and arabinose are neutral sugars, which are commonly involved in proteoglycan and glycoprotein linkages in plant materials (Clarke et al. 1979; Brown and Kimmins 1977). The major sugar in the alcohol-insoluble fraction is glucose (97.0%). This sugar is not very common in plant glycoproteins (Brown and Kimmins 1977). No reports in the literature have unequivocably demonstrated glucose in a bond compound of a glycoprotein.

Considering the above strong indications that a covalent linkage between the carbohydrate and protein components may exist in the alcohol-soluble fraction I, a number of experiments were designed to test for a specific linkage. These experiments are described in the following section.

2. Testing for the Presence of a Hydroxyproline-Arabinogalactan Linkage in the Alcohol-Soluble Fraction I

Considering the suggestive evidence for a covalent linkage between the protein and the carbohydrate in the alcohol-soluble fraction I already discussed, it seemed reasonable that an arabinose- and galactose-containing glycoprotein or proteoglycan may be present. Only serine and hydroxyproline have been found to be glycosylated in the arabinose- and galactose-containing proteoglycans and glycoproteins (Clarke et al. 1979). Accordingly, the carbohydrate containing fraction was tested for the presence of the hydroxyproline-arabinogalactan linkage.

Galactose and arabinose are the sugars found most frequently in plant glycoproteins and proteoglycans (Knee 1975; Brown et al. 1975;

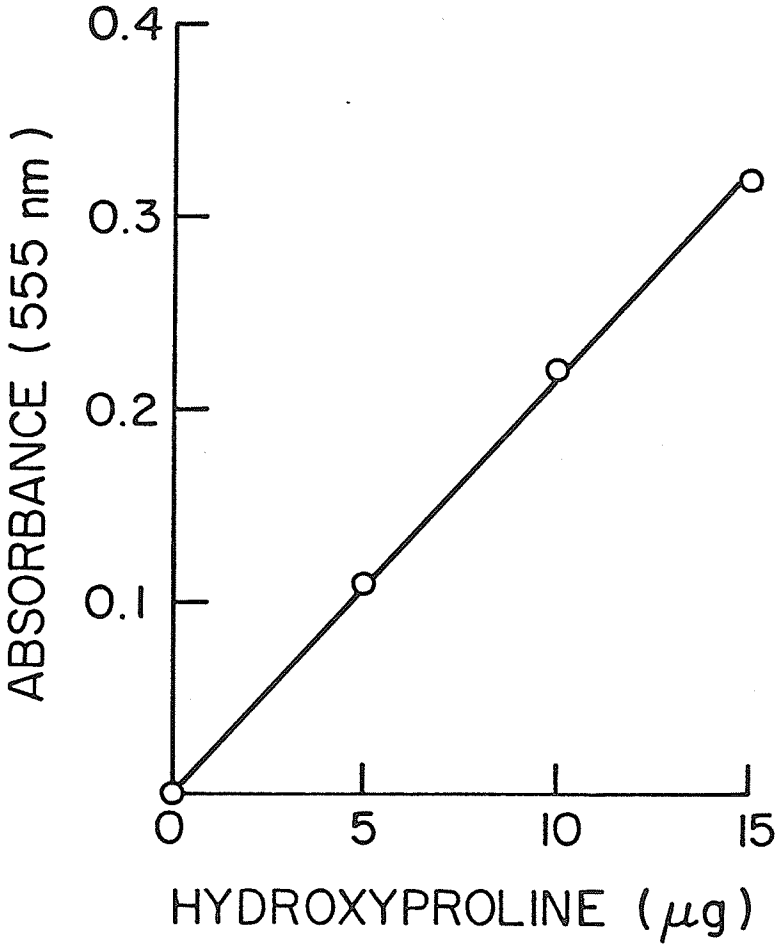
Monro et al. 1976). Three types of carbohydrate-protein linkages have generally been proposed for plant proteoglycans and glycoproteins that contain arabinose and galactose (Clarke et al. 1979). These include a galactopyranosyl 4-O-hydroxyproline linkage, a galactopyranosyl-O-serine linkage, and an arabinofuranosyl 4-O-hydroxyproline linkage. A water-soluble hydroxyproline-arabinogalactan peptide has been isolated from wheat endosperm (Fincher and Stone 1974) and the carbohydrate-protein linkage confirmed as galactose glycosydically linked to 4-hydroxyproline (Fincher et al. 1974).

In testing for the presence of a hydroxyproline linked arabinogalactan, two approaches were used. Firstly, both acid and alkali digestions of the sample were directly assayed for hydroxyproline content. Secondly, use was made of the characteristic resistance of hydroxyproline galactopeptides to alkali hydrolysis.

a. Hydroxyproline content of alcohol-soluble fraction I and the alcohol-insoluble fraction. Hydroxyproline content of the samples was determined using the colorimetric method of Neuman and Logan (1950) as modified by Leach (1960). This method was preferred over the ninhydrin method because of its increased sensitivity. The extinction coefficient for the ninhydrin-hydroxyproline complex is low compared with that of other amino acids (Leggett Bailey 1967). A calibration curve of absorbance at 555 nm versus micrograms of hydroxyproline is shown in Figure 19. From this curve, it can be seen that the lower detection limit of the assay is in the vicinity of 3 or 4 micrograms of hydroxyproline. Above 15 micrograms hydroxyproline, the assay becomes non-linear.

Samples (10 mg) of the alcohol-soluble fraction I and the alcohol-insoluble fraction were digested in acid (6 N HCl, 1.0 ml, 6 hr, 100° C)

Figure 19. Calibration curve for hydroxyproline assay showing absorbance at 555 nm as a function of the quantity of hydroxyproline. The method was that of Neuman and Logan (1950) as modified by Leach (1960).


0.4
0.3
0.2
0.1
ABSORBANCE (555 nm)
0
5
10
15
HYDROXYPROLINE (μg)

and alkali (0.22 M $Ba(OH)_2$, 1.0 ml, 8 hr, 100° C). The digests were neutralized, centrifuged, and the supernatants evaporated to dryness. The digests were dissolved in 1.0 ml of 0.1% NaCl solution and 100 μl aliquots assayed for hydroxyproline.

No hydroxyproline was detected in any of the digests of alcohol-soluble fraction I protein or the alcohol-insoluble fraction protein. Considering that 5 μg of hydroxyproline is the detection limit of the assay and that no hydroxyproline was detected, then it may be concluded that if hydroxyproline is present, its proportion in the protein is less than 0.6% ($\frac{5}{809}$ x 100) of the amino acids by weight (100 μl of sample contained 809 μg protein).

Similarly, the hydroxyproline content of the alcohol-insoluble fraction can be estimated to be below the detection limit of 0.6% by weight of the amino acids present in the digest. These calculations assume that little destruction of amino acids occur during acid hydrolysis. Accordingly, these results indicate that the involvement of hydroxyproline in the covalent linkage between the carbohydrate and protein is very unlikely.

b. Beta-alkoxy elimination reaction of the alcohol-soluble fraction I and alcohol-insoluble fraction. Tentative identification of the amino acid involved in the carbohydrate-protein linkage can be made on the basis of its alkali lability. For example, the glycosyl-serine (or threonine) linkage is usually quite labile in mild alkaline conditions (Neuberger et al. 1972). The release of the glycosyl group from the peptide material by treatment with alkali is termed the β-alkoxy elimination reaction (Brown and Kimmins 1977; Clarke et al. 1979). On the other hand, glycosidic linkages to hydroxyproline (and hydroxylysine) exhibit

a unique property in that they are quite stable to alkaline hydrolysis. The structure of this linkage does not favor the β-alkoxy elimination reaction (Clarke et al. 1979). This property has often been utilized in the isolation of hydroxyproline glycosides from glycoproteins and proteoglycans (Lamport and Miller 1971; Brown and Kimmins 1977; Fincher et al. 1974). Solutions of barium, sodium, or potassium hydroxides at numerous temperatures and extraction times, have been used in the extraction of glycoproteins from plant tissues, especially those of cell walls (Brown and Kimmins 1977). Alkali extractions at high temperatures ($\sim 90^{\circ}$ C) has allowed the demonstration of hydroxyproline arabinosides in the cell walls of numerous plant species (Lamport and Miller 1971). Fincher et al. (1974) demonstrated the resistance of the hydroxyproline-arabinogalactan peptide from wheat endosperm to the β-alkoxy elimination reaction upon incubation with 5 M NaOH at 110° C for 24 hr.

In the context of the above discussion, samples of the alcohol-soluble fraction I and the alcohol-insoluble fractions were subjected to alkaline hydrolysis as previously described (see Materials and Methods). The level of carbohydrate remaining after hydrolysis was monitored to test for resistance to the β-alkoxy reaction or hydrolysis from the reducing end of the sugar. The recovery of carbohydrate for each fraction is given in Table 18. Low recoveries of carbohydrate were noted for both fractions, 1.5% and 1.65% for the alcohol-soluble fraction I and the alcohol-insoluble sample, respectively. The low recoveries indicate that the sugars present in the fractions have readily accessible reducing ends rendering the carbohydrate susceptible to digestion in alkali. Thus, it appears that none of the carbohydrates'

TABLE 18. Recovery of carbohydrate after alkaline hydrolysis of the alcohol-soluble fraction I and the alcohol-insoluble fraction.

Sample	Weight of sample hydrolyzed (mg)	Carbohydrate content (μg)	Carbohydrate after hydrolysis (μg)	Recovery (%)
Alcohol-soluble fraction I[a]	20	600	9	1.50
Alcohol-insoluble fraction[b]	25	4250	70	1.65

[a] Digestion in saturated $Ba(OH)_2$; (approx. 0.22 M).

[b] Digestion in NaOH (2.5 M).

free reducing ends are protected by a glycosydic linkage to 4-hydroxyproline.

Because there was a small amount of carbohydrate remaining in the digests, they were chromatographed on a Biogel P-6 column to ascertain whether the carbohydrate was polymeric. If this were the case, then a careful check for a residual, alkali-stable glycopeptide would be considered. This experiment is described in the next section.

3. Gel Filtration Chromatography of the Alkaline Digest of the Alcohol-Soluble Fraction I on Biogel P-6

In a study of the chemical and physical properties of an arabinogalactan-peptide from wheat endosperm, Fincher et al. (1974) showed that the peptide was polydisperse with an average molecular weight of 22,000 daltons. Furthermore, the arabinogalactan-peptide, after hydrolysis in strong alkali solution (5 M NaOH, 100° C, 24 hr) eluted at the void volume of a Biogel P-6 column (exclusion limit - 6,000 daltons).

In the present study, a sample (20 mg) of alcohol-soluble fraction I was subjected to alkaline hydrolysis (see Methods) and the resulting digest chromatographed on a Biogel P-6 column. An hydroxyproline-arabinogalactan peptide would be expected to chromatograph in the void volume of the column due to its resistance to the β-alkoxy elimination reaction as previously mentioned. The fractions obtained were assayed for carbohydrate, protein, and hydroxyproline. Since it is quite likely that under these conditions the protein component would be hydrolyzed to amino acids and small peptides, the protein content of the column fractions was measured by two methods. The absorbance at 280 nm was monitored to estimate the content of tryptophan and tyrosine and the content of α-amino groups was determined with ninhydrin reagent to estimate

total peptide and amino acid content (Mertz et al. 1974). The calibration curve showing absorbance at 570 nm versus micrograms of the amino acid glutamate is given in Figure 20. In routine measurement of protein content of column fractions 50 microlitres of sample was assayed. Carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (1956) using 1.0 ml aliquots.

The elution profile of the alkaline digest of the alcohol-soluble fraction I sample on Biogel P-6 is shown in Figure 21. Protein, as measured by absorbance at 280 nm, elutes in three major peaks from the column. The ninhydrin assay revealed two major protein peaks which eluted between the first and second A_{280} nm peaks. No protein eluted in the void volume of the column.

Low levels of carbohydrate were detected and found to elute with the second ninhydrin protein peak. No carbohydrate polymers eluted at the void volume of the column. Thus, the alkaline hydrolysis effectively hydrolyzed both the protein and the carbohydrate components of the alcohol-soluble fraction I sample. This indicates that the arabinogalactan-hydroxyproline peptide is probably not present in this fraction.

The carbohydrate detected in the column fractions eluted at a volume very close to that of glucose. This would suggest that the remaining carbohydrate present in the digest is composed of monosaccharides (galactose and arabinose).

In the early phase of this study, the total volume of the column, determined as the elution volume of tryptophan, was 290 ml. If indeed this was the true value, then the small amount of carbohydrate detected would have to be polymeric in nature (see Figure 21). The molecular weight fractionation range of the Biogel P-6 matrix is between 6,000

Figure 20. Calibration curve for the determination of α-amino group showing absorbance at 570 nm versus micrograms of glutamic acid.

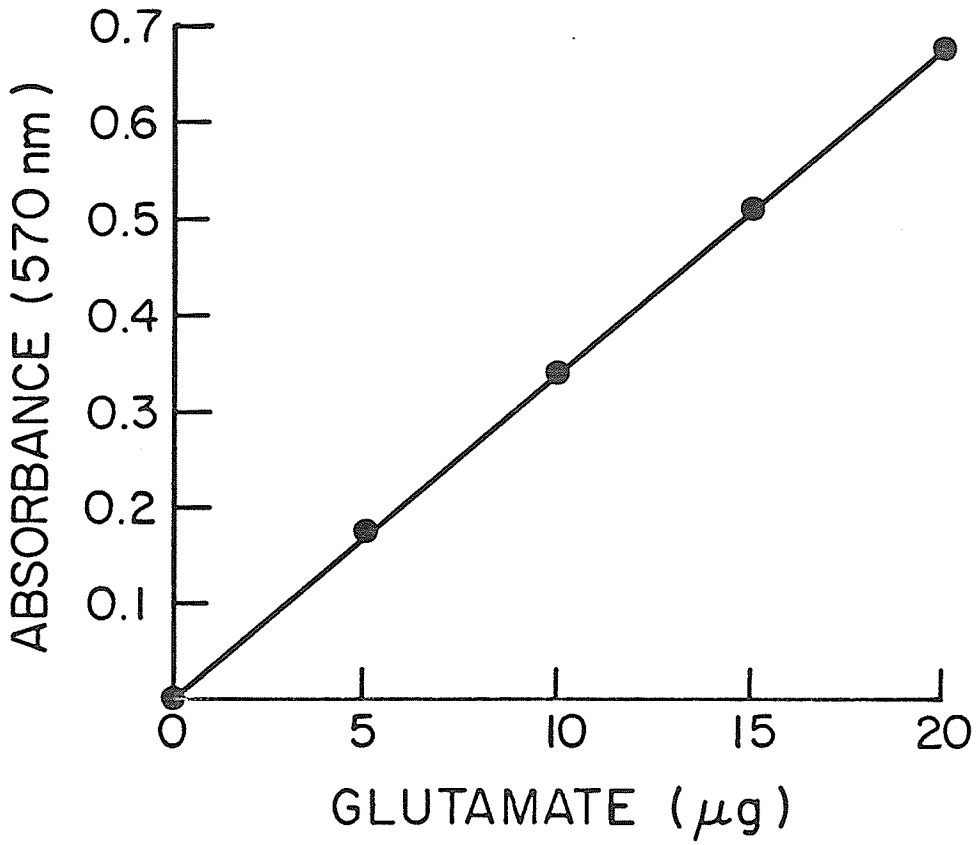

ABSORBANCE (570 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
GLUTAMATE (μg)

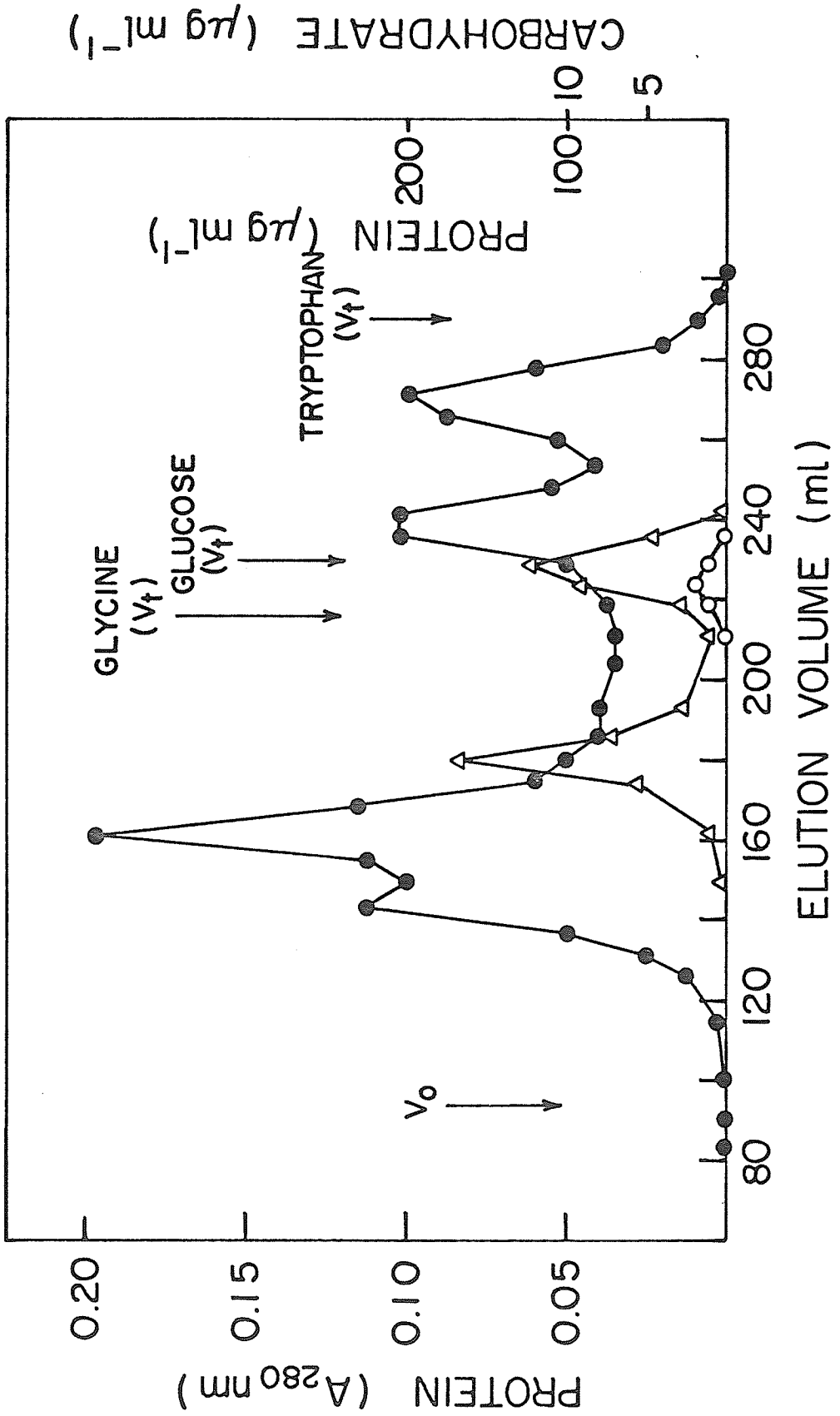
Figure 21. Elution profile from gel filtration chromatography of the alkali digest of the alcohol-soluble fraction I on Biogel P-6.

Conditions:

Column size : 1.6 x 117 cm
Bed height : 110 cm
Solvent : NaCl (0.1% w/v), NaN_3 (0.02% w/v)
Head pressure: 20 cm
Flow rate : 8.0 ml hr^{-1}
Fraction size: approx. 6.0 ml
Sample volume: 1.0 ml

Carbohydrate : o——o
Protein (A_{280} nm) : ●——●
Protein (ninhydrin reagent): △——△

Sample digestion conditions: 20 mg sample incubated with saturated $Ba(OH)_2$ (approx. 0.22 M, 1.0 ml) at 100° C for 9 hr.


PROTEIN ($A_{280\,nm}$)
0.20
0.15
0.10
0.05
CARBOHYDRATE ($\mu g\ ml^{-1}$)
200
100
10
5
PROTEIN ($\mu g\ ml^{-1}$)
V_o
GLYCINE (V_t)
GLUCOSE (V_t)
TRYPTOPHAN (V_t)
80
120
160
200
240
280
ELUTION VOLUME (ml)

and 1,000 daltons. In an attempt to confirm the total volume of the column, two other low molecular weight substances (glucose and glycine) were subjected to gel filtration chromatography under the same conditions as used for tryptophan. Glucose eluted at a volume of 230 ml, 60 ml ahead of tryptophan, and glycine eluted at 216 ml, 74 ml ahead of tryptophan (see Figure 21). The carbohydrate in the alkaline digest eluted at a volume close to that of glucose. Thus, it can be concluded that the carbohydrate was of molecular weight ($<$ 1,000 daltons) and probably comprised monosaccharides, disaccharides, or trisaccharides. It was, therefore, concluded that the tryptophan molecule was, due to its aromaticity, hydrophobically retarded on the Biogel matrix giving an elution volume that did not correspond to its molecular weight. This phenomena has been reported for Sephadex matrices (G-10, G-15, G-25, and G-50), cellulose, and starch matrices (Janson 1967).

The column fractions were also assayed for hydroxyproline. Each of three consecutive fractions were pooled (approximate volume 15.0 ml total), evaporated to dryness and the residue resuspended in a small volume of distilled water (0.5 ml) and assayed for hydroxyproline. A positive hydroxyproline assay was noted in three fractions, corresponding to the elution volume of the carbohydrate. The absorbance level was low, however, (A_{555} nm = 0.073) corresponding to 3.5 μg hydroxyproline which is just above the detection limit of the assay. Due to the extremely low levels of hydroxyproline present, no further attempts were made to confirm the presence of hydroxyproline. If this absorbance was indeed due to hydroxyproline, it is presumed that hydroxyproline occurs in a non-glycosylated form for two reasons:

i. The elution volume of hydroxyproline corresponds to

that of glycine indicating that it occurred as a small molecule and that no sugars were attached to the amino acid.

ii. The expected ratio of hydroxyproline to carbohydrate in the wheat endosperm arabinogalactan-peptide is approximately 1.7 to 100 (calculated from the data of Fincher et al. 1974). Thus, provided that the hydroxyproline present was linked glycosydically to the galactose residues in alcohol-soluble fraction I, then a vastly larger amount of carbohydrate would have been detected.

Assuming that the alcohol-soluble fraction I was composed of 100% protein, then the level of hydroxyproline would have been 1.7 μg per 20,000 μg protein or 0.008% of the protein by weight. Clearly, this is below the detection limit of the original assay procedure used when testing for the presence of hydroxyproline.

This experiment provided further evidence that an arabinogalactan-hydroxyproline peptide was not present in the alcohol-soluble fraction I.

4. Gel Filtration Chromatography of the Alkaline Digest of the Alcohol-Insoluble Fraction on Biogel P-6

The alcohol-insoluble fraction was also subjected to strong alkaline hydrolysis and the resulting digest chromatographed on a Biogel P-6 column. A sample (20 mg) of the fraction was digested in sodium hydroxide (2.5 M, 1.0 ml, 100° C, 12 hr). The digest was prepared as described in the Methods section and subjected to gel filtration chromatography in a solvent of 0.1% (w/v) NaCl and 0.02% (w/v) NaN_3.

The gel filtration profile of the alcohol-insoluble digest is shown

in Figure 22. This profile is, in general, quite similar to that of the alcohol-soluble fraction I digest (compare Figure 21). No material was observed to chromatograph in the void volume of the column, indicating that both the protein and carbohydrate components were extensively hydrolyzed. Protein, as measured by absorbance at 280 nm, gave three major peaks. As measured by ninhydrin reagent, protein occurred as two major peaks eluting between the first and second A_{280} nm peaks. A small amount of carbohydrate was detected which eluted at the same volume as the second ninhydrin peak. This elution volume was between that of glycine and glucose, indicating that the carbohydrate was of low molecular weight and probably comprised monosaccharides, disaccharides, or trisaccharides.

Although the elution volumes of the protein and carbohydrate peaks in this profile differ slightly from those in the profile of the alcohol-soluble fraction I digest (Figure 21), the two profiles are generally very similar. It was, therefore, concluded that the patterns of hydrolysis under alkaline conditions was also very similar for both fractions.

The column fractions were also assayed for hydroxyproline content. Each of three consecutive fractions were pooled (approximate volume: 15.0 ml) evaporated to dryness and the residue resuspended in a small volume of distilled water (0.5 ml) and assayed for hydroxyproline. A positive hydroxyproline assay was noted in the three fractions, corresponding to the elution volume of the carbohydrate in the column. The level of hydroxyproline appeared to be slightly higher than that of the alcohol-soluble fraction I, however, was still quite low (A_{555} nm = 0.133). From Figure 19 this corresponds to approximately 6.25 μg of

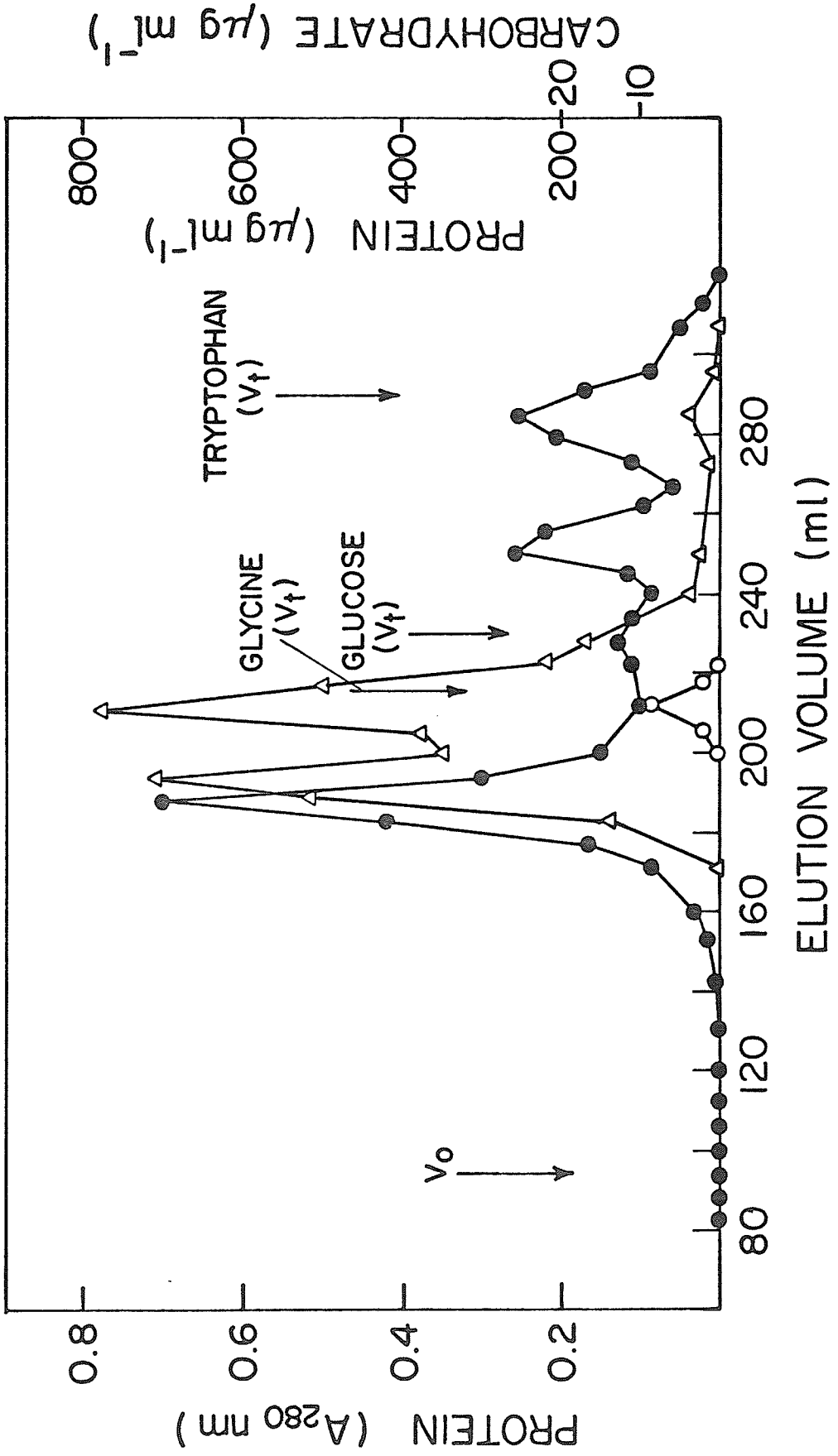
Figure 22. Elution profile from gel filtration chromatography of the alkali digest of the alcohol-insoluble fraction on Biogel P-6.

Conditions:

Column size : 1.6 x 117 cm
Bed height : 110 cm
Solvent : NaCl (0.1% w/v), NaN_3 (0.02% w/v)
Head pressure: 20 cm
Flow rate : 8.0 ml hr^{-1}
Fraction size: 6.0 ml
Sample volume: 1.0 ml

Carbohydrate : o——o
Protein (A_{280} nm) : ●——●
Protein (ninhydrin reagent): △——△

Sample digestion conditions: 26 mg sample incubated with NaOH (2.5 M, 1.0 ml) at 100° C for 12 hr.


PROTEIN (A_{280} nm)
0.2
0.4
0.6
0.8
V_o
80
120
160
200
240
280
ELUTION VOLUME (ml)
GLYCINE (V_t)
GLUCOSE (V_t)
TRYPTOPHAN (V_t)
PROTEIN ($\mu g\ ml^{-1}$)
200
400
600
800
CARBOHYDRATE ($\mu g\ ml^{-1}$)
10
20

hydroxyproline. The percentage (dry weight) of hydroxyproline present in the protein component, assuming no loss during hydrolysis was calculated to be approximately 0.03% ($\frac{6.25}{20,750}$ x 100; 20,750 micrograms protein present in original sample). This level of hydroxyproline was also well below the detection limit of the original assay procedure used to test for the presence of hydroxyproline.

It was concluded from this experiment that under alkaline digestion conditions used, the patterns of hydrolysis for the alcohol-soluble fraction I and the alcohol-insoluble fraction were similar.

5. General Conclusions on the Association of the Protein and Carbohydrate in the Alcohol-Soluble Fraction I

The results summarized in the previous sections indicate that:

i. more than 98% of the carbohydrate component of the alcohol-soluble fraction I (and the alcohol-insoluble fraction) is destroyed by hot alkaline digestion (see Table 18). This indicates that the carbohydrate is not protected from hydrolysis via a glycosidic linkage involving the reducing end of the sugars and 4-hydroxyproline.

ii. gel filtration chromatography of the alkaline digests of both fractions showed that no polymers (> 6,000 daltons) of either protein or carbohydrate remain after alkaline digestion. The carbohydrate component chromatographs as a single peak of low molecular weight (Figures 21 and 22).

iii. the pattern of hydrolysis for both fractions as determined by gel filtration chromatography of the

alkaline digests is very similar. This further indicates that a carbohydrate-hydroxyproline linkage is not present in the alcohol-soluble fraction I.

iv. only extremely low levels of hydroxyproline were detected in the fractions that were analyzed. The hydroxyproline present in these fractions occurs in the free form and does not appear to be glycosylated.

Thus, it is concluded that a carbohydrate-hydroxyproline linkage is not present in the alcohol-soluble fraction I gluten protein. The water-soluble arabinogalactan-peptide isolated from wheat endosperm (Fincher et al. 1974) is, therefore, not involved in the association or aggregation of the protein and carbohydrate in this fraction.

However, there is strong evidence (discussed previously) that the carbohydrate and protein component in this fraction may be covalently linked. The low carbohydrate content and the tedious preparation procedure required makes this fraction difficult to work with. Consequently, no further investigations were performed with this fraction. Two other possibilities do exist that may explain the tight association of the carbohydrate and protein in this fraction. Firstly, the carbohydrate may be linked to the protein via a linkage to serine. This possibility could be worthy of further investigation. In this context, it would be useful to characterize the carbohydrate component to see if the polymer is an arabinogalactan. If this is indeed the case, then a linkage to serine would be a strong possibility.

Secondly, a lipid component may be present in this fraction (see Table 2). Considering that the major sugar present in the carbohydrate

component is galactose, then there is a possibility of the presence of a glycolipid. The presence of digalactosyl lipids have been reported in wheat endosperm (Morrison 1978). A complex consisting of protein, carbohydrate, and lipid may also have to be considered in future work in this area.

J. The Nature of the Association of Protein and Carbohydrate Components in the Alcohol-Insoluble Fraction

It has been shown in this study that the carbohydrate component of the alcohol-insoluble fraction is associated with the aggregating polypeptides of wheat gluten. This component, on gel filtration chromatography on Sephadex G-200, eluted as a high molecular weight peak in the void volume of the column (refer to Figure 9). It was also shown that this component comprised 17% (w/w) of the alcohol-insoluble fraction. Monosaccharide composition analysis of the carbohydrate component indicated glucose was the major sugar present (97%; see Table 16). However, several questions remain unanswered regarding the association of carbohydrate and protein in this fraction. The experiments described in this section were designed to evaluate the nature of the association of these components and determine whether they were associated by a covalent or non-covalent linkage.

1. Enzymic Hydrolysis of the Protein Component of the Alcohol-Insoluble Fraction with Proteinase K

As described previously in this study, partial chemical modification of the protein component of this fraction via reduction of disulfide bonds and alkylation of the exposed sulfhydryl groups, did not result in the release of the carbohydrate component from the complex. Therefore, the highly purified Proteinase K enzyme was used to effect a major

modification of the protein structure. This enabled a more detailed investigation on the released carbohydrate component.

A sample of the alcohol- insoluble fraction (10.4 mg) was incubated with a solution of Proteinase K in a buffer containing Tris-HCl (100 mM), $CaCl_2$ (5 mM), pH 8.3 for 16 hr at 40° C. An enzyme protein to substrate protein ratio of 1 to 30 was used. Following the 16 hr incubation period, a further 0.2 ml of enzyme solution was added and the mixture incubated for a further period of 4 hr. The digest was then centrifuged (10,000 x g, 15 min, 20° C) and the supernatant assayed for total carbohydrate using the phenol-sulfuric acid method. The remaining pellet was washed with Tris-HCl buffer and the suspension again centrifuged. The supernatant was assayed for carbohydrate. The pellet was homogenized by vortexing in a solvent containing acetic acid (0.1 N) and urea (3 M) and assayed for total carbohydrate. The suspension was again centrifuged and the supernatant assayed for total carbohydrate. The distribution of carbohydrate of the alcohol-insoluble fraction amongst the various fractions analyzed is summarized in Table 19.

Hydrolysis of the protein component by the protease released 930 μg of carbohydrate into the Tris-HCl buffer. This represents 55% of the recovered carbohydrate. A further 20 μg of carbohydrate was released by washing the pellet with Tris-HCl buffer. A total of 750 μg carbohydrate remained in the pellet representing 44% of the recovered carbohydrate. An overall recovery of 96% of carbohydrate was obtained in the experiment. A further 250 μg of carbohydrate was released by treatment of the pellet with the acetic acid-urea solvent. Thus, a total of 1180 μg of carbohydrate representing 70% of the recovered carbohydrate could be solubilized using this procedure.

TABLE 19. Solubilization of carbohydrate[a] by the action of Proteinase K on the protein component of the alcohol-insoluble fraction.[b]

		Carbohydrate (μg)	Percentage of carbohydrate recovered
Carbohydrate solubilized by Proteinase K digestion	(A)	930	55
Buffer wash of pellet	(B)	20	1
Resuspended pellet in 0.1 N acetic acid + 3 M urea	(C)	750	44
Acetic acid-urea soluble carbohydrate in pellet	(D)	250	15
Total carbohydrate solubilized	(A + B + D)	1180	70
Total carbohydrate recovered[c]	(A + B + C)	1700	100

[a]Carbohydrate determined as "total carbohydrate" by the phenol-sulfuric acid method of Dubois et al. (1956).

[b]10.4 mg of alcohol-insoluble fraction containing 1768 μg of carbohydrate was subjected to digestion with Proteinase K.

[c]Overall recovery of carbohydrate: $\frac{1700}{1768} \times \frac{100}{1} = 96\%$.

It was concluded from this experiment that treatment of the alcohol-insoluble fraction with Proteinase K resulted in a release or solubilization of over half of the carbohydrate component. A further 15% of the carbohydrate was solubilized by extraction with acetic acid-urea solvent. These results indicate that the carbohydrate is strongly bound in the glutenin complex. It was also concluded that the structure of the protein plays a major role in the stabilization of the complex.

The release of the carbohydrate component by modification of the protein structure by enzymic hydrolysis allowed further investigation of the properties of this component. These are described in the next section.

2. Analysis of the Linkage Between the Monosaccharide Units of the Carbohydrate Component of the Alcohol-Insoluble Fraction

The linkage of the glucose units (α- or β-anomeric) of the carbohydrate component of the alcohol-insoluble fraction was investigated. Two approaches were used.

a. Enzymic hydrolysis of the carbohydrate component with amyloglucosidase. The supernatant of the Proteinase K digest (discussed above) was desalted using an Amberlite MB-1A mixed bed resin and the desalted supernatant assayed for carbohydrate. It contained 930 μg of carbohydrate. The supernatant was reduced to a final volume of 0.2 ml and 15 μl (60 μg) of the resulting solution was spotted on a paper chromatogram (Whatman No. 3). Sodium acetate buffer (25 mM, pH 4.5) containing amyloglucosidase (0.2 mg ml^{-1}) was added and the mixture incubated at 45° C for 60 min. Ion-exchange resin was again added to remove the buffer salts, separated by filtering on sintered glass, and the filtrate and washings evaporated to dryness. The material was

resolubilized in distilled water (1.0 ml) and assayed for total carbohydrate. A total of 830 μg of carbohydrate was detected indicating that no loss occurred during the desalting and digestion procedures. The supernatant was again concentrated to a final volume of 0.2 ml and 15 μl spotted on the paper chromatogram. Fifteen μl of the enzyme solution was also spotted to check for extraneous sugars in the preparation. A standard solution of glucose, maltose, and maltotriose was also spotted. The chromatogram was run (26 hr) by the downward flow technique in a solvent mixture of n-propanol, ethyl acetate, and water in a ratio of 14:2:7, and developed as described in the Materials and Methods section. No carbohydrate was detected in the protease enzyme preparation or the original protease digest of the alcohol-insoluble fraction. However, glucose was observed as the major sugar, together with trace amounts of maltose and maltotriose, in the protease digest which had been incubated with amyloglucosidase enzyme. This result indicates that the carbohydrate released from the complex by the action of Proteinase K was polymeric (no sugars detected in the original digest). Hydrolysis of the carbohydrate by amyloglucosidase to form glucose demonstrates that the sugar components are linked in α-anomeric configuration forming α-glucan polymers.

b. Partial acid hydrolysis of the carbohydrate component. A sample of the alcohol-insoluble fraction (10 mg) was digested in H_2SO_4 (0.3 N, 1.0 ml, 110° C, 60 min). The mixture was centrifuged (10,000 x g, 10 min, 20° C) to remove insoluble material and the supernatant neutralized by addition of barium carbonate. The mixture was again centrifuged to remove precipitated barium sulfate and the neutralized supernatant concentrated and spotted on a paper chromatogram. Paper chromatography was

performed as described above. Upon development of the chromatogram, glucose, maltose, maltotriose, and maltotetrose spots were visible, corresponding in relative mobility to the mixture of standard sugars (glucose, maltose, and maltotriose). Higher oligosaccharides were possibly present, but if so, were too faint to clearly identify. There was no indication of the presence of cellobiose or higher β-oligosaccharides. This experiment confirms that the carbohydrate component of this fraction is an α-glucan.

This information was very important as it then allowed a thorough check for any possible contaminating α-carbohydrases that may have occurred in the original Proteinase K preparations. This will be discussed in the next section.

3. Analysis of the Commercial Preparation of Proteinase K for Contaminating Carbohydrase Activity

Proteinase K was chosen for the cleavage of gluten proteins in this study, because of its high purity and high activity compared with other commercial preparations of Pronase (Ebeling et al. 1974). The fact that this enzyme was an endoprotease was also important. To date, there has been no reports in the literature of contaminating carbohydrases or nucleases in the commercial preparation of this enzyme. However, given that the carbohydrate component of the alcohol-insoluble fraction is an α-glucan, the preparation of Proteinase K used in this study was investigated for the presence of carbohydrases capable of hydrolyzing α1,4 and α1,6 glucose linkages. The significance of these tests was to ascertain that the carbohydrate component (released by the action of Proteinase K on the alcohol-insoluble fraction), was not modified by any contaminating carbohydrase activity.

Enzyme preparations of Proteinase K and thermolysin were tested against two substrates: soluble starch, because it contains both α1,4 and α1,6 linkages and the Phadebas substrate, a dye labelled carbohydrate substrate that is specific for α-amylase activity.

a. Incubation with soluble starch substrate. Soluble starch substrate was prepared by pouring a cold slurry into boiling Tris-HCl buffer (100 mM, $CaCl_2$ 5 mM). Upon cooling, the volume of solubilized starch solution was adjusted to give a substrate concentration of 1.0% w/v. The pH of the buffer at the incubation temperature (40° C) was 8.3. At this pH, the Tris-HCl buffer is at its maximum buffering capacity. Thermolysin solution (0.5 mg ml^{-1}) was added to an equal volume of soluble starch substrate and aliquots (0.5 ml) were withdrawn at intervals of 1.0 min and assayed for reducing sugar by the method of Roybt and Whelan (1968).

A release of significant amounts of reducing sugar from the soluble starch substrate was evident in the thermolysin-containing solution. The rate of substrate hydrolysis was equivalent to 140 μg glucose per ml reaction mixture per min. Accordingly, thermolysin contains contaminating α-glucanase activity and would be unsuitable for use in the present study. On the other hand, Proteinase K enzyme solution (0.34 mg ml^{-1}), when incubated with soluble starch substrate under the same conditions did not release any reducing sugar. In fact, extension of the incubation period for 24 hr did not result in the production of reducing sugar from the substrate. Thus, it is concluded that the Proteinase K preparation used in this study did not contain any α-glucanase activity.

b. Incubation with Phadebas substrate. Four ml of thermolysin solution (0.5 mg ml^{-1}) in Tris-HCl buffer (100 mM, 5 mM $CaCl_2$, pH 8.3) was incubated with one tablet of Phadebas substrate for 2 hr at 50° C. Total release of dye was noted, indicating complete hydrolysis of the substrate. This indicates that the thermolysin enzyme is heavily contaminated with α-glucanase activity, probably α-amylase.

Incubation with Proteinase K (2.0 ml, 40° C, 24 hr, pH 8.3) did not release any dye. The absence of α-glucanase in this preparation was, therefore, confirmed. Thus, the Proteinase K enzyme was deemed suitable for the experimental requirements of hydrolysis of the protein components of the fraction under study. That is, the carbohydrate (α-glucan) component released by Proteinase K should remain unmodified retaining its native polymeric structure.

4. Observations on the Molecular Weight of the Carbohydrate Component of the Alcohol-Insoluble Fraction

On gel filtration chromatography of the reduced-alkylated alcohol-insoluble fraction on G-200, the carbohydrate component eluted as a single peak in the void volume of the column (see Figure 9). This result invokes the question as to whether the carbohydrate is of high molecular weight and independent of the co-eluting protein component or whether the carbohydrate eluted at that volume because it is "associated" with the protein component. The same two approaches used to resolve this question vis a vis the alcohol-soluble fraction I, were applied to the alcohol-insoluble fraction.

Firstly, the content of reducing end-groups was determined. Secondly, the protein component of the fraction was selectively cleaved with Proteinase K and the resulting digest chromatographed on Sephadex G-200.

a. Estimation of reducing end-groups. Reducing sugar content in the alcohol-insoluble fraction was determined by the method of Roybt and Whelan (1968) using glucose as the standard sugar (see Figure 15). This value was expressed as a percentage of the total carbohydrate as determined by the phenol-sulfuric acid method. Because of the solubility problems encountered with this fraction and the added problem of urea interference with the reducing sugar assay (see Figure 15), the assay was made on the carbohydrate component after release from the insoluble fraction by Proteinase K. This short cut is justified as the enzyme preparation used, contained no α-glucanase activity (previously shown), which would cause an increase in the level of reducing sugar. Peak III proteins, obtained from the gel filtration of the alcohol-soluble fraction on Sephadex G-200 (see Figure 4) were used as a protein blank. This blank did not possess any detectable reducing power in the assay.

The reducing sugar content of the carbohydrate component of the alcohol-insoluble fraction was 10.8%. A value of this magnitude indicates that average molecular weight of the α-glucan is low with an estimated chain length of about 10 glucose residues. It must be stressed, however, that this is an average value only, and if the preparation contained a small amount of carbohydrate in the form of oligosaccharides or simple sugars, then the value may not be a reliable estimate of the chain length of the carbohydrate polymers. Nevertheless, this experiment does provide evidence that the α-glucan present is not of extremely high molecular weight and certainly not comparable to that of amylose or starch molecules.

b. Gel filtration chromatography of the Proteinase K digest of the alcohol-insoluble fraction on Sephadex G-200. The protein component of the alcohol-insoluble fraction was selectively hydrolyzed with Proteinase K as described previously. To ensure maximum release of carbohydrate from the fraction, the digest was dissolved in 0.1 N acetic acid and 3 M urea (see Table 19) prior to gel filtration. If the carbohydrate component of this fraction was of high molecular weight and simply co-incident with the protein component on gel filtration chromatography, then it would be expected to elute in the void volume of the column upon chromatography of the proteolytic digest.

For the purposes of comparison, a sample (40 mg) of the reduced-alkylated alcohol-insoluble fraction that had not been treated with Proteinase K was subjected to gel filtration under the same conditions. The elution profiles of these two samples are shown in Figure 23. Protein was monitored as absorbance at 280 nm and carbohydrate was assayed by the phenol-sulfuric acid method.

The profile of the Proteinase K digest of the fraction (Figure 23B) shows that no carbohydrate or protein eluted in the void volume of the column. Two peaks of carbohydrate were detected: a small broad included peak and a large peak at the total volume of the column. Protein was detected as a single peak also eluting at the total volume of the column.

The results of this experiment show that the carbohydrate component of the alcohol-insoluble gluten fraction is not of high molecular weight. Accordingly, it must be tightly associated with the aggregating protein (peak I in Figures 9 and 23A) which causes it to elute in the void volume of Sephadex G-200. The fact that the carbohydrate and protein

Figure 23. A. Elution profile from gel filtration chromatography of reduced-alkylated alcohol-insoluble gluten fraction on Sephadex G-200.

Conditions:

Column size	: 2.0 x 100 cm
Bed height	: 90 cm
Solvent	: acetic acid (0.1 N) + urea (3 M)
Flow rate	: 8.0 ml hr^{-1}
Fraction size	: 3.5 ml
Sample volume	: 2.0 ml
Sample concentration	: 12.0 mg ml^{-1}

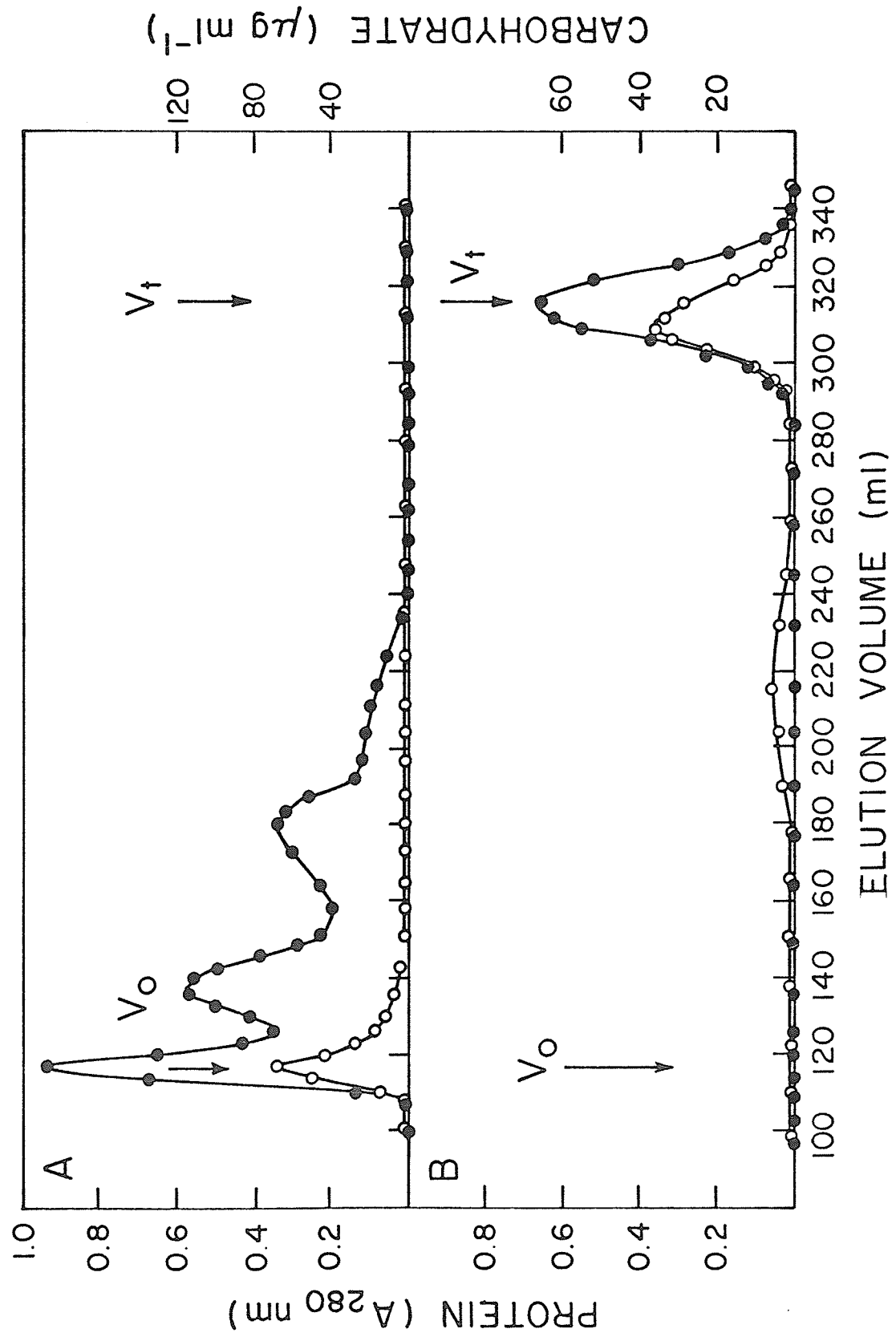
Carbohydrate: o———o
Protein : ●———●

B. Elution profile from gel filtration chromatography of Proteinase K digest of alcohol-insoluble gluten fraction on Sephadex G-200.

Conditions:

Column size	: 2.0 x 100 cm
Bed height	: 90 cm
Solvent	: acetic acid (0.1 N) + urea (3 M)
Flow rate	: 8.0 ml hr^{-1}
Fraction size	: 3.5 ml
Sample volume	: 2.0 ml

Carbohydrate: o———o
Protein : ●———●


A
B
V_o
V_t
PROTEIN ($A_{280\ nm}$)
CARBOHYDRATE ($\mu g\ ml^{-1}$)
ELUTION VOLUME (ml)
1.0
0.8
0.6
0.4
0.2
120
80
40
60
40
20
100 120 140 160 180 200 220 240 260 280 300 320 340

components elute co-incidentally at the total column volume upon chromatography of the proteolytic digest simply indicates that the molecular weight of each component is below that equivalent to the V_t (total volume) of the column. Consequently, the molecular weight of each is less than 12,000 daltons. The Sephadex G-200 column does not resolve components with molecular weights below this minimum limit.

5. Gel Filtration Chromatography of a Proteinase K Digest of the Alcohol-Insoluble Fraction on Sephadex G-15

A sample (15.2 mg) of the alcohol-insoluble fraction digested Proteinase K, was examined by gel filtration chromatography on Sephadex G-15 to obtain a more accurate estimation of the molecular weight of the components. Carbohydrate was monitored in the column fractions using the phenol-sulfuric acid method. Protein was assayed using ninhydrin reagent following the method of Mertz et al. (1974) using glutamate as the standard amino acid (Figure 19).

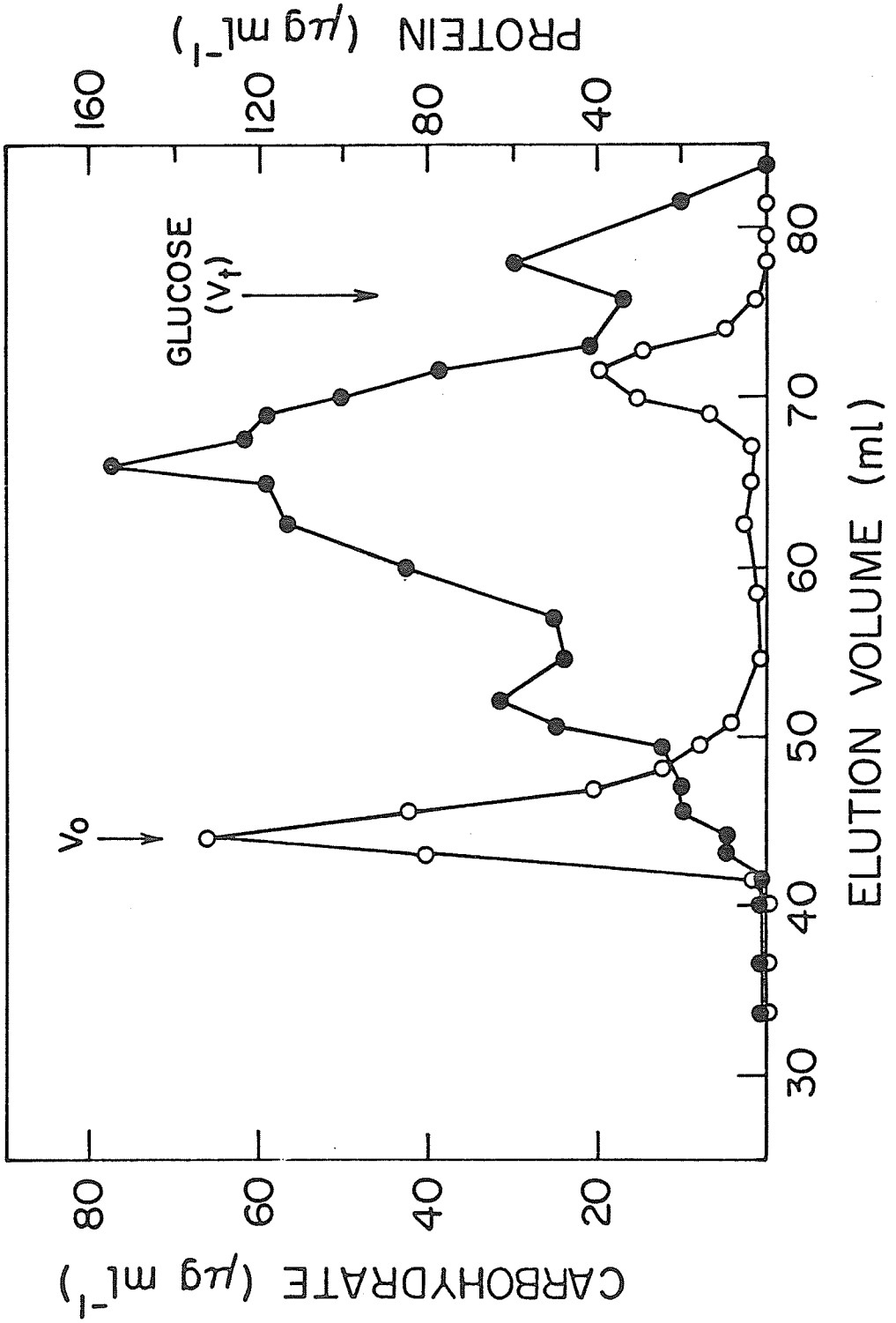
The elution profiles are shown in Figure 24. An almost complete separation of the carbohydrate and protein is evident. The carbohydrate chromatographed as two peaks in the profile. The major peak eluted at the void volume of the column and a minor included peak eluted at an elution volume very close to that of glucose which was used to determine the V_t of the column. Protein chromatographed as a broad, heterogenous profile that is mainly included in the column, indicating a heterogenous mixture of small peptides as would be expected for a proteolytic digest. A trace amount of protein eluted in the void volume of the column with the major carbohydrate peak, however, did not occur as a distinct coincident peak. It was assumed that the protein that is present in the void volume is not associated with the carbohydrate. The exclusion

Figure 24. Gel filtration chromatography of the Proteinase K digest of the alcohol-insoluble fraction on Sephadex G-15.

Conditions:

Column size: 1.5 x 100 cm
Bed height : 90 cm
Solvent : NaN_3 (0.02%)
Flow rate : 9.6 ml hr^{-1}

Carbohydrate: o———o
Protein : ●———●


CARBOHYDRATE (μg ml⁻¹)
PROTEIN (μg ml⁻¹)
ELUTION VOLUME (ml)
Vo
GLUCOSE (Vt)

limit of the Sephadex G-15 matrix is 1,500 daltons for both globular proteins and dextrans (manufacturers' specification). Thus, the majority of the protein occurs as peptides that have molecular weights below 1,500 daltons. The main carbohydrate component has a molecular weight greater than 1,500 daltons.

On the basis of the results of this experiment (almost complete separation of the carbohydrate and protein) it appeared to be a strong possibility that the association between these components in the alcohol-insoluble fraction may be non-covalent. However, because a small amount of protein eluted with the major carbohydrate component, the proteolytic digest was chromatographed on a Biogel P-6 matrix which has a higher exclusion limit than Sephadex G-15. This experiment is described and the question of covalent or non-covalent association of protein and carbohydrate discussed in detail in the next section.

6. Gel Filtration Chromatography of a Proteinase K Digest of the Alcohol-Insoluble Fraction on Biogel P-6

A sample (15.4 mg) of the alcohol-insoluble fraction was treated with Proteinase K (as previously described) and the resulting digest subjected to gel filtration chromatography on Biogel P-6. The elution profiles obtained are shown in Figure 25. The carbohydrate and protein content of the column fractions were determined as previously described.

A total separation of the carbohydrate and the hydrolyzed protein component was observed. The carbohydrate component chromatographed as two peaks: a major peak which eluted in the void volume of the column and a minor, included peak which eluted at an elution volume close to glucose. The elution of the major carbohydrate peak at the void volume of the Biogel P-6 column indicates that the molecular weight of the

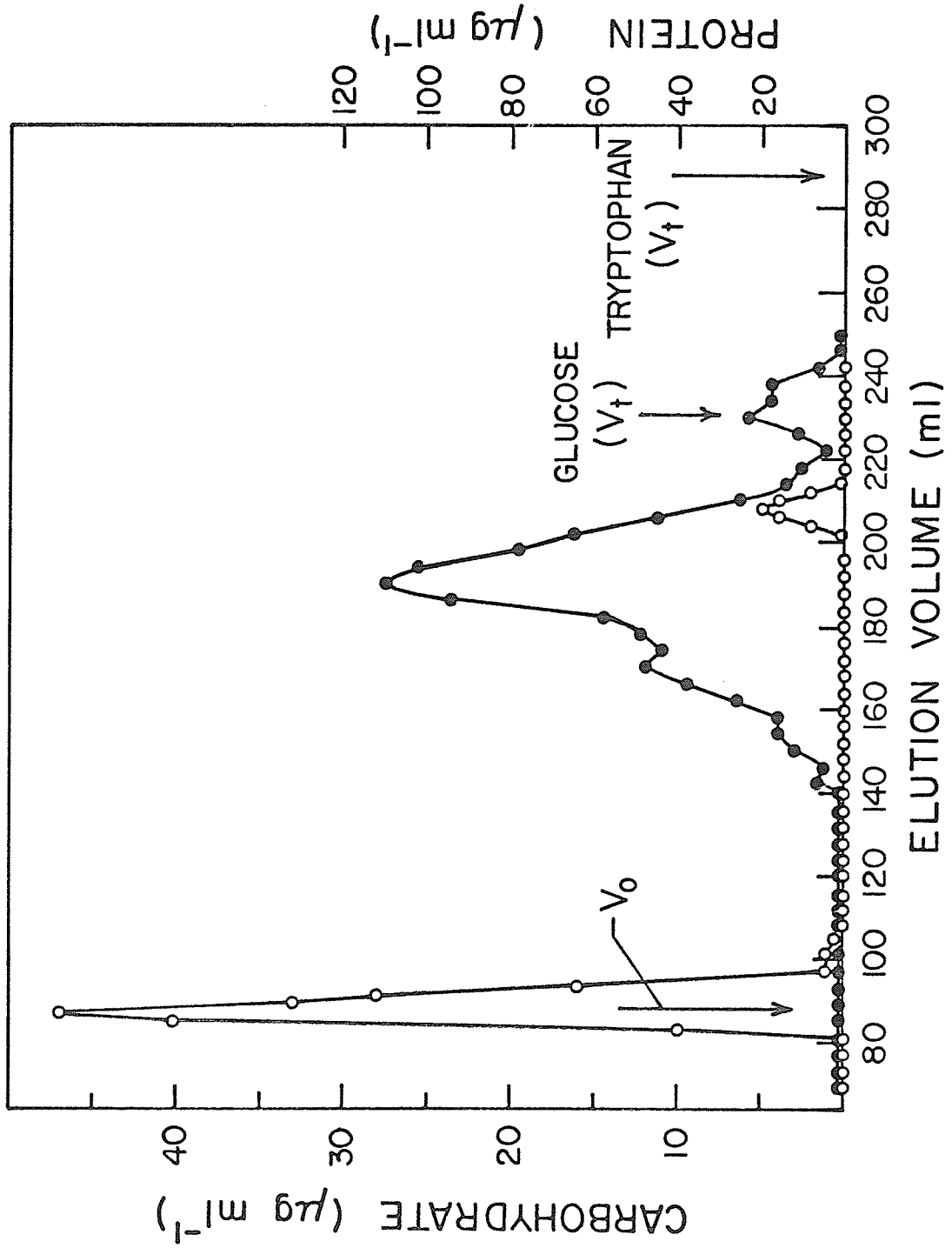
Figure 25. Elution profile from gel filtration chromatography of Proteinase K digest of alcohol-insoluble gluten fraction on Biogel P-6.

Conditions:

Column size	: 1.6 x 117 cm
Bed height	: 110 cm
Solvent	: Tris-HCl buffer (10 mM, pH 8.3) + 0.02% NaN_3
Flow rate	: 8.0 ml hr^{-1}
Fraction volume:	2.0 ml

Carbohydrate: o———o

Protein: ●———●


CARBOHYDRATE (μg ml⁻¹)
PROTEIN (μg ml⁻¹)
ELUTION VOLUME (ml)
GLUCOSE (V_t)
TRYPTOPHAN (V_t)
V_o

carbohydrate component is greater than 6,000 daltons (manufacturers' specifications).

Protein, as measured by ninhydrin reagent, eluted as several broad peaks in the included volume of the column. No protein was detected in the void volume of the column. The overall elution profile of the protein and the carbohydrate on Biogel P-6 are similar to those obtained with Sephadex G-15 (compare with Figure 24). However, the higher exclusion limit of the Biogel P-6 matrix resulted in a complete separation of the carbohydrate and protein component. This result provides very strong evidence that these components are not covalently associated when in the gluten complex. This observation is key to the conclusion of this study, hence will be considered in more detail at this point.

The carbohydrate content of the peak fraction eluting in the void volume of the profile shown in Figure 25 is 47 μg ml^{-1}. As protein was not detected in these fractions, it was concluded that if protein was present, then the content was below the detection limit of the assay (2 μg). For the sake of argument, the hypothesis was made that the protein was present at this level and was a part of a hypothetical glycoprotein. It was, therefore, of interest to calculate the expected molar ratio of amino acids to glucose residues to determine the theoretical length of a hypothetical carbohydrate side chain.

Firstly, consider the number of molecules of amino acids that comprise the weight of protein at the detection limit of the assay. Assuming the molecular weight for an average amino acid is 120, then 2 μg of amino acid contain 0.017×10^{-6} moles. The number of amino acid molecules present was calculated by multiplying this value by Avogadro's Number (6.02×10^{23}). This gave a value of 0.102×10^{17}

molecules of amino acids.

Similarly, 47 μg of glucose is equivalent to 0.29×10^{-6} moles (molecular weight of anhydrous glucose equals 160). This represents a total of 1.75×10^{17} molecules of glucose when multiplied by Avogadros Number. Therefore, the ratio of amino acid molecules to glucose molecules is:

$$\frac{0.102 \times 10^{17}}{1.750 \times 10^{17}}$$

$$= \frac{1}{17}$$

At this point it may be concluded that in the situation of one amino acid residue being attached to an α-glucan, then the detection limit would be 17 glucose units. In other words, a hypothetical glycoprotein with a ratio of 1 amino acid residue to anything greater than 17 glucose units would not be detected under the assay conditions described.

Further, consider the enzymic action of Proteinase K on a hypothetical glucopeptide structure in this fraction. The endoprotease could not be expected to hydrolyze the polypeptide chain completely to a single amino acid residue that was covalently linked to an α-glucan molecule. Apart from the limitation posed by an endo mode of attack, the enzyme would be subject to severe steric hindrance at the location of the hypothetical bond compound in the polypeptide chain. Thus, it is not unreasonable to assume that the endoprotease could only hydrolyze to within a margin of no less than five amino acid residues either side of the bond compound. This arbitrary value is in all probability a conservative estimate, however, can be used in this study. Therefore, at the hypothetical linkage site, a polypeptide of at least 10 amino acid residues attached to the carbohydrate component would be expected

to remain after proteolytic digestion. Therefore, this would serve to increase the detection limit ratio calculated above by tenfold, increasing it from 1 amino acid to 17 glucose residues, to 1 amino acid residue to 170 glucose residues for the experimental conditions described in Figure 25.

Thus, a hypothetical glycopeptide, if present, would have to possess a ratio of 1 amino acid residue to greater than 170 glucose units. Clearly this experiment presents very strong evidence that a covalent linkage between carbohydrate and protein is not present. It was concluded that the association of protein and carbohydrate components in the gluten complex is non-covalent in nature and involves very strong secondary forces.

In addition to the above evidence for a non-covalent association of protein and carbohydrate, it must be pointed out that there has been no reported glycopeptide structure involving a glucose-amino acid linkage directly. A structure involving glucose residues directly attached to protein has been proposed to explain the mechanism of "unprimed" starch synthesis (Whelan 1976; Krisman and Barengo 1975). However, this structure has not been isolated and the bond compound linkage has not been characterized or confirmed to date.

7. Release of Carbohydrate by Partial Acid Hydrolysis of the Alcohol-Insoluble Fraction

It has been noted earlier in this thesis that alteration of the tertiary structure of the protein component of this fraction, via reduction of disulfide bonds and alkylation of exposed sulfhydryls did not release the carbohydrate component from the gluten complex. Therefore, it must be assumed that the bonding forces involved in the stabilization

of the gluten complex are localized at the lower levels of protein structure (i.e. either secondary or primary). It has also been demonstrated in this study that the forces responsible for the association of carbohydrate and protein components in the gluten structure are not covalent but are secondary in nature. Results from the gel filtration chromatography experiments on Proteinase K digests of this fraction showed that disruption of the protein component at all levels of structure (i.e. tertiary, secondary, and primary) was necessary to release the carbohydrate from the gluten complex.

To obtain some further information on the nature of the protein-carbohydrate interactions the alcohol-insoluble fraction was treated with various mild acids at high temperatures. These experiments were basically designed to determine if a partial hydrolysis of the protein component would result in a release of the α-glucan from the gluten complex.

In the first experiment, samples of the alcohol-insoluble fraction were incubated with a weak acid, oxalic acid (0.0125 M, pH 2.15) at a temperature of 110° C for 3 hr. Little digestion of the protein occurred and at the neutral pH the protein components precipitated. No carbohydrate was released from the complex into the supernatant.

In the next experiment, samples of the alcohol-insoluble fraction were incubated with increasing concentrations of hydrochloric acid (0.1 M, 0.25 M, and 1.0 M) for 4 hr at 110° C. A release of carbohydrate from the complex into the supernatant was noted at all three concentrations of acid. Upon neutralization of the partial digests, the protein in the 0.1 M HCl digest precipitated. There was very little carbohydrate in the precipitate separated by centrifugation.

In the third experiment, a time study of the release of carbohydrate from the alcohol-insoluble fraction during hydrolysis of the fraction with 0.3 N H_2SO_4 was carried out. The sample (10 mg) was incubated with H_2SO_4 (0.3 N, 110° C) for 15 and 60 min. The resulting suspension was centrifuged (15,000 x g, 15 min, 4° C), the supernatant retained, and the pellet resuspended in H_2SO_4 (0.3 N). The release of carbohydrate at the two times of hydrolysis in the weak acid is given in Table 20. Surprisingly, no carbohydrate was released from the sample after 15 min of acid hydrolysis. However, after 60 min incubation, essentially all of the carbohydrate was released. After 60 min of acid hydrolysis, the carbohydrate component was comprised of 43% reducing sugar, indicating that it was also extensively hydrolyzed. Results of this experiment indicated that release of the carbohydrate from the gluten complex was dependent on a certain degree of prior hydrolysis of the protein component. Obviously, in an experiment of this nature on an insoluble mass, poor solvent accessibility would be an important factor during the initial stages of hydrolysis. Nevertheless, a small amount of carbohydrate would be expected to be released under these conditions. It was concluded that an extensive breakdown of the protein component was required before the non-covalent forces of association in the complex were sufficiently weakened to allow release of the carbohydrate.

Paper chromatography of the neutralized digest after 60 min hydrolysis (method described previously) revealed the presence of glucose, maltose, and maltotriose. This pattern of hydrolysis is consistent with the products of partial acid hydrolysis of α-glucan polymers under the conditions of the experiment.

TABLE 20. Temporal release of carbohydrate from the alcohol-insoluble fraction upon hydrolysis with mild acid.[a]

Time of hydrolysis (min)	Percentage of carbohydrate[b] recovered in		Percent reducing sugar[c] present in supernatant
	Supernatant	Pellet	
15	0	100.0	-
60	95.4	4.6	43.0

[a] 0.3 N H_2SO_4, 110° C.

[b] Carbohydrate determined as "total carbohydrate" by the phenol-sulfuric acid method of Dubois *et al.* (1956).

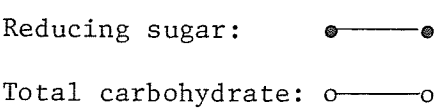
[c] Reducing sugar determined by the copper-reduction method of Roybt and Whelan (1968).

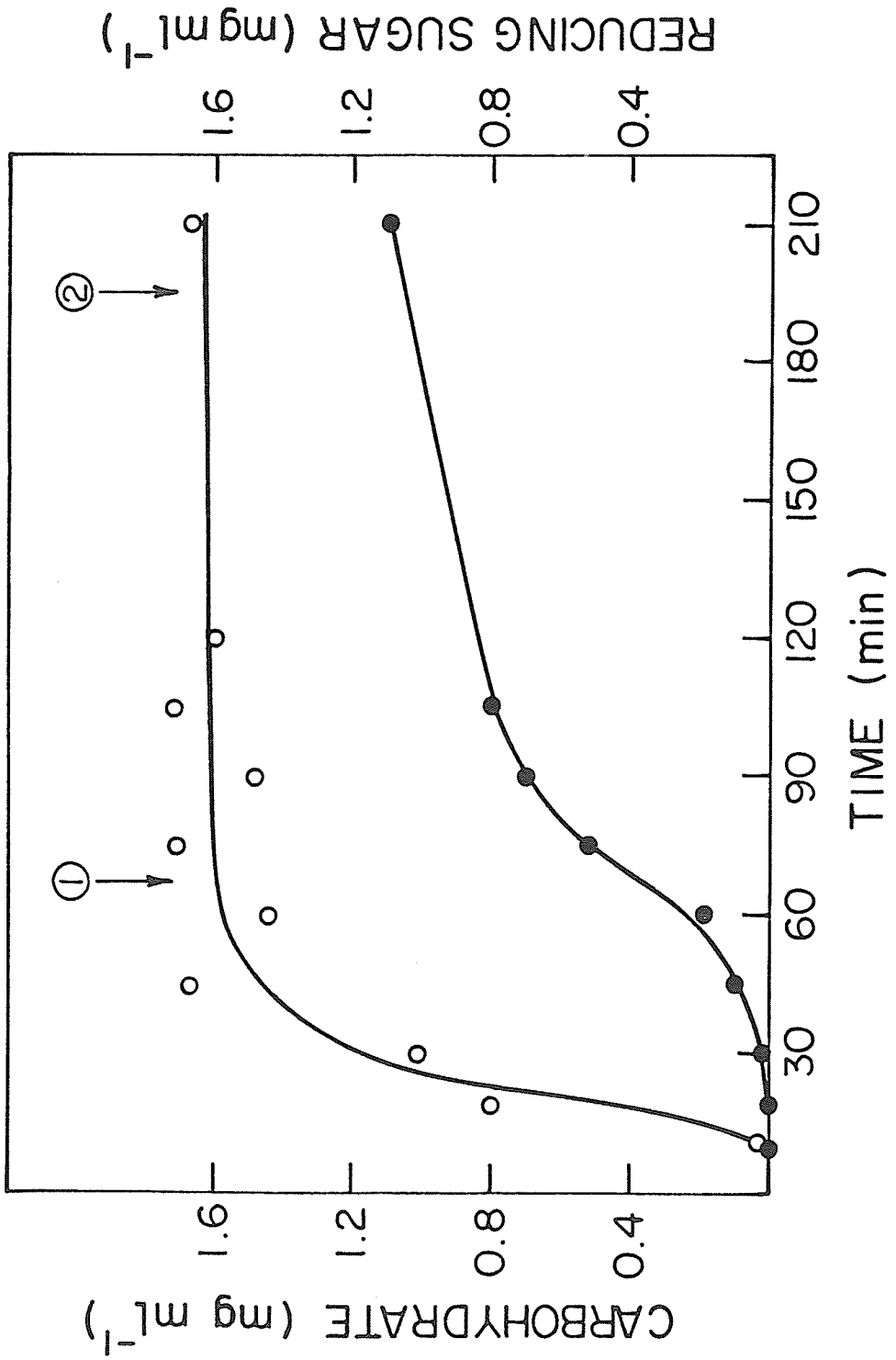
In the fourth experiment, a second time course study of the release of carbohydrate from the alcohol-insoluble fraction upon partial acid hydrolysis was carried out. A sample (50 mg) of the fraction was suspended in 5.0 ml of 0.3 N H_2SO_4 and incubated in an oven at 110° C. Aliquots (300 μl) were withdrawn at various time intervals, rapidly cooled, and neutralized by addition of 30 μl of 3 N NaOH. The mixtures were then centrifuged (8,000 x g, 5 min, 20° C) to remove insoluble material. The supernatants were assayed for total carbohydrate by the phenol-sulfuric acid method and the reducing sugar contents by the method of Roybt and Whelan (1968). The curve for the solubilization of carbohydrate under these conditions is shown in Figure 26.

Initially, no carbohydrate was released from the alcohol-insoluble gluten complex. After 15 min hydrolysis period carbohydrate began to solubilize from the complex and after 60 min the carbohydrate was totally released. At point 1 in the reaction curve (see Figure 26), the reaction mixture appeared as a very fine suspension, while at point 2 it was a clear solution (i.e. no precipitate was obtained on centrifugation). No change in the total solubilized carbohydrate was observed for the period of hydrolysis between these two points. Accordingly, it was concluded that complete solubilization (hydrolysis) of the protein component is not a requirement for total carbohydrate release.

A distinct lag phase was observed in amount of reducing sugar in the supernatant with time of hydrolysis (see Figure 26). This suggests that the α-glucan may have to be first released from the gluten complex before hydrolysis will proceed under conditions of mild acid hydrolysis. Under the experimental conditions the hydrolysis of the α-glucan to its constituent glucose residues proceeds very slowly as the reducing

Figure 26. Release of carbohydrate and reducing sugar from the alcohol-insoluble gluten complex with time of mild acid hydrolysis (0.3 N H_2SO_4, 110° C).




CARBOHYDRATE (mg ml⁻¹)
REDUCING SUGAR (mg ml⁻¹)
TIME (min)
0.4
0.8
1.2
1.6
30
60
90
120
150
180
210
①
②

sugar content after 210 min of hydrolysis is approximately 65%.

The results from these experiments indicate that the carbohydrate component of the alcohol-insoluble fraction can be released upon mild acid hydrolysis of the protein. A partial, but not total, disruption of the protein structure is required for the solubilization of the carbohydrate.

The results of these experiments are consistent with the hypothesis that the association of carbohydrate components with the aggregating polypeptides of glutenin is stabilized by extremely strong secondary (non-covalent) forces.

V. GENERAL DISCUSSION

The principal aims of the research undertaken for this thesis were to investigate the nature and the role of the carbohydrate components of wheat gluten and to evaluate the contribution of these components to the structure of functional gluten in bread doughs. The overall strategy of the research plan was to extend the available knowledge on the physicochemical structure of the carbohydrate and the protein components of gluten on the assumption that such information is key to the structure of gluten. As will be evident from the discussion that follows, some of the aims of this project have been realized more fully than others.

The study confirmed that carbohydrate is present in significant levels in the "protein" fractions obtained from standard gluten preparations. The techniques used in this study yielded three carbohydrate-containing fractions; an alcohol-soluble fraction containing 0.6% carbohydrate, an alcohol-soluble fraction I with 2.9% carbohydrate, and an alcohol-insoluble fraction with 17.0% carbohydrate. The alcohol-soluble fraction I was obtained from the total alcohol-soluble fraction of gluten by gel filtration.

Analysis of the alcohol-soluble and the alcohol-insoluble fractions by gel filtration chromatography on Sephadex G-200 showed that the carbohydrate components were exclusively associated with the fractions that eluted in the void volume (i.e. high apparent molecular weight). It

should be noted that use of the term "high molecular weight" is purely operational in relation to gel filtration and does not carry the fundamental implication that the particles are true molecules (i.e. all atoms covalently linked). Indeed, as will be indicated later, some of these so-called high molecular weight fractions are aggregates of smaller molecules.

Partial modification (i.e. reduction of disulfide bonds and alkylation of exposed sulfhydryls) of the protein components of two of the carbohydrate-containing fractions, the alcohol-soluble fraction I and the alcohol-insoluble fraction, altered the elution profiles of the protein components of these fractions but did not produce any changes in the profiles of the carbohydrate components on gel filtration chromatography on Sephadex G-200. The carbohydrate eluted in the void volume of the column with the high molecular weight protein peak as in the case of the unmodified fractions.

Examination of the peak fractions (obtained by gel filtration chromatography) by SDS-PAGE demonstrated that the high molecular weight protein fraction (peak I) comprises aggregates of polypeptides of molecular weight 68,000 daltons and lower. The carbohydrate components were associated with specific protein subunits in the peak I fraction. Furthermore, it was shown that the largest polypeptides of gluten ($>$ 68,000 daltons) are released from the gluten complex on reduction and alkylation and are eluted as peak II fraction. These large subunits show no tendency to associate either with themselves or with the carbohydrate under the conditions of this study.

The results on the protein components obtained by SDS-PAGE and gel filtration chromatography did not provide a clear differentiation between

the alcohol-soluble fraction I and the alcohol-insoluble fraction. The two fractions differed slightly in the proportion of the large protein subunits (peak II proteins). These large subunits were present in substantially lower amounts in the alcohol-soluble fraction I. From these results, it would appear that the classical differentiation of gliadin and glutenin on the basis of solubility is not strictly applicable to these fractions. The large protein subunits ($>$ 68,000) are generally associated with the glutenin (alcohol-insoluble) fraction.

The two fractions (alcohol-soluble fraction I and alcohol-insoluble fraction) are clearly different in the monosaccharide composition of their carbohydrate components. This component of the alcohol-soluble fraction I contains galactose as the major sugar (70%), whereas glucose was the main sugar (97%) of the carbohydrate component of the alcohol-insoluble fraction. It is quite possible that the carbohydrate component of the alcohol-soluble fraction I contributes in some way to its solubility in alcohol.

As found by other researchers, significant differences in amino acid composition of the gluten protein fractions were observed. In general, the alcohol-soluble protein fractions contained more hydrophobic residues than the alcohol-insoluble fractions. Accordingly, the former fraction possessed a much lower charge density per residue than the alcohol-insoluble fraction.

The peak fractions obtained by gel filtration of the reduced-alkylated alcohol-soluble fraction I and the reduced-alkylated alcohol-insoluble fraction also showed some interesting differences in amino acid composition. The peak I fractions (contain carbohydrate) are more hydrophobic compared with peak II and peak III fractions (do not contain

carbohydrate). The hydrophobicity of the protein subunits of peak I fractions may be involved in the aggregation mechanism. In contrast, peak II proteins are not very hydrophobic and in this aspect are similar to other structural proteins such as fibroin and collagen. The low hydrophobicity of these proteins could well be exploited in the future for purification using hydrophobic interaction chromatography.

Considering the overall amino acid compositions of the gluten proteins examined in this study, some comments can be made on the possible secondary structures of these proteins. The high proportion of glycine and proline residues, if at all randomly distributed in the amino acid sequence, would almost totally preclude the nucleation of the α-helix conformation. A lack of α-helicity in the secondary structure of gluten proteins may also explain the poor solubility properties of these proteins.

The amino acid compositions of gluten proteins are generally consistent with the β-sheet secondary structure. Proline and glycine, whilst being the strongest α-helix "breakers", are the strongest β-turn "formers". The hydrophobic amino acids are also strong β-sheet "formers".

The probability of the existence of the β-sheet in the secondary structure of the gluten proteins was investigated by applying the predictive scheme of Chou and Fasman (1978a, b) to the 25 N-terminal amino acid residues of A_2-gliadin. A folded β-sheet structure involving a β-turn appears highly probable. No α-helix, or random coil structure could be predicted for the same sequence. A closer examination of the structure showed that the amide amino acids occur in a "close cluster" around the β-turn, forming a region of high hydrogen bonding potential.

It is likely that this region could serve to stabilize the β-turn structure. Addition of hydrogen bond breaking agents such as urea would have the effect of destabilizing the β-turn and cause unfolding. Characteristic of the predicted structure is a highly hydrophobic region involving hydrophobic amino acid residues in both β-sheets, which are in close proximity in the folded structure. Also, it is interesting to note that the two charged amino acids that occur at the extremity and opposite sides of this "hydrophobic core" can serve to stabilize this region through ionic interactions.

A β-sheet secondary structure (stabilized mostly by secondary forces - hydrogen and hydrophobic bonds) in gluten proteins would explain technologically important rheological properties of dough systems such as elasticity and plasticity. For example, the mixing and dough development process may well involve the unfolding and realignment of more extended, stable sheet structures in the protein matrix. As more sequence data becomes available, more secondary structures of gluten proteins can be predicted and eventually generalized structures developed and confirmed.

Detailed investigation of the carbohydrate-containing fractions showed that the carbohydrate was associated with the "aggregating" polypeptide subunits of wheat gluten. Selective cleavage of the protein component by proteolytic digestion of the fractions and rechromatography of the digest on Sephadex G-200, demonstrated that disruption of the primary structure of the protein component resulted in the release of the carbohydrate component. It was shown that the carbohydrate component of both the alcohol-soluble fraction I and the alcohol-insoluble fraction are relatively low molecular weight. Accordingly, it was concluded that

the carbohydrate components occur in the "high molecular weight" fractions (by gel filtration chromatography) because they are closely associated with the protein component. It is also apparent that the primary structure of the protein is necessary for stabilization of the carbohydrate-protein complex. Partial modification of the protein structure by reduction of disulfide bonds did not release the carbohydrate component.

Examination of the alcohol-soluble fraction I by electrophoretic techniques showed that coincident protein and carbohydrate stains occurred in the agarose-acrylamide gels. This suggests that the carbohydrate of this fraction is covalently linked to a protein component. Because the major sugars of the carbohydrate in this fraction are galactose and arabinose, the possibility of a hydroxyproline-arabinogalactan linkage was investigated. A proteoglycan involving a hydroxyproline-galactose linkage has been previously isolated in wheat endosperm by Fincher et al. 1974 (see Literature Review). However, only a trace of hydroxyproline was detected and the fraction was not resistant to alkali digestion (β-elimination reaction). Accordingly, it was concluded that the linkage between the carbohydrate and protein was not a glycosidic linkage involving 4-hydroxyproline. More research is needed to identify the nature of the carbohydrate-protein linkage in this fraction. In such research, the carbohydrate component should be completely characterized. If it is shown to be an arabinogalactan, as suggested by the monosaccharide composition, then it is possible that the carbohydrate may be covalently linked to serine. There is also the possibility that lipids may be involved in the carbohydrate-protein binding (interaction) of this fraction. The possible involvement of lipids or glyco-

lipids (galactosyl glycerides) in the aggregation of the alcohol-soluble fraction I could be a fruitful area of future research.

Research on the nature of the association of carbohydrate and protein in the alcohol-insoluble fraction produced more definitive results. Gel filtration chromatography of the protease digest of this fraction on Biogel P-6 facilitated complete separation of the protein and carbohydrate components. Accordingly, it was concluded that the association of protein and carbohydrate in this fraction was non-covalent. However, it is obvious that the association is very strong; destruction of the primary structure of the protein was necessary to release the carbohydrate component. In fact, the association of these components, although non-covalent, could easily be incorrectly interpreted as being covalent due to the strength of association. An association of protein subunits and carbohydrate of this nature may well be unique and restricted to wheat flour proteins. These results emphasize the importance of detailed, careful experimentation before a claim of covalent attachment of carbohydrate to protein (glycoprotein) is rendered. In this context, the results which led to the conclusion on the presence of glucose-containing glycoproteins in wheat flour reported by Graveland et al. (1979) should be re-examined.

The carbohydrate component released from this fraction by the action of protease was partially characterized. Enzymic hydrolysis of the polymer with amyloglucosidase yielded glucose as the major product. Partial acid hydrolysis produced glucose, maltose, maltotriose, but not cellobiose. It was, therefore, concluded that the glucose residues in the carbohydrate were linked in α-anomeric configuration to form an α-glucan.

Another important and interesting aspect of the results on the carbohydrate of the alcohol-insoluble (glutenin) fraction is its molecular size. Although somewhat heterogenous by gel filtration chromatography, the molecular weight is relatively low and the range is narrow. The molecular weight of the major portion of the carbohydrate is between 6,000 and 12,000 daltons. Considering that a high level of protein structure is a prerequisite for the interaction with the carbohydrate component, it is reasonable to speculate that the molecular size and type of carbohydrate may also be important.

This suggestion is in general agreement with the published results (see Literature Review) of rheological measurements which showed that molecular size of the "clinical dextrans" added to wheat flour doughs determined its effect on the rheological properties. These observations were explained by the hypothesis that changes in rheological properties were due to specific interactions between the added dextrans and gluten proteins. This hypothesis is supported by the results obtained in this study which showed that specific glutenin subunits interact strongly with an α-glucan to form large aggregates.

The involvement of α-glucans in the formation of large complexes with gluten proteins suggests a crucial role for this minor component in the rheological properties of bread doughs. The results expressed in this thesis open up challenging new directions in research on biochemical aspects of breadbaking quality of wheat flour. Foremost in these new directions are the interactions between certain minor components with the major components that have received most of the research and individual attention in the past.

The major contribution of this thesis is the demonstration that some

wheat flour polysaccharides interact strongly with specific gluten polypeptides to form high molecular weight complexes in the gluten matrix. More research is required to elucidate the exact nature of the interaction and its precise rheological significance. Whilst it was demonstrated that carbohydrate interacts specifically with the aggregating polypeptides of gluten, the precise involvement of the polysaccharide component is not known.

The studies reported in this thesis have been performed on flour derived from one wheat genotype of the hard red spring class, which is of good breadmaking quality. Analogous studies using wheat cultivars of diverse genotype and breadmaking quality are needed to extend the findings of this study to bread wheats, in general.

The research described should also serve to stimulate further research in the area of modification of rheological properties of wheat flours with added polysaccharides. The strength and nature of the interactions that produce these rheological modifications may well depend on the molecular size of the polysaccharide. Furthermore, specific chemical modification of the polysaccharide of the desired molecular weight range may serve to enhance or diminish the interaction between the components. Further research in this area may provide a unique biochemical approach to improving the rheological properties of wheat flours derived from higher yielding, but poorer quality bread wheats that are often discarded in wheat breeding programs.

In conclusion, the future development of cereal chemistry research should prove extremely interesting, challenging, and hopefully beneficial to mankind.

VI. CONTRIBUTIONS TO KNOWLEDGE

This study was carried out to investigate the associative properties of the protein and carbohydrate and their possible involvement or role in the overall structure of wheat gluten. The major contributions to knowledge arising from this research are listed below:

1. Three carbohydrate-containing fractions were isolated from wheat gluten : the alcohol-soluble fraction, the alcohol-soluble fraction I (obtained by gel filtration of the alcohol-soluble fraction), and the alcohol-insoluble fraction. These fractions contained 0.6%, 2.9%, and 17.0% (w/w) carbohydrate, respectively.
2. Gel filtration chromatography on Sephadex G-200 showed that the carbohydrate component of these fractions was exclusively associated with the "high molecular weight" fraction.
3. Partial chemical modification of the protein component (reduction of disulfide bonds and alkylation of exposed sulfhydryls) did not release the carbohydrate component from the high molecular weight fractions.
4. The SDS-PAGE patterns of the peak fractions obtained by gel filtration of the reduced-alkylated alcohol-soluble fraction I and the reduced-alkylated

alcohol-insoluble fraction showed that the carbohydrate components were associated with specific glutenin subunits of intermediate molecular weight (< 68,000 daltons) which were aggregated under the conditions of gel filtration.

5. The highest molecular weight subunits of glutenin (68,000 to 110,000) did not show any tendency to associate with the carbohydrate.
6. Amino acid composition studies showed distinct differences between the peak I subunits (< 68,000) and peak II subunits (68,000 to 110,000) obtained from gel filtration of the reduced-alkylated samples of alcohol-soluble fraction I and the alcohol-insoluble fractions. Peak I subunits (contain carbohydrate) were more hydrophobic than the peak II subunits. The average hydrophobicity values of peak II subunits were extremely low, similar to those of collagen.
7. The SDS-PAGE patterns of the alcohol-soluble fraction I and the alcohol-insoluble fraction were similar, however, these fractions were clearly differentiated by the monosaccharide compositions of their carbohydrate components. Galactose (70%) was the major sugar present in the alcohol-soluble fraction I, whereas glucose (97%) was the major sugar in the alcohol-insoluble fraction.

8. Coincident stains for carbohydrate and protein were obtained after disc electrophoresis of the alcohol-soluble fraction I on agarose-acrylamide gels, indicating that the carbohydrate is probably covalently linked to the protein in this fraction.
9. Chromatography of the Proteinase-K digest of the alcohol-soluble fraction I showed that the carbohydrate component was of relatively low molecular weight ($<12,000$).
10. A glycosidic linkage involving 4-OH proline and galactose was tested for in the alcohol-soluble fraction I, but was not detected. It was postulated that the linkage between carbohydrate and protein in this fraction may involve serine.
11. Gel filtration chromatography of a proteolytic digest of the alcohol-insoluble fraction on Biogel P-6 led to a separation of the carbohydrate component from the protein. The molecular weight of the major portion of the carbohydrate was between 6,000 and 12,000 daltons (by gel filtration).
12. Since the protein and carbohydrate components of the proteolytic digest of the alcohol-insoluble fraction were completely separated by gel filtration, it was concluded that the association between the carbohydrate is non-covalent.
13. Hydrolysis of the released carbohydrate component with amyloglucosidase yielded glucose, indicating

that the glucose residues are linked in α-anomeric conformation forming an α-glucan.

14. Analysis of the secondary structure of the 25 N-terminal amino acids of A_2-gliadin using the predictive methods of Chou and Fasman (1978a, b, 1977) predicts a β-sheet structure involving residues 1 to 11 and residues 16 to 25.

15. Analysis of the A_2-gliadin sequence for β-turn secondary structure using the above method, showed the presence of a β-turn at residues 12, 13, 14, and 15.

16. In the proposed secondary structure of the 25 N-terminal sequence for A_2-gliadin, it was postulated that the β-turn structure was stabilized by a "close cluster" of amide amino acids (occurring immediately before and after the β-turn in the sequence) forming a region of high hydrogen bonding potential between the β-sheet structures.

17. An additional stabilizing zone a hydrophobic "core" region was also proposed involving hydrophobic amino acid residues of both β-sheets which, in the folded structure, would be in close proximity.

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APPENDIX I: THE EFFECT OF IONIC STRENGTH ON THE GEL FILTRATION BEHAVIOR OF GLUTEN PROTEINS

It was noted in this study that incorporation of small amounts of salt to increase the ionic strength of the solvents used, had a pronounced effect on the results obtained for some of the gluten protein fractions by gel filtration chromatography. Generally, solvents of very low ionic strength have been used in experiments with gluten proteins due to the decreased solubility of these proteins in salt solutions.

The alcohol-soluble peak III proteins from gel filtration of the alcohol-soluble fraction (refer to Figure 4) were chosen for further investigation. The gel filtration profile of the alcohol-soluble fraction under solvent conditions of very low ionic strength shows that peak III fraction elutes as a broad peak. A distinct "leading edge" or adsorbtive effect is evident suggesting that the fraction is apparently heterogeneous. Close examination of the elution profile of this peak shows that a "shoulder" may be present. Accurate determination of the peak elution volume from a profile of this nature is difficult and consequently the molecular weight estimated from such results can only be approximate.

When examined by PAGE at pH 3.1 (refer to Figure 5, pattern 6), peak III fraction produced a pattern with a number of bands of high mobility. SDS-PAGE results, however, showed that the proteins in that fraction migrate as a single band and, therefore, are of the same molecular weight (refer to Figures 7 and 8). The observed heterogeneity on PAGE can, therefore, be attributed to differences in the charge density of the proteins.

Accordingly, it was concluded that protein-protein interactions, or ionic interactions involving the protein molecules and the unproton-

ated (charged) carboxyl groups on the Sephadex G-200 matrix, may be responsible for the irregular gel filtration profile of this fraction under conditions of very low ionic strength. Sephadex matrices are known to possess a low content of carboxyl groups (0.1 to 0.2 mequiv g^{-1} dry Sephadex) (Morris and Morris 1976). Because the pK_a of carboxyl groups is approximately at pH 1.8 to 2.2, the majority of these groups would be unprotonated (i.e. negatively charged) at the running pH of the 0.1 N acetic acid - 3 M urea solvent (pH 4.0).

Proteins of peak III column fractions were pooled as indicated by horizontal bars in Figure 4 and the pooled peak III fraction dialyzed against frequent changes of distilled water, frozen and freeze dried. A sample of the peak III fraction was redissolved in the same solvent used in the original fractionation (0.1 N acetic acid, 3 M urea) and rechromatographed on Sephadex G-200. Approximately the same amount of the fraction (290 mg) as obtained from the original profile (Figure 4) was loaded onto the column in a volume (30 ml) approximately equal to that used in the original procedure. The elution profile of this fraction, under conditions of very low ionic strength[1] (I approximately equal to

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[1] Ionic strength (I) calculated from the formula

$$I = \frac{1}{2} \sum m.z^2$$

where m = molality of ion species
z = valence of ion

for CH_3COOH, $K_a = 1.76 \times 10^{-5}$

therefore for 0.1 M CH_3COOH, $I = 1.33 \times 10^{-3}$

for 0.020 M K CL, $I = 0.02$

The ionic strength of the urea solution was assumed to be zero. However, in practice, urea may confer a small amount of ionic strength to the solvent due to ionic contaminants.

0.00133), is shown in Figure 27.

On rechromatography the elution profile of the peak III fraction, under these conditions, was similar to that obtained on the gel filtration of peak III proteins in the original alcohol-soluble fraction (compare Figure 27 with Figure 4). The same "leading edge" effect was noted indicating a heterogeneous peak. Comparison of the elution profiles of peak III proteins in Figure 4 and Figure 27 shows that rechromatography of this fraction results in a lower elution volume (50 ml). This effect was also noted when other gluten fractions were individually subjected to rechromatography.

A second sample of the peak III fraction was dissolved in a solvent (of slightly increased ionic strength) containing 0.1 M acetic acid, 3 M urea and 20 mM KCl. The Sephadex G-200 column was also re-equilibrated in this solvent and the same volume and concentration of sample as above was chromatographed. The elution profile of peak III proteins under conditions of slightly increased ionic strength is also given in Figure 27. The elution profile in the presence of KCl was distinctly different from that obtained without KCl. The peak was symmetrical with an elution volume of 850 ml, some 100 ml lower than the value obtained with the salt-free solvent. Because of the increased symmetry of the elution profile of peak III when KCl was present in the solvent, the elution volume, and hence the molecular weight of the peak III proteins, could be determined more accurately.

It was concluded from this experiment that addition of 20 mM KCl to the solvent system increased the ionic strength sufficiently to overcome the effects of ionic interactions that apparently interfere in the chromatography of the peak III proteins on Sephadex G-200. This

Figure 27. Elution profile of gel filtration chromatography of alcohol-soluble Peak III proteins under conditions of zero and low ionic strength on Sephadex G-200.

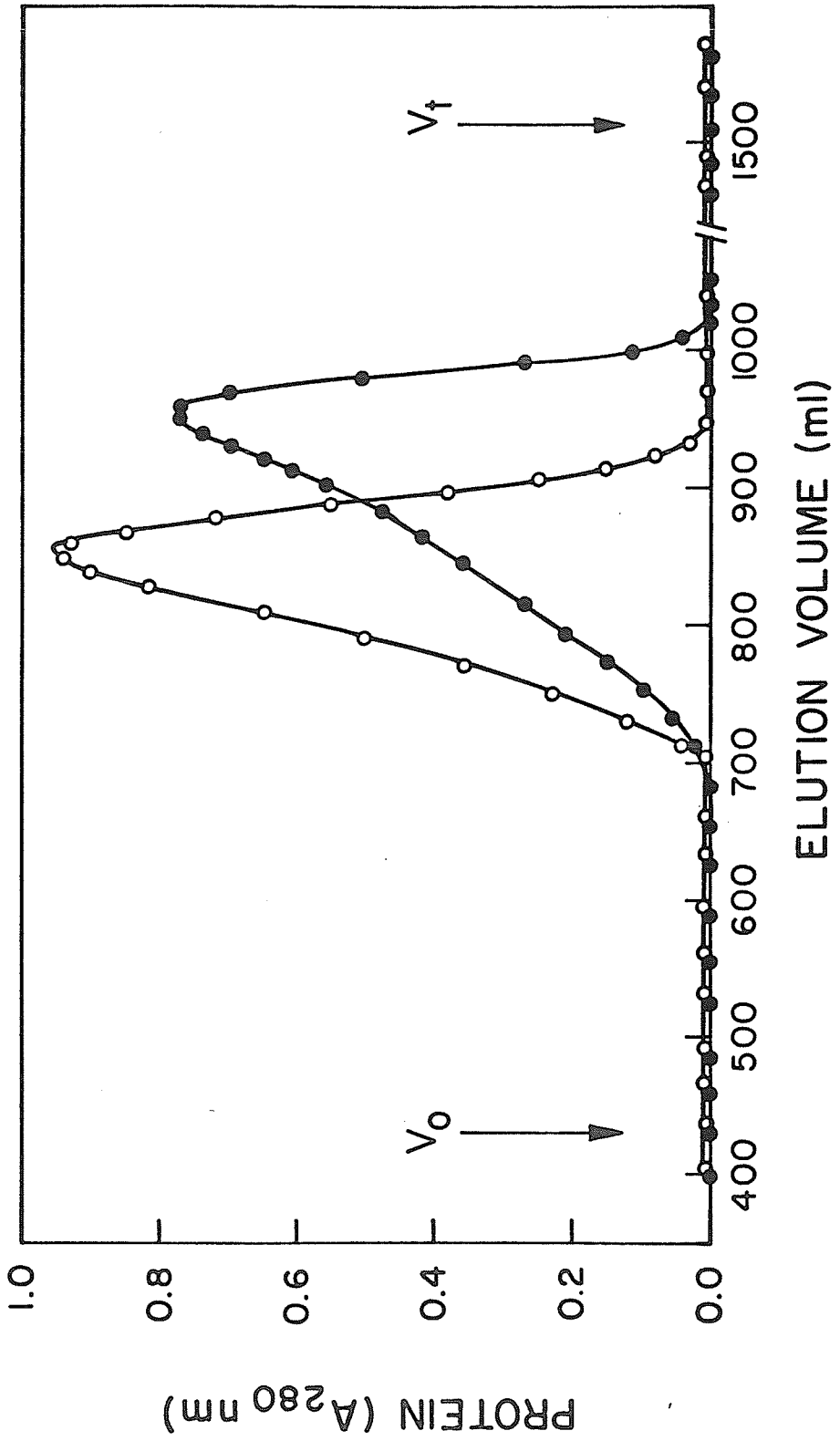
Conditions:

Column size	:	5.0 x 100 cm
Bed height	:	80 cm
Flow rate	:	28.0 ml hr^{-1}
Fraction size	:	9.0 ml
Sample volume	:	30 ml
Sample concentration	:	9.6 mg ml^{-1}

Solvent:

o———o : acetic acid (0.1 N) + urea (3 M) + KCl (20 mM); I = 0.02

●———● : acetic acid (0.1 N) + urea (3 M); I = 0


PROTEIN ($A_{280\ nm}$)
1.0
0.8
0.6
0.4
0.2
0.0
V_o
V_t
400
500
600
700
800
900
1000
1500
ELUTION VOLUME (ml)

experiment also demonstrated the sensitivity of this gluten fraction to weak ionic interactions. A question that arises at this point is whether the effects demonstrated in the above experiment are due to ionic interaction between the protein molecules themselves (protein-protein interactions), or to an ion-exchange interaction between protein molecules and negatively charged carboxyl groups on the Sephadex matrix mentioned previously. It may also be possible that both types of interactions are present during gel filtration chromatography on Sephadex matrices under conditions of low ionic strength in the solvent.

The second experiment was designed to demonstrate the presence of protein-protein interactions in peak III fraction under conditions of low ionic strength. Equal amounts of the fraction were dissolved in two equal volumes of 0.1 N acetic acid. The ionic strength of one of the solutions was increased slightly by adding KCl to a final concentration of 20 mM. The two samples were placed in double sector cells and centrifuged in a Model E ultracentrifuge as described in the Methods section. Photographs were taken of the Schlieren patterns and the sedimentation coefficient determined for each sample. The Schlieren patterns obtained are shown in Figure 28 at progressive time intervals of the centrifugation run. The upper pattern in each photograph is for the solvent containing KCl and the lower pattern for the KCl-free solvent.

The Schlieren pattern for the solvent containing KCl remained symmetrical throughout the run. This result indicates that the protein in the sample is homogeneous. This is in good agreement with the results obtained by gel filtration chromatography in the presence of KCl and also by subunit analysis by SDS-PAGE. The sedimentation coefficient ($s_{20,w}$) for peak III proteins under these conditions is 2.17 S. A concentration

Figure 28. Schlieren patterns obtained for the alcohol-soluble peak III proteins in solvents of zero ionic strength and low (I = 0.02) ionic strength.

Upper Schlieren pattern - 0.1 N acetic acid

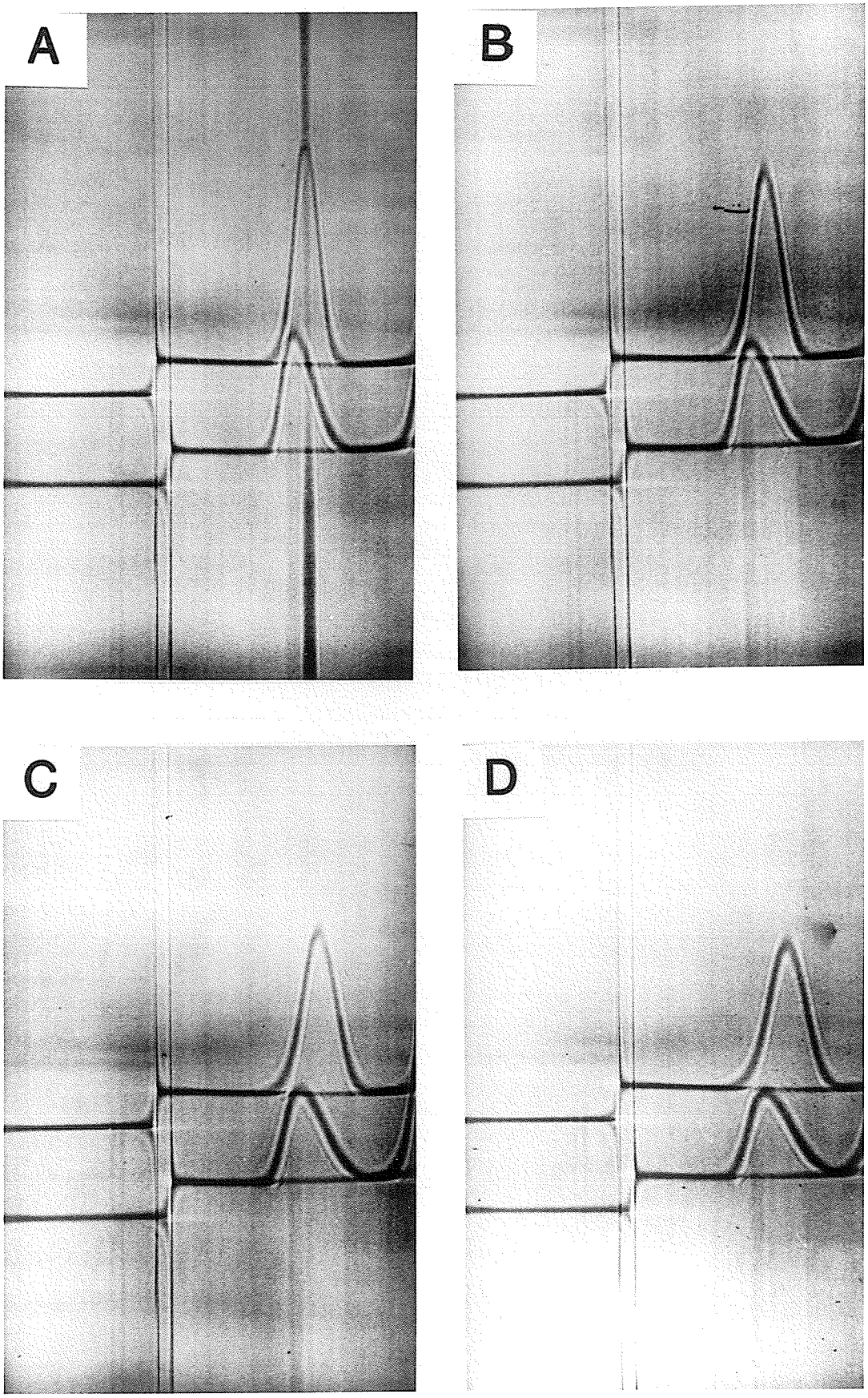
Lower Schlieren pattern - 0.1 N acetic acid
+ 20 mM KCl

A - 16 min run time

B - 24 min run time

C - 32 min run time

D - 40 min run time


A
B
C
D

study was also performed on this sample and the sedimentation coefficient determined for each protein concentration. By extrapolation, the sedimentation coefficient ($s_{20,w}$) at infinite dilution is 2.0 S.

The Schlieren pattern in the absence of KCl was quite different from that obtained for the solvent with KCl. Instead of a symmetrical pattern, a skewed Schlieren peak with a "leading edge" was obtained for all centrifugation times. This effect was similar to that observed when these proteins were subjected to gel filtration chromatography in the absence of KCl. A Schlieren pattern of the shape obtained indicates that the sample may be polydisperse. The sedimentation coefficient ($s_{20,w}$) in the absence of KCl was 0.96 S, approximately half the volume obtained when the solvent contained KCl (higher ionic strength).

These sedimentation results indicate the presence of strong protein-protein interaction in the absence of salt. It was, therefore, concluded that both protein-protein interactions and interactions between protein molecules and unprotonated carboxyl groups on the Sephadex matrix are likely altering the elution profiles obtained by gel filtration of these proteins under conditions of very low ionic strength.

Clearly, the addition of very small amounts of salt, to provide a finite but low ionic strength, has a pronounced effect on the behavior of peak III proteins under the conditions investigated. Neutral salts must, therefore, be added to prevent ionic interactions involving charged groups on native protein molecules and thereby reduce anomolous results that may occur in biochemical studies on gluten proteins.

APPENDIX II. Deleterious effects of increased gluten protein concentration on resolution obtained by gel filtration chromatography.

In this study, anomalous results were obtained when gluten proteins were subjected to gel filtration chromatography at relatively high concentrations. The anomalies were particularly noticeable for the higher molecular weight gluten proteins which eluted at, or immediately following the void volume of a Sephadex G-200 matrix. Accordingly, several exploratory experiments were carried out to obtain additional information on the anomalous behaviour.

In the first experiment, samples of the alcohol-soluble fraction I were subjected to gel filtration chromatography at higher concentrations on Sephadex G-200. The protein was dissolved in 2.0 ml of solvent containing 0.1 N acetic acid and 3 M urea and loaded onto the column which was previously equilibrated in this solvent. The elution profiles that were obtained are shown in Figure 29 (A, B, and C).

The first elution profile (A) for the lowest concentration (5 mg ml^{-1}) shows that the protein elutes as a single symmetrical peak, eluting at the void volume of the column. The absorbance at 280 nm of the peak column fraction was 0.35. The second elution profile (B) shows the effect of increasing the concentration to 11 mg ml^{-1}. A distinct "shoulder" is observed forming a trailing edge on the peak eluting at the void volume. No significant increase in the absorbance at 280 nm for the peak column fraction was observed. By further increasing the concentration to 16 mg ml^{-1} (profile C) the shoulder seen in the previous profile has developed into what appears to be included protein peaks in the elution profile. Clearly, the included peaks in this profile are artifacts which have arisen due to a break-

Figure 29. Elution profiles from gel filtration chromatography of increasing concentrations of alcohol-soluble fraction I on Sephadex G-200.

Conditions:

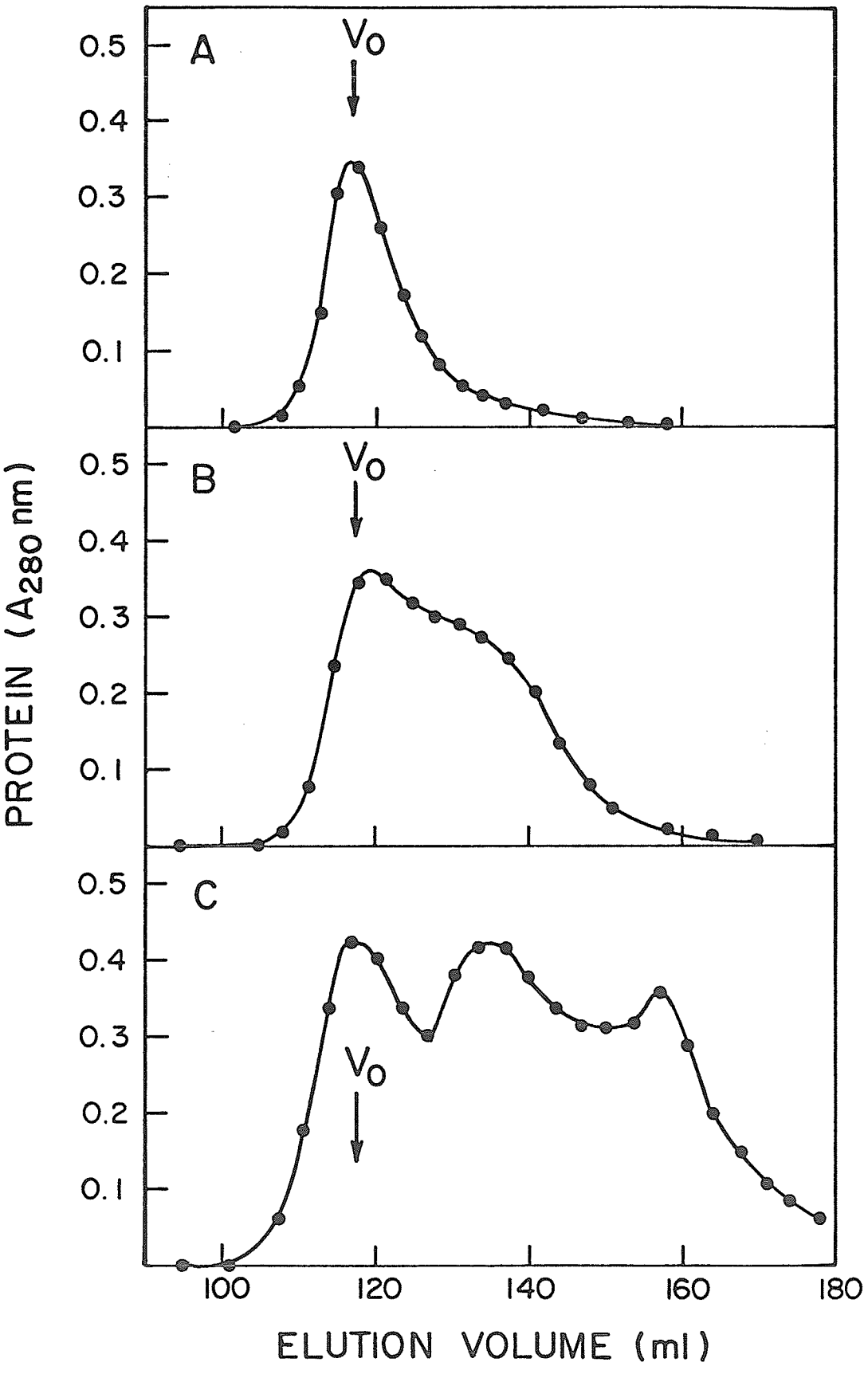
Column size	:	2.0 x 100 cm
Bed height	:	90 cm
Solvent	:	acetic acid (0.1 N) + urea (3 M)
Flow rate	:	8.0 ml hr^{-1}
Fraction size	:	approx. 3.5 ml
Sample volume	:	2.0 ml

Sample concentration:

A : 5 mg ml^{-1}

B : 11 mg ml^{-1}

C : 16 mg ml^{-1}


A
B
C
V_O
V_O
V_O
0.5
0.4
0.3
0.2
0.1
PROTEIN ($A_{280\,nm}$)
100
120
140
160
180
ELUTION VOLUME (ml)

down in the fractionation characteristics of the Sephadex G-200 matrix at high concentrations of sample. Those anomalous peaks may be more accurately described as being retarded by, but not included, in the Sephadex matrix.

Another example of this effect was obtained with the reduced-alkylated alcohol-insoluble fraction. Samples of this fraction, at two different concentrations, were subjected to gel filtration chromatography on Sephadex G-200, in the same solvent (0.1 N acetic acid and 3 M urea) under the same operating conditions. The elution profiles that were obtained are shown in Figure 30 (A and B).

The elution profile in Figure 30A was obtained for 230 mg of sample dissolved in 19.2 ml of solvent (i.e. 12 mg ml). Good resolution of eluted peaks was obtained indicating that the sample concentration was appropriate for the experimental conditions used. However, when the concentration was increased to 20 mg ml^{-1}, the resolution of the void volume peak and the first included peak decreased markedly (see Figure 30B). In fact, at this concentration, another protein peak eluted immediately following the void volume. It is presumed that this peak is an artifact produced by the higher concentration employed.

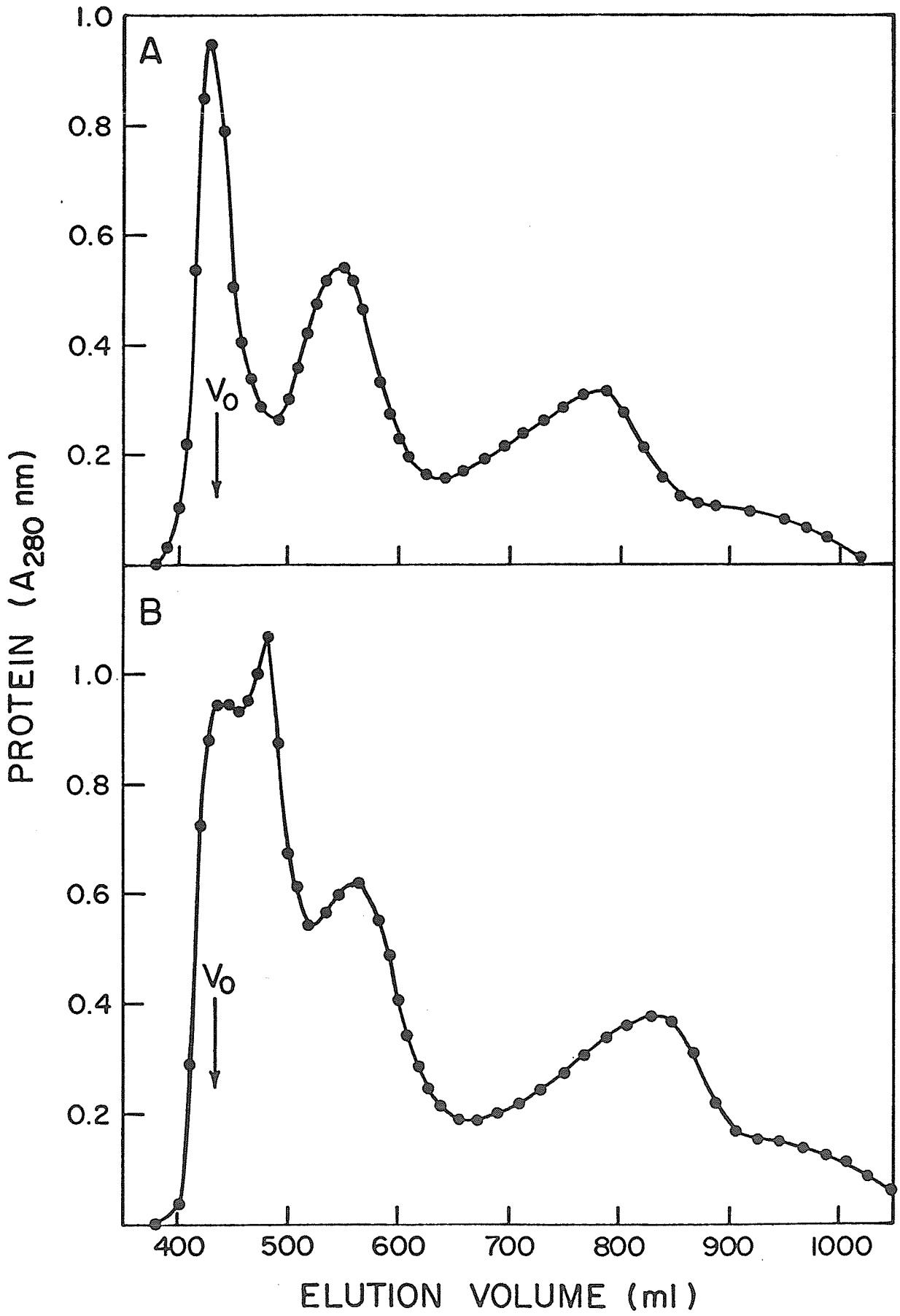
Why these effects occur on gel filtration is not completely clear. However, because they tend to occur with the higher molecular weight proteins and at high concentrations of these proteins, it is not unreasonable to speculate that the viscosity of the samples loaded onto the column may be responsible for the anomalies that were observed. The results obtained indicate that caution must be exercised in gel filtration experiments with gluten proteins. Unfortunately, the effects described in this Appendix appear to be quite widespread in the cereal

Figure 30. Elution profiles from gel filtration chromatography of reduced-alkylated alcohol-insoluble fraction on Sephadex G-200.

Conditions:

Column size	:	5.0 x 100 cm
Bed height	:	80 cm
Solvent	:	acetic acid (0.1 N) + urea (3 M)
Flow rate	:	28.0 ml hr^{-1}
Fraction size:		9.0 ml

A.	Sample volume	:	19.2 ml
	Sample concentration:		12 mg ml^{-1}
B.	Sample volume	:	17.0 ml
	Sample concentration:		20 mg ml^{-1}


A
B
V_o
V_o
1.0
0.8
0.6
0.4
0.2
PROTEIN (A_{280} nm)
400
500
600
700
800
900
1000
ELUTION VOLUME (ml)

chemistry literature. Every effort must be made to ascertain that optimum conditions have been achieved for the separation of these proteins by gel filtration chromatography to ensure that resolved peaks represent different molecular species and not experimental artifacts.