PHYSICOCHEMICAL STUDIES OF THE POLYPEPTIDE SUBUNITS OF GLUTENIN

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Khalil Khan

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

Department of Plant Science

October 1977

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KHALIL KHAN

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

To my daughter

LISA

ACKNOWLEDGMENTS

I wish to thank Dr. W. Bushuk for giving me the opportunity to study under his supervision. His friendly nature, his sincere concern for his students, and above all his unflagging interest in this thesis project created the ideal atmosphere for scientific research.

I also wish to thank Dr. J. C. Jamieson, Dept. of Chemistry, University of Manitoba, for providing a set of plans for the designing of the isoelectric focusing equipment used in this study. Thanks are also extended to Dr. B. L. Jones (formerly of the Dept. of Plant Science, University of Manitoba), U.S. Grain Marketing Research Centre, Manhattan, Kansas, for his numerous helpful suggestions during the latter stages of this thesis project.

I also wish to extend my gratitude to other members, both staff and students, of the Dept. of Plant Science, University of Manitoba, for their kind services in many ways.

I also wish to thank Mrs. S. Kusmider for her dedication to work and for the careful typing of the thesis.

Special thanks are extended to my wife for her moral support, for being the breadwinner, for raising our family, and above all for maintaining her sanity, during the very trying years of my graduate studentship.

The financial assistance of the National Research Council of Canada is gratefully acknowledged.

ABSTRACT

Khan, Khalil, Ph.D., The University of Manitoba, October 1977.
Physicochemical Studies of the Polypeptide Subunits of Glutenin.
Major Professor: Dr. W. Bushuk.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Sephadex gel-filtration chromatography, preparative isoelectric focusing, and amino acid composition analyses were used to study the subunit composition of glutenin from various wheat cultivars.

SDS-PAGE showed that the reduced glutenin from bread wheats consists of approximately 17 subunits ranging in molecuar weights (mol wts) from 12,000 to 134,000 daltons, while that of durum wheats consists of approximately 15 subunits ranging in mol wt from 12,000 to 110,000.

SDS-PAGE at pH 7.3 and pH 8.9 revealed that most bread wheats contain 5 high mol wt glutenin subunits of 134,000, 132,000, 110,000, 98,000, and 90,000. The cultivar Chinese Spring contains only 4 high mol wt subunits; the 132,000 mol wt subunit is absent in the glutenin of this cultivar.

Stewart 63, a natural AABB tetraploid wheat, contains only the 110,000 and 98,000 mol wt subunits of the 5 high mol wt subunits found in most bread wheats. The extracted AABB tetraploids, Tetraprelude and Tetrathatcher showed 3 high mol wt subunits of 132,000,

110,000 and 98,000; the 134,000 and 90,000 mol wt subunits having been lost on removal of the D genome. Tetrarescue, on the other hand, lost the 132,000, instead of the 134,000, and the 90,000 mol wt subunits.

SDS-PAGE, with buffers of different ionic strength, showed that some of the high mol wt subunits of bread wheat glutenin responded differently to changes in ionic strength. Subunits 1 and 2 (mol wts 134,000 and 132,000, respectively) were resolved in 0.02M phosphate buffer, pH 7.3 and 8.0, for most cultivars while subunits 4 and 5 (mol wts 98,000 and 90,000, respectively) were not resolved for most cultivars under these conditions. On the other hand, subunits 4 and 5 were resolved for most cultivars with the 0.125M and 0.06M Tris-borate, pH 8.9, system while subunits 1 and 2 were not resolved for most cultivars at these conditions. As the ionic strength of the phosphate buffer system was increased subunits 4 and 5 began to separate on SDS-gels. On the other hand, resolution of subunits 4 and 5 improved as the ionic strength of the Tris-borate buffer was decreased.

SDS-PAGE also revealed qualitative and quantitative differences in subunit composition of glutenin prepared by different extraction procedures. Glutenin purified by the pH precipitation procedure of Jones *et al.* (1959) showed more intensely stained subunits in the mol wt region below 68,000 than glutenin purified by pH precipitation followed by purification with the ion-exchanger, sulfoethyl-Sephadex (Orth and Bushuk, 1973a). Extensively purified glutenin, when subjected to SDS-PAGE in the unreduced state, still showed many prominent protein bands that entered the SDS-gel.

The reduced acetic acid (HoAc)-soluble and the mercuric chloride

($HgCl_2$)-soluble glutenins from the Osborne procedure of Bietz and Wall (1975) were qualitatively identical by SDS-PAGE. However, when these two extracts were examined in the unreduced state by SDS-PAGE, many qualitative and quantitative differences were evident.

Glutenin treated with 1 mM $HgCl_2$ in Tris-borate-SDS-buffer at pH 8.9 gave the same subunit pattern by SDS-PAGE as glutenin treated with 1% ($^{v}/v$) β-mercaptoethanol. Ethylenediamine tetraacetic acid (EDTA) at a 1.0×10^{-2}M concentration seemed to reverse the dissociation effect of $HgCl_2$ on glutenin.

A glutenin fraction was isolated from the gliadin fraction from the Osborne procedure of Bietz and Wall (1975) by the pH precipitation procedure of Jones *et al.* (1959). Approximately 10% (by weight) of the gliadin fraction was recovered as glutenin. SDS-PAGE showed that the reduced and unreduced subunit patterns of this glutenin were different from both the reduced and unreduced gliadin and the HoAc-soluble glutenin.

The HoAc-soluble glutenin treated with a number of different reducing agents [β-mercaptoethanol, dithiothreitol (Cleland's reagent), L-cysteine, and sodium sulfite] gave the same SDS-PAGE patterns at pH 8.9.

Gel-filtration chromatography on Sephadex G-200 in acetic acid-urea solvent was used to fractionate glutenin that was reduced and alykylated with 4-vinylpyridine. The elution profile showed 3 peaks. Peak I eluted with the void volume and showed subunits with mol wts ranging from 68,000 and lower. Peak II, however, showed subunits with mol wts of 68,000 to 134,000 while peak III showed those subunits with mol wts in the range of 60,000 to 35,000. Amino acid compositions showed

differences in certain amino acids among the three peaks.

The reduced and alkylated glutenin of four cultivars of widely different mixing and baking properties were subjected to gel-filtration on Sephadex G-200. Three peaks were obtained as before. The SDS-PAGE patterns of each peak showed both qualitative and quantitative differences (between cultivars) in subunit composition. Amino acid compositions showed intercultivar differences especially in peak II proteins.

The two highest mol wt subunits of glutenin (134,000 and 132,000) were isolated together from SDS-gels from the hexaploid cultivars Chinese Spring, Manitou, Ponca, Red River 68 and Talbot. Amino acid composition data showed differences among cultivars especially in the basic and acidic amino acids. Chinese Spring showed a higher lysine content but a lower glutamic acid content than the other cultivars.

Reduced and alkylated glutenin from the cultivar Manitou was subjected to isoelectric focusing on a 3-10 pH gradient of carrier ampholytes in 6M urea. The elution profile showed approximately eleven peaks in the pI range 4.5 to 9.

Peak II subunits from gel-filtration chromatography of Manitou glutenin was subjected to isoelectric focusing on a 6-8 pH gradient of carrier ampholytes in 6M urea. The elution profile showed approximately 12 peaks. SDS-PAGE of each peak revealed that peak II subunits consist of approximately 20 different protein species in the pI range 4 to 9. These different species could be assigned one of the mol wt values of 134,000, 132,000, 110,000, 98,000, 90,000, 80,000, and 68,000 (determined for unfractionated, reduced glutenin). Two homogeneous subunits of 134,000 and 90,000 mol wt were isolated by this

method. The N-terminal amino acid of the 134,000 mol wt subunit was aspartic acid while that of the 90,000 mol wt subunit was glutamic acid. The amino acid composition of each peak from isoelectric focusing showed that greatest variation was in the basic and acidic amino acids, and in glycine, proline, and phenylalanine contents. Methionine content was generally very low.

Based on the physicochemical data from this study a modified definition of the glutenin protein fraction is presented. Also, a descriptive molecular model of glutenin is presented to explain most of the observed rheological properties of dough.

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I. INTRODUCTION

Wheat flour proteins are classified, on the basis of solubility, into four major groups, namely, albumins (water-soluble), globulins (salt-soluble), gliadins (alcohol-soluble), and glutenin (dilute acid- or alkali-soluble). The glutenin fraction is extremely important in the breadmaking quality of wheat flour.

Glutenin is the protein fraction that contributes the elastic component of the viscoelastic properties of dough. This fraction plays an important functional role in the entire breadmaking process, from dough mixing to the baking of the dough into a loaf of bread.

A number of studies on the physicochemical properties of glutenin, using the SDS-PAGE technique, have been carried out. The results from those studies point to the complex nature of this protein. It is generally agreed that glutenin of bread wheats consists of approximately 17 polypeptide subunits. However, the manner in which these subunits are joined together has not been elucidated completely. The major objective of all physicochemical studies on glutenin, therefore, has been to formulate a molecular model that would explain the influence of this protein fraction in determining the breadmaking quality of wheat flour. Certain researchers have, however, put forward working models of glutenin, based on available data from physicochemical studies in an attempt to explain the known rheological properties of dough.

Not all published results on glutenin are in agreement. For example, there are conflicting reports on the molecular weight values of the high molecular weight subunits of glutenin as determined by SDS-PAGE. There are also conflicting reports on the subunit composition of glutenin from certain genetically modified wheat cultivars.

This study was undertaken, first of all, to try and reconcile the conflicting reports on the subunit composition of glutenin. A second aim of this study was to broaden the knowledge on the physicochemical properties of the subunits of glutenin in order to better understand its functionality in the breadmaking process. The approach taken here was to examine some physicochemical properties of the subunits of glutenin by SDS-PAGE, and to isolate, purify, and characterize some of the high molecular weight subunits in order to obtain information at the molecular level that might lead to a better understanding of the structure to function relationship of glutenin.

The major technique used in this study is SDS-PAGE. The versatility, sensitivity and resolving power of this technique were found to be ideally suited for both analytical and preparative purposes. Other analytical and preparative techniques used were Sephadex gel-filtration chromatography, isoelectric focusing and amino acid composition analyses.

This thesis will be divided into two sections. The first section will cover results of the SDS-PAGE experiments. The second section will cover the results related to the purification and isolation of certain of the high molecular weight subunits of glutenin. SDS-PAGE was used in this part of the study as an analytical technique.

The functional implications of the results from the two sections will be discussed in detail in the General Discussion section of the thesis.

II. LITERATURE REVIEW

A. Introduction

The major objective of this thesis project was to extend the knowledge on the physical and chemical properties of glutenin, to further characterize this unique protein, and to provide additional information on its functional role in breadmaking. Therefore, this review of the literature will cover in greater detail those publications that are pertinent to the thesis project such as the preparative procedures for isolating and purifying glutenin, the physical and chemical characterization of the prepared glutenin in relation to its structure. Since the ultimate aim of this and already published research on glutenin was to obtain information on its role in breadmaking quality of wheat flour, a short section reviewing some of the relevant research on the functional properties of glutenin in breadmaking will be included in this review.

Finally a short section will be devoted to a review of the sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) technique since it was used extensively in this study as an analytical and preparative method.

B. Definition

Osborne (1907) was the first cereal chemist to propose a comprehensive fractionation scheme for wheat proteins based on differential solubility in various solvents. He classified wheat proteins into four major groups:

(a) Albumins - soluble in water

(b) Globulins - soluble in salt solutions

(c) Gliadins - soluble in 70% to 90% alcohol

(d) Glutenins - soluble in dilute acid or alkali

Glutenin, therefore, is that portion of wheat proteins that is soluble in dilute acid or alkali after sequential extraction of wheat flour with salt and alcohol solutions.

C. Preparative Procedures

The following section will review some of the purification procedures devised to isolate and characterize glutenin.

Jones *et al.* (1959) developed two methods for isolating glutenin. They first defatted flour with n-butanol and then prepared gluten by washing a dough ball in 0.1% sodium chloride solution. Their first procedure involved dispersing the washed gluten in 70% ($^{v}/v$) ethanol to dissolve the gliadin proteins. The suspension was then centrifuged to separate the glutenin which remained in the precipitate. Their second procedure involved dispersing the washed gluten in 0.01M acetic acid and adding ethanol to make the suspension 70% ($^{v}/v$) in ethanol. The pH was then adjusted to 6.5 with 2N NaOH and the glutenin was allowed to precipitate at 0^{o} to $4^{o}C$. Precipitated glutenin was then

separated by centrifugation.

Meredith and Wren (1966) found that the solvent comprising 0.1M acetic acid, 3M urea, and 0.01M cetyltrimethylammonium bromide (AUC) extracted about 95% of the total protein of flour. They then subjected these AUC extracts of flour to gel-filtration on a 2.3 x 43 cm column of Sephadex G-200 and obtained four major, fairly-well resolved peaks. They categorized their peaks on the basis of elution volume. Glutenins were considered to be those proteins eluting at 85 ml (highest molecular weight fraction); gliadins at 85 to 145 ml; albumins at 145 to 180 ml; and non-protein material at 180 ml and beyond.

Danno *et al.* (1974) compared the effectiveness of sodium dodecyl sulfate (SDS) and AUC as solubilizing agents for flour proteins. They reported that 1% SDS in 0.05M phosphate buffer (pH 6.8) was more effective in solubilizing flour proteins than AUC solvent. In their experiments their SDS buffer extracted 75% of total proteins whereas their AUC solvent extracted only 72%. The lower extraction with the AUC solvent compared with that of Meredith and Wren (1966) could be due to the fact that the extraction conditions used were somewhat different. Meredith and Wren (1966) used vigorous homogenization whereas Danno *et al.* (1974) used gentle stirring in the first step of the solubilization procedure.

Cluskey and Dimler (1967) solubilized flour, gluten, and acetic acid-insoluble proteins (of flour) in the binary solvent, hydrochloric acid-2-chloroethanol. They reported that 98% of the flour proteins, 95% of the gluten proteins, and 86% of the insoluble residue proteins (insoluble glutenin) were dissolved by this solvent.

Glutenin can also be obtained by the pH precipitation procedure of Orth and Bushuk (1973a). According to this procedure, the flour is suspended in AUC, centrifuged, and the supernatant decanted. Ethanol is then added to the supernatant to a concentration of 70% ($^{v}/v$). The pH of the resulting solution is then adjusted to 6.4 with 1N NaOH, and the glutenin is allowed to precipitate slowly at 0^{o} to $4^{o}C$. Precipitated glutenin is separated by centrifugation, dispersed in 0.01M acetic acid, dialyzed against distilled water, and finally freeze-dried to give "crude" glutenin. Crude glutenin (0.2 g) is stirred overnight in 50 ml AUC solvent with sulfoethyl-Sephadex ion-exchanger (0.1 g). The suspension is then centrifuged, the supernatant dialyzed against distilled water, and finally freeze-dried to give "purified" glutenin.

Wasik and Bushuk (1974) purified glutenin from AUC extracts of flour by stepwise precipitations with ammonium sulfate. The proteins obtained from the first precipitation with 1.5 g ammonium sulfate were redissolved in AUC solvent and precipitated once more with 1.5 g ammonium sulfate to give purified glutenin.

The criterion of purity used by both Orth and Bushuk (1973a) and Wasik and Bushuk (1974) was that glutenin should not migrate into the resolving portion of an analytical polyacrylamide disc-gel during electrophoresis (Chen and Bushuk, 1970b).

Bietz and Wall (1975) used a series of solvents to solubilize total glutenin (proteins remaining after salt and alcohol extractions), applied in the following order: 1) acetic acid; 2) acetic acid-mercuric chloride; and 3) acetic acid-β-mercaptoethanol. The glutenin

fractions from the acetic acid and mercuric chloride extracts were further purified by the pH precipitation procedure of Jones *et al.* (1959). They concluded that a series of solvents must be used for complete extraction of glutenin.

Another variation in wheat protein extraction derives from the use of various organic solvents to extract lipids before preparing gluten or other protein fractions. For example, Simmonds and Wrigley (1972) found that 6M urea extracted about 95% of the protein from a preparation of "storage" protein obtained by flotation of dry flour particles in mixtures of chloroform and benzene ranging in density from 1.32 to 1.34. In contrast, 6M urea extracted only 80% of the protein from a gluten ball prepared from defatted flour, and about 85% of the protein from "storage" protein to which extracted flour lipids had been added. They suggested that these differences resulted from the formation of relatively insoluble lipid-protein complexes.

In a recent article Mecham (1973) reviewed the findings of some workers which cast some doubt as to whether the glutenin that is isolated by solubility fractionation procedures, such as the Osborne procedure, actually exists as such in wheat and flour. When phenol: acetic acid:water (1:1:1) solvent was used to solubilize flour proteins, a mol wt of 52,000 was found by ultracentrifugation for the least soluble protein fraction, while the more soluble fractions ranged down to a molecular weight of 13,000. These results suggested that the high molecular weights of glutenin reported by other workers indicate that in many solvents glutenin exists as an incompletely dissociated micelle or particle. It should be noted, however, that phenol (Folin-Ciocalteau) reagent may possess reducing activity, which might explain the low mol wt values obtained.

It is inevitable, therefore, that these many somewhat different fractionation procedures would give rise to qualitative and quantitative differences in the protein fractions obtained. For example, Bietz and Wall (1975) found both qualitative and quantitative differences in SDS-gel electrophoretic patterns of reduced glutenin prepared by five different fractionation procedures. Amino acid compositions showed differences between preparations, especially in the glutamic acid and proline contents. Kasarda *et al.* (1975) have classified glutenins on the basis of amino acid composition into a high glutamic acid group (250 to 350 glutamic acid residues per 10^5 g protein) and a low glutamic acid group (150 to 200 residues per 10^5 g).

There still is a need for a precise and reproducible characterization of wheat proteins, especially the gliadins and the glutenins. Despite this setback, however, a great deal of research has been done on glutenin; research on its physical and chemical composition, on its structure, and on its genetics, in an attempt to discover the unique properties that control its functionality in the breadmaking process.

D. Physico-Chemical Studies of Glutenin

The idea that glutenin was made up of polypeptide chains joined together by disulfide bonds came from the work of Pence and Olcott (1952) on the effect of reducing agents on the viscosity of gluten. Some 12 years later, Woychik *et al.* (1964) used starch-gel electrophoresis to show that some of the subunits obtained by reducing glutenin with mercaptoethanol had similar mobilities to gliadin proteins. They introduced the term "subunit" to refer to the polypeptides

obtained when glutenin is treated with a reducing agent such as β-mercaptoethanol. On the basis of their results, they suggested that glutenin might be a polymer of gliadin components joined through disulfide bonds. This hypothesis was supported by the fingerprinting experiments of Ewart (1966) who observed many similarities between glutenin and gliadin fingerprints (peptide maps).

On the other hand, Bietz and Rothfus (1970) showed, by chromatography of enzymatic digests, that glutenin and gliadin were distinctly different proteins. The peptides obtained from peptic hydrolysis had average mol wts of 1,990 and 1,150 for glutenin and gliadin, respectively. Later work by Bietz and Rothfus (1971) showed that, although several peptides were common to gliadin and glutenin, many were also unique to each protein. They also noted that glycine occurred more frequently in the peptides of glutenin, and proline more frequently in those of gliadin.

Weight-average-mol wt of glutenin and gliadin obtained with the analytical ultracentrifuge (Jones *et al.*, 1961) also showed differences between these two protein fractions. The two major gliadin components had mol wts of 47,000 and 42,000, respectively. Glutenin, on the other hand, had a mol wt of 2 to 3 million, but also contained some small molecules of about 50,000 mol wt. Since the glutenin was dissolved in highly dissociating solvents such as aluminum lactate-lactic acid containing 1M urea, 80% aluminum lactate-lactic acid-20% dimethylsulfoxide, and 4M guanidine thiocyanate, they concluded that the mol wts obtained with these solvents represented values for stable molecules and not aggregated particles.

But, perhaps, the most convincing evidence for the uniqueness of glutenin came from the work of Bietz and Wall (1972) on the comparison of reduced glutenin and reduced gliadin, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; method to be discussed later). They reported that most gliadin molecules were single-chained with mol wts of 78,100, 69,300, 44,200, 36,000, and 11,400. Glutenin, on the other hand, contained subunits with mol wts ranging from 11,600 through to 133,000. Two glutenin subunits had the same mol wts of 36,000 and 44,000 as the major gliadin polypeptides.

Amino acid compositions of gliadin and glutenin also revealed differences between these proteins. Ewart (1967) found that glutenin contained a higher proportion of lysine, tryptophan, and glycine whereas gliadin contained more proline, glutamic acid, cystine, isoleucine, phenylalanine, and amide nitrogen. Both proteins contain low proportions of basic and acidic amino acids but have relatively high proportions of hydrophobic amino acids. These amino acid compositions are generally consistent with the high insolubility of these proteins in aqueous solvents of high ionic strengths.

The isoelectric points (pI's) of the gluten proteins, as determined by isoelectric focussing in polyacrylamide gels, fall in the pH range from 6 to 9 (Wrigley, 1968a,b). Mita and Yonezawa (1970) using preparative column isoelectric focussing, showed that cyanoethyl glutenin contained various polypeptides with pI's in the pH range 6 to 9. Danno *et al.* (1976) cut out seven protein bands of reduced glutenin separated by SDS-PAGE. Each band was electrofocussed on a 3.5 to 10 pH gradient of carrier ampholytes on polyacrylamide gel. The pI's of

the seven bands were in the pH range from 4 to 9.

Huebner and Wall (1974) subjected pyridylethylated-glutenin to gel-filtration chromatography on Sephadex G-200 and obtained 3 fractions. Fraction A eluted with the void volume and, upon SDS-gel electrophoresis, showed protein bands with mol wts ranging from 60,000 to 30,000 and lower. Fraction B showed protein bands with mol wts of 87,000 to 133,000. The third fraction C showed protein bands of 37,000, 43,000 and 45,000 mol wts. Huebner *et al.* (1974) subjected each of the three fractions to ion-exchange chromatography on sulfoethyl-cellulose and succeeded in isolating two homogeneous sub-fractions, B-1 and HC-3, of mol wts 133,000 and 42,000 respectively. Amino acid composition analyses showed that B-contained much more glutamic acid, glycine and proline than HC-3. The HC-3 sub-fraction resembled gliadin proteins in mol wt and amino acid composition.

Orth *et al.* (1973a) used the scanning electron microscope to demonstrate that glutenin, obtained from gel-filtration chromatography on Sephadex G-100 of AUC extracts of flour, has a typical ultrastructure but one that appears to be highly heterogeneous. On the other hand, the glutenin fraction obtained by their pH precipitation procedure (Orth and Bushuk, 1973b) showed highly regular fibrous ultrastructure. They concluded, from amino acid composition analyses and disc-gel electrophoresis, that this latter glutenin preparation was much purer than their earlier preparation.

E. Structure of Glutenin

Apart from showing that glutenin was a unique protein, different from gliadin, a great deal of research has been carried out on glutenin *per se* with the hope of elucidating its structure. The ultimate aim of most of this work is to relate the structure to functionality of breadmaking.

Wu and Dimler (1964) subjected glutenin to viscosity, sedimentation velocity, ultraviolet difference spectra, and optical rotatory dispersion studies. From these studies, they concluded that glutenin was a highly assymetric molecule.

From optical rotatory dispersion results, Wu and Cluskey (1965) concluded that glutenin had a low α-helix (secondary structure) content when dispersed in urea solution and somewhat higher content in hydrochloric acid solution. Cluskey and Wu (1966), applying this same technique to gliadin and glutenin in aluminum-lactate buffer, concluded that gliadin contained more α-helix than glutenin. When urea was added they noticed a decline in α-helicity for glutenin. They postulated that the secondary structure of glutenin was a mixture of random coil and α-helix, with the former being the predominant structure.

Wu *et al.* (1967) studied the influence of ionic strength on the physico-chemical properties of wheat gluten proteins dispersed in 3M urea solutions by osmotic pressure, optical rotatory dispersion, viscosity and sedimentation velocity measurements. They found that the helical content and the intrinsic viscosity of glutenin increased with increasing ionic strength whereas the sedimentation coefficient did

not change for ionic strengths between 0.0025 and 0.5.

It has been known for a number of years that reduction of the disulfide bonds of glutenin enhances its solubility. Beckwith and Wall (1966) investigated the nature of the disulfide bonds in glutenin and the factors that influence their reformation by oxidation. They observed a drastic drop in viscosity immediately after reduction followed by a slight increase on subsequent standing. The initial decrease in viscosity was attributed to interpolypeptide disulfide bond cleavage while the subsequent small increase was attributed to unfolding of the polypeptide chains after slower reduction of intra-polypeptide disulfides or to non-covalent aggregation of the products. Oxidation of reduced glutenin at 5% concentration gave a product that resembled native glutenin in viscosity, mol wt, and elasticity. They concluded that an appropriate ratio of intra- and inter-polypeptide disulfide bonds is essential for the visco-elastic properties of glutenin required for optimum functionality in the breadmaking process.

Rothfus and Crow (1968) subjected aminoethylated glutenin to fractional precipitation with cupric nitrate and obtained four fractions. The fraction precipitated by 0.023M cupric nitrate accounted for 22% of the glutenin protein and comprised slow-moving electrophoretic components when subjected to starch-gel electrophoresis. The amino acid composition of this fraction showed unusually high glutamic acid, glycine, and tyrosine contents, quite different from the other three fractions which resembled gliadin proteins in amino acid composition.

An unusual phenomenon results when the residue proteins (acetic acid-insoluble glutenin from Osborne procedure) are extracted repeatedly

with 0.01M acetic acid. Mecham *et al.* (1972) reported that a characteristic "gel" is left after these repeated extractions. These workers found that the "gel" protein could be solubilized with 0.01M acetic acid containing 0.04M mercuric chloride. They concluded that rupture of disulfide bonds was not involved in this solubilization as the glutenin retained its rubbery cohesiveness. In contrast, Danno *et al.* (1975) have reported that mercuric chloride can cleave disulfide bonds of glutenin. They reported a maximum dissociation of glutenin's subunits at a mercuric chloride concentration of 10^{-3}M; dissociation into subunits being depressed below or above this concentration.

Cole *et al.* (1976) used the SDS-PAGE technique to characterize the "gel" proteins. They found a similar subunit pattern as for the acetic acid-soluble glutenin but certain quantitative differences, as indicated by staining intensity, were evident between the two patterns.

Bietz and Wall (1972) applied the high resolution technique of SDS-PAGE to both gliadin and glutenin proteins. They showed that glutenin consisted of about 15 subunits apparently joined through disulfide bonds. These subunits ranged in mol wts from 11,000 to 133,000. Orth and Bushuk (1973b) found approximately the same number of subunits by SDS-PAGE for reduced glutenin from bread wheats.

Bietz and Wall (1973) found that about 62% of pyridylethylated glutenin was soluble in 70% ethanol. SDS-PAGE analysis of this fraction showed that it consisted mainly of gliadin-like subunits of 44,000 and 36,000 mol wts. However, its amino acid composition was different from that of gliadin proteins.

Dalek-Zawistowska *et al.* (1975) reported some interesting and

apparently controversial results that may be pertinent to glutenin structure. They subjected a gluten dispersion (in acetic acid) to gel-filtration chromatography on Sephadex G-200 and obtained the normal glutenin (I) and gliadin (II) peaks. Rechromatography of peak I also yielded two peaks, the second peak eluting at the same position as peak II of gluten solution. Amino acid composition analyses showed that peak II from glutenin (peak I) was different from peak II (gliadins) from the first chromatographic separation of the gluten solution. When peak II proteins were radioactively labelled and incubated with unlabelled peak I proteins, radioactivity was found in the peak I proteins. On the basis of these results, they postulated that in solution an equilibrium exists between a high molecular weight glutenin (peak I) and a low molecular weight glutenin (peak II) by way of disaggregation or aggregation.

Besides the physico-chemical studies of glutenin reviewed above, there have been a number of publications on its ultrastructure as discerned by electron microscopy.

Seckinger and Wolf (1970) obtained transmission electron micrographs of particles and surface dispersions of proteins from hard and soft wheats. A purified gliadin fraction contained particles 20 to 80 A^{o} in diameter, representing mol wts between 17,000 and 216,000, whereas a glutenin fraction did not form discrete particles.

Simmonds (1972) also used the transmission electron microscope to demonstrate the presence of residual structures such as endoplasmic reticulum and amyloplast membranes in mature endosperm cells. He concluded that part of the glutenin fraction was derived from the endoplasmic reticulum. The remainder of the glutenin was derived from a

high mol wt protein fraction present in the endosperm of developing and mature grain (Bushuk and Wrigley, 1971), and from association of protein and lipid molecules when flour is mixed with water to form dough (Simmonds, 1972).

Buttrose (1963), Graham *et al.* (1962), and Jennings *et al.* (1963) used the electron microscope to demonstrate that proteins are deposited and concentrated in wheat endosperm in discrete areas within lipoprotein membranes called protein bodies. These protein bodies are formed throughout grain development and their number and size increase as the grain matures. This increase in number and size was paralleled by an increase in the proportion of the acetic acid-soluble glutenin (Jennings, 1968; Jennings and Morton, 1963a,b; Jennings *et al.*, 1963).

Bernardin and Kasarda (1973a,b) used both scanning and transmission electron microscopy to study wheat endosperm proteins. On the basis of their observations they suggested that storage proteins (gliadins and glutenins) are deposited in laminar form in the protein bodies of developing grain. This laminar structure is converted to a sheeted structure when the protein comes in contact with water. Under stress (as in dough mixing) these sheets rupture forming fibrillar webs of proteins which are composed of fibrils ranging in diameter from fifty to several thousand Angstrom units.

At the present stage of our knowledge it is not possible to assign a definite molecular structure to glutenin. However, there have been a number of working models postulated based on the data available. Articles on this subject will be reviewed next.

An analysis of the content of the disulfide bonds of glutenin

(Redman and Ewart, 1967a,b) led to the first model for glutenin by Ewart (1968). He proposed that glutenin was a linear molecule made up of polypeptide chains (subunits) joined to one another by disulfide bonds. The observed rheological properties of dough (or gluten) could not be explained on the basis of this linear model. Subsequently, Ewart (1972b) proposed a more complex concatenated structure for glutenin in which extensively folded polypeptide chains were joined by two interchain disulfide bonds to each neighboring subunit. Greenwood and Ewart (1975) modified the concatenated model to include unbranched molecules with a variable number of polypeptide chains, not necessarily of the same type, joined together by disulfide bonds to form supermolecules possessing linear, unbranched, configuration. They postulated that the rubber-like elasticity, characteristic of glutenin, derives from strong secondary forces at regions of interaction called nodes. This hypothesis was further modified (Ewart, 1977) by placing less emphasis on these regions of interaction called nodes and more emphasis on a sequential build up of secondary forces to explain the phenomenon of elasticity.

In contrast, Kasarda *et al.* (1975) have proposed that there are no interchain disulfide bonds in the gluten proteins but only intrachain disulfide bonds. This idea came from their studies on a fraction of gliadin called A-gliadin (aggregable α-gliadin). They observed that A-gliadin, depending on pH and ionic strength, aggregated to form particles with particle molecular weights in the millions (Bernardin *et al.*, 1967). Transmission and scanning electron microscopy of aggregated A-gliadin showed that it possessed a characteristic microfibrillar

structure (Kasarda *et al.*, 1967). Aggregated A-gliadin can be readily dissociated into its monomeric subunits (31,000 mol wt) in 0.001M HCl. On the basis of their observations on the aggregation-disaggregation of A-gliadin, Bernardin *et al.* (1975) have proposed that glutenin is a complex aggregate of subunits held together by specific interacting secondary forces (hydrogen bonds and ionic and hydrophobic interactions). The formation of stable glutenin "micelles" apparently requires a specific conformation and arrangement of the subunits. These authors suggested that the aggregate can be readily dissociated into subunits by reducing the intrachain disulfide bonds and thereby destroying the specific conformation required for aggregation. Recent work by Kobrehel and Bushuk (1977), which showed that insoluble glutenin can be solubilized by the addition of soaps such as sodium stearate, supports the hypothesis that functional glutenin may be an aggregate instead of a large molecule.

F. Genetics of Glutenin Subunits

This section on the genetics of glutenin will be divided into two parts. The first part will deal with the research involving the deletion of the D genome chromosomes from bread wheats. The second part will review the research involving the addition of the D genome chromosomes into natural tetraploid wheats. The effects of these deletions and additions on the functional properties of the cultivar and on the physicochemical properties of glutenin will be discussed. The reader is referred to a recent article on glutenin (Bushuk, 1974) for an explanation of the genetic terms such as genomes, chromosomes and genes.

1. Deletion of the D genome

Kaltsikes *et al.* (1968a) produced a high quality durum-type wheat (extracted tetraploid) from the hexaploid cultivar Prelude by the method of Kerber (1964). The flour of the extracted tetraploid had higher protein content, and baking absorption, and produced bread of larger loaf volume than its hexaploid counterpart. The superior quality of this extracted tetraploid was attributed to the higher protein content and to the beneficial effects of a translocation involving the long arm of chromosome 1D (Kaltsikes *et al.*, 1968b).

Dronzek *et al.* (1970), using the hexaploids Prelude (Kaltsikes *et al.*, 1968a), Rescue, Thatcher and their extracted AABB tetraploids, studied the protein solubility distribution of these cultivars. All the cultivars contained similar proportions of albumins and globulins, whereas Tetrarescue and Tetrathatcher contained more gliadin, more acetic acid-soluble glutenin and less insoluble glutenin (residue) than their hexaploid counterparts. These two tetraploid cultivars were inferior in breadmaking quality to their hexaploid counterparts. In contrast, Prelude and Tetraprelude contained similar amounts of gliadin, acetic acid-soluble glutenin and residue proteins. The baking quality of these two cultivars were quite similar. It was postulated that breadmaking quality of wheat flour is determined by a critical balance of the proteins comprising the gluten complex.

A number of electrophoretic studies have also demonstrated differences in protein composition between extracted AABB tetraploids and their hexaploid counterparts.

Starch-gel electrophoresis (Boyd and Lee, 1967; Boyd *et al.*, 1969) was used successfully to demonstrate that the protein patterns of Tetracanthatch, an extracted AABB tetraploid, was different from the patterns of the natural hexaploids Chinese Spring and Canthatch. Tetracanthatch lacked four protein bands present in the hexaploids.

Dronzek *et al.* (1970) studied the disc-gel electrophoretic patterns of the four protein solubility fractions obtained from the Osborne fractionation procedure from the hexaploids Prelude, Rescue, and Thatcher, and their extracted AABB tetraploids. They found qualitative intercultivar differences in the gel patterns but the pattern for each hexaploid and extracted tetraploid pair were qualitatively similar.

Orth and Bushuk (1973c) used SDS-PAGE to identify the subunits of glutenin that are controlled by the D genome. Removal of the D genome from the cultivars Canthatch, Prelude, Rescue and Thatcher resulted in the deletion of the highest mol wt subunit and also the 90,000 mol wt subunit. The extracted tetraploids showed poor breadmaking quality except Tetraprelude (Kaltsikes *et al.*, 1968a).

In contrast to the findings of Orth and Bushuk (1973c), Bietz *et al.* (1975), using the same cultivars and the same SDS-PAGE analytical technique but at pH 8.9, found a high molecular weight subunit for the extracted tetraploids. This subunit had either lower or higher mobility than the corresponding subunit of the hexaploid counterpart. However, Khan and Bushuk (1977), using the SDS-PAGE technique both at pH 7.3 and 8.9, showed that the hexaploid cultivars used in these studies contained two high molecular weight subunits of 134,000 and 132,000 instead of one as reported by Orth and Bushuk (1973c) and Bietz *et al.* (1975). Removal

of the D genome results in the deletion of the 134,000 (Prelude and Thatcher) and the 132,000 (Rescue) molecular weight subunits. Chinese Spring, on the other hand, showed only one subunit of 134,000 molecular weight at both pH's in this molecular weight region.

2. Addition of the D Genome

Other researchers have produced synthetic hexaploids by adding the D genome into tetraploid cultivars to observe the effect of this added genome on breadmaking quality. Kerber (1964), and Kerber and Tipples (1969) crossed Tetracanthatch (AABB) with several varieties of *Aegilops squarrosa* (D genome donor to bread wheats) to obtain synthetic (AABBDD) hexaploids. They found that these synthetic hexaploids were superior in breadmaking quality to the tetraploid Tetracanthatch, but were inferior to Canthatch, the natural hexaploid, in breadmaking quality.

Recently a disomic-1D addition line of the durum cultivar, Langdon, was developed by Joppa *et al.* (1975) using the 1DL ditelosomic (ditelocentric) line of Chinese Spring wheat as the chromosome donor. SDS-PAGE of glutenin from this addition line contained two high mol wt glutenin polypeptide subunits not present in the durum cultivar, Langdon. These two high mol wt subunits, however, are present in the cultivar Chinese Spring (Orth and Bushuk, 1974; Bietz *et al.*, 1975). The addition line also showed superior mixing properties and higher protein content than its durum counterpart, Langdon. However, the amino acid composition of glutenin from this addition line was similar to that of most durum wheats.

G. Glutenin and Breadmaking Quality

Although it has not yet been possible to delineate the molecular structure of glutenin (and relate the structure to function in breadmaking), there has, nevertheless, been a great deal of research on the functionality of glutenin demonstrating that it is, perhaps, the most important flour component in breadmaking quality.

Solubility fractionation techniques have been used extensively to identify the contribution of glutenin to breadmaking quality. Pomeranz (1965) investigated the dispersibility of flour proteins of wheat cultivars of widely different baking quality in 3M urea - 0.01M pyrophosphate buffer (pH 7.0). He found that poor breadmaking cultivars contained more urea-dispersible proteins (mostly gliadin and soluble glutenin) than good breadmaking cultivars which contained more urea-insoluble proteins (mostly insoluble glutenin or residue proteins).

Shogren *et al.* (1969) obtained various fractions of gliadin and glutenin by stepwise pH precipitation of gluten proteins dissolved in 0.005N lactic acid. When the protein fractions were reconstituted singly and in their original proportions to a base flour, it was found that a decrease in glutenin resulted in a large decrease in dough mixing time and water absorption, and an increase in gliadin resulted in a large increase in oxidation requirement for optimum loaf volume.

Huebner (1970) studied the response of glutenin isolated by gel-filtration from eleven wheat cultivars to precipitation after the addition of salt (NaCl). The response of glutenin to salt precipitation suggested that the quality of gluten (for breadmaking) may be related to the sensitivity of its glutenin proteins to changes in ionic strength.

Cultivars that showed higher breadmaking quality were characterized by steeper precipitation curves than poor quality cultivars.

A more comprehensive protein fractionation study relative to breadmaking quality was carried out by Orth and Bushuk (1972) and Orth *et al.* (1972) using the flours from 26 cultivars of diverse baking quality grown in four locations in Western Canada. They reported that the proportion of acetic acid-soluble glutenin was negatively correlated to loaf volume (index of breadmaking quality). On the other hand, the insoluble glutenin (residue protein) was positively correlated loaf volume.

Mixing experiments have demonstrated the functional importance of glutenin in breadmaking. In general, flours that show a long mixing time in the farinograph have better breadmaking potential than short mixing flours.

Mecham (1968) demonstrated a definite functional role for glutenin in dough mixing. Doughs (freeze-dried) contained more protein that is soluble in dilute acetic acid than the original flours. The proportion of acetic acid-soluble proteins (glutenin) increased with increasing mixing time. Tanaka and Bushuk (1973b,c) showed that the amount of residue proteins (insoluble glutenin) decreased with increased mixing beyond dough development in the farinograph. They proposed a depolymerization of glutenin through disulfide interchange as an explanation of dough breakdown, in contrast to Tsen (1967) who proposed a disaggregation mechanism.

Lee and MacRitchie (1971) studied the effect of flour protein fractions on the rheological properties of dough using the mixograph and

alveograph. They extracted flour sequentially with water, 2M urea, 4M urea, and 0.1N NaOH. The 2M urea extract (gliadin and soluble glutenin), when added to a base flour, decreased mixing stability and produced weaker more extensible doughs. The 4M urea and NaOH extracts (mainly glutenin) increased mixing stability and produced stronger doughs.

Gel-filtration chromatography and electrophoretic studies have also demonstrated the importance of glutenin in breadmaking. Bushuk and Wrigley (1971) investigated the possibility that glutenin might have arisen from aggregation of low mol wt proteins during the drying and ripening 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 mol wt glutenin was present in the hexaploid (bread) wheats but not in durum wheats in the late stages of grain maturity. They suggested that this low mol wt glutenin might be related to breadmaking quality.

Since the results of Bushuk and Wrigley (1971) were based on gel-filtration chromatography elution profiles, Khan and Bushuk (1976) extended this study by examining the subunit composition of glutenin by SDS-PAGE from an early stage of grain development to maturity from a bread and durum wheat cultivar. Qualitative and quantitative intra- and intercultivar differences were observed in the subunit patterns at different stages of maturity. However, it is not yet known whether these differences are related to observed differences in breadmaking

quality.

Bietz and Wall (1972), using the SDS-PAGE technique, showed that certain quantitative differences existed in the subunit composition of reduced glutenin from good and poor breadmaking cultivars. However, the exact relationship, if there is one, between these differences and breadmaking quality is still not understood.

Huebner and Wall (1976) fractionated glutenin using Sepharose 2B and 4B into two fractions, I and II, from good and poor breadmaking cultivars. Fraction I comprised the very high mol wt glutenin while fraction II comprised low mol wt glutenin. Amino acid compositions and SDS-PAGE results showed that these two fractions were quite similar in these respects. The ratio of fraction I to fraction II was higher for good breadmaking cultivars. They postulated that a sufficient total amount of protein and suitable ratio of the two glutenin fractions were necessary for a good bread flour. These findings are in agreement with the earlier work of Orth and Bushuk (1972) who showed that the acetic acid-soluble glutenin was negatively correlated while the residue protein (insoluble glutenin) was positively correlated with loaf volume.

Electron microscopy studies have also demonstrated a possible relationship between the ultrastructure of glutenin and breadmaking quality. Orth *et al.* (1973b) observed that glutenin from two hard red spring wheat cultivars and a synthetic hexaploid, all of good breadmaking quality, showed fibrous structures with long, thick strands. On the other hand, a durum cultivar, and a spring rye, both of poor breadmaking quality, showed broad ribbon-like and short rod-like structures, respectively. A triticale, of intermediate breadmaking quality,

derived from durum and rye parents, showed both ribbon- and rod-like structures. The features of molecular structure of glutenin that are important to the ultrastructure that is characteristic of high quality bread wheats have not been identified.

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

Some of the relevant publications on SDS-PAGE will be reviewed because it has been the basic method used in the present study and because it has been extensively applied by a number of other researchers to wheat proteins.

SDS-PAGE was first introduced as an analytical tool by Shapiro *et al.* (1967) for the rapid estimation of molecular weights of proteins and their subunits. Since that time this technique has become one of the most widely used methods in protein chemistry.

Analytical polyacrylamide gel electrophoresis, according to the Davis method (1964), separates proteins on the basis of both size and charge. SDS-gel electrophoresis, however, separates proteins, solely on the basis of size or molecular weight. The elimination of charge differences is achieved by the binding of the anionic detergent, SDS, to the protein molecule, thereby converting the protein molecule to a highly negatively charged SDS-protein complex. Furthermore, if disulfide bonds are reduced to disrupt the secondary structure of the molecule, the entire polypeptide chains can be exposed to maximum SDS binding. In an electric field, the negatively charged molecule will migrate to the positive electrode. Since polyacrylamide gels are

sieving mediums having approximately constant pore sizes, large molecules will migrate slower than small molecules. If the relative mobility of protein molecules of different molecular weights is measured on SDS-gels and plotted against the log of their molecular weights, a linear relationship is obtained. In this manner, the molecular weight of an unknown protein molecule can be determined from a standard curve obtained with proteins of known molecular weight.

This technique, although introduced by Shapiro *et al.* (1967), is now commonly identified with Weber and Osborn (1969) who used the method of Shapiro *et al.* (1967) and determined the molecular weights of 40 widely different proteins. They found that the reliability of the technique was good to within ±10% for the molecular weight range 15,000 to 200,000, as compared with more precise and accurate methods such as amino acid composition, amino acid sequence, osmotic pressure and sedimentation equilibrium. Somewhat better accuracy of ±5% was achieved by Dunker and Rueckert (1969) who electrophoresed both standard and unknown proteins on the same disc-gel. The SDS-PAGE technique also lends itself, like the standard analytical method of Davis (1964), to modifications both in pore size of the gel by varying the acrylamide or bisacrylamide concentrations, or to variations in types and concentrations of buffer (Neville, Jr., 1971).

The theory of SDS binding to proteins was developed after the introduction of the method. Reynolds and Tanford (1970a,b) observed that in an aqueous solution, SDS can exist as monomers and as micellar aggregates, the concentration of each depending upon the total SDS concentration, the ionic strength, and the temperature. All of the

investigated proteins bound 1.4 g SDS per g of protein at monomer concentrations above 5×10^{-4}M. The nature of the binding was thought to be mainly hydrophobic. The degree of binding was dependent only on the SDS monomer concentration and not on the number and size of the micelles present. From intrinsic viscosity and optical rotatory measurements, they concluded that SDS-bound proteins behaved as prollate ellipsoids or relatively rigid rods. The short axis of such complexes is constant, of the order of 18 Å, while the long axis is a function of the length and, therefore, mol wt of the polypeptide chain. Thus, since SDS-treated protein molecules migrate as rods through SDS-polyacrylamide gels, they would encounter more frictional resistance through the gel than untreated protein molecules which are globular in shape.

In a recent review, Weber and Osborn (1975) have dealt exhaustively with SDS-PAGE, its uses and pitfalls. For molecular weight determination this method offers many advantages over other methods. Because of its high resolution, highly heterogeneous mixtures of proteins can be analyzed and characterized. Also very little sample is required; usually microgram quantities are sufficient. The technique is rapid; one electrophoretic run lasting approximately 3 hours. Also a large number of samples can be analyzed in a single run. Measurement of electrophoretic mobility is highly reproducible. The equipment and materials required by the method are inexpensive and readily available.

There are, however, a number of pitfalls to the SDS-PAGE method of mol wt determination and caution must be exercised in the

interpretation of results. There is the possibility that SDS might bind abnormally to certain proteins. For example, Pitt-Rivers and Impiombato (1968) found that glycoproteins and proteins whose disulfide bonds (S-S) were not reduced bound less SDS per g of protein than other proteins. Also the introduction of chemical cross-links appears to restrict the binding of SDS and lower the electrophoretic mobilities (Davies and Stark, 1970). The primary structure of a protein molecule may influence SDS-binding, for example, the highly charged polylysyl-glutamic acid bound only 0.4 g SDS per g of polypeptide (Pitt-Rivers and Impiombato, 1968).

The amino acid composition of a protein can affect the behaviour of the protein on SDS-gels. Furthmayr and Timpl (1971) reported that certain collagen proteins exhibited higher mol wts by the SDS-PAGE technique than by other methods. They attributed this abnormal behaviour to a very high imino acid (proline) content which, in turn, results in a more rigid structure for the polypeptide chains, thus resulting in impaired mobility on SDS-gels.

The net charge of a protein can influence its electrophoretic mobility on SDS-gels. Histones, protein molecules with exceptionally high net charges, bind the usual amount of SDS, but have atypically low electrophoretic mobility (Panyim and Chakley, 1971), resulting in higher mol wt estimation.

Proteins with mol wts below 15,000 may show abnormal behaviour on SDS-gels. SDS-binding converts proteins to rod-shaped molecules, but at this low mol wt, rod-like particles approximate the shape of spheres since their length is similar to their diameter (Fish *et al.*,

1970). Thus, some calibration curves may change shape in this mol wt region (Dunker and Ruekert, 1969) from changes in electrophoretic mobility of these low mol wt proteins.

Precautions must also be taken to destroy proteolytic activity from protein preparations. Weber *et al.* (1972) and Pringle (1970) found that several proteolytic enzymes failed to give bands on SDS-gels if incubated at room temperature with SDS and β-mercaptoethanol. Thus, in order to destroy proteolytic activity, SDS-protein solutions should be heated to 100°C for at least 3 minutes before electrophoresis.

Despite the shortcomings indicated above in mol wt estimation of some proteins, SDS-PAGE can be used as a preparative method for isolating small quantities of proteins (Weiner *et al.*, 1972) for subsequent analysis and more detailed characterization. Weber and Kuter (1971) have provided a method for the quantitative removal of SDS from SDS-bound proteins by using the ion-exchange resin Dowex 1X2 (200-400 mesh, Bio-Rad). Proteins recovered from SDS-gels have been subjected to peptide mapping (Udenfriend *et al.*, 1972), amino terminal sequence by a modified Edman method (Weiner *et al.*, 1972), and amino acid composition analyses. Weber and Kuter (1971) have also described a method for restoration of biological activity to certain SDS-denatured enzymes.

Perhaps, the full potential of the SDS-PAGE technique as applied to protein chemistry has not yet been fully realized.

III. MATERIALS AND METHODS

A. Wheat Cultivars

The following wheat cultivars were used in this study:

Cultivar	Class	Comments
Chinese Spring	Hard Red Spring	-
Manitou	" " "	-
Prelude	" " "	-
Red River 68	" " "	-
Rescue	" " "	-
Thatcher	" " "	-
Ponca	Hard Red Winter	Supplied by J. A. Bietz, U.S.D.A., Peoria.
Talbot	Soft White Winter	-
Stewart 63	Amber Durum	-
TetraPrelude	Synthetic Tetraploid	AABB component of Prelude
TetraRescue	" "	" " " Rescue
TetraThatcher	" "	" " " Thatcher

B. Flour Samples

Tempered wheat (15.5% moisture) was milled into flour on a Buhler pneumatic experimental mill. Flour extraction ranged from 65 to 70%.

C. Protein Determination

Total nitrogen was determined by the Kjeldhal method (American Association of Cereal Chemists, 1970). Protein content was obtained by multiplying the nitrogen content by the conversion factor 5.7 (Tkachuk, 1969).

D. Defatting of Samples

For the protein extraction procedure of Bietz and Wall (1975), flour was defatted as follows: First, 60 g of flour was extracted 3 times, each time for 20 min, with 300 ml of 1-butanol by stirring at room temperature, and filtering on a Buchner funnel. Each extraction was followed by extraction with 300 ml of petroleum ether. The flour was air-dried.

E. Protein Extraction Procedures

Glutenin was prepared by five different extraction procedures, as follows:

Procedure 1

Glutenin from whole meal, flour, and gluten was extracted and purified by the pH precipitation procedure of Orth and Bushuk (1973a). According to this procedure the proteins are first solubilized with AUC solvent and the preparation centrifuged. The supernatant is then adjusted to 70% ($^{v}/v$) in ethanol and the pH raised to 6.4 with 1N NaOH. The mixture is kept at 0 to 4^{o}C overnight to allow the glutenin to precipitate. Precipitated glutenin is separated by centrifugation, dispersed in 0.01N acetic acid, dialyzed against distilled water, and

finally freeze-dried to give a crude glutenin preparation. Crude glutenin is purified by stirring overnight in AUC solvent with sulfoethyl-Sephadex C-50 (Pharmacia Chemicals, Sweden) ion-exchanger and separating the undissolved material by centrifugation. The supernatant is dialyzed against distilled water, and freeze-dried to give purified glutenin.

Procedure 2

Glutenin was isolated and purified according to the pH precipitation procedure of Jones *et al.* (1959). In this procedure wet gluten is dispersed in 0.1N acetic acid-70% ethanol and the pH adjusted to 6.6 with 2N NaOH. Glutenin is allowed to precipitate overnight at 0^{o} to 4^{o}C. Precipitated glutenin is separated by centrifugation, redissolved in 0.1N acetic acid-70% ethanol and precipitated once more with 2N NaOH to pH 6.6. Reprecipitated glutenin is separated by centrifugation, dispersed in 0.01N acetic acid, and freeze-dried to give twice precipitated purified glutenin.

Procedure 3

Glutenin was also isolated from whole meal or flour according to the modified Osborne procedure of Chen and Bushuk (1970a). The meal or flour is extracted sequentially with 0.5N NaCl, 70% ethanol, and 0.05N acetic acid to give albumins and globulins, gliadins, and acetic acid-soluble glutenin, respectively.

Procedure 4

Three types of glutenins were isolated according to the modified Osborne procedure of Bietz and Wall (1975). In this procedure, defatted flour was extracted sequentially with 0.04N NaCl, 70% ethanol,

0.1N acetic acid, 0.01N acetic acid-0.2mM mercuric chloride, and 0.1N acetic acid-0.1% β-mercaptoethanol, to give albumins and globulins, gliadins, acetic acid-soluble glutenin, mercuric chloride-soluble glutenin, and β-mercaptoethanol-soluble glutenin, respectively. The acetic acid- and mercuric chloride-soluble glutenins were purified by the pH precipitation procedure of Jones *et al.* (1959) (Procedure 2).

Procedure 5

Glutenin was also isolated according to the single-kernel procedure of Bietz *et al.* (1975). In this procedure, the embryo end of a single kernel of wheat is removed, and the rest of the kernel ground with a mortar and pestle. The meal is extracted twice, each time for 1/2 hr, by stirring at room temperature in 5 ml of 0.04N NaCl. The suspension is centrifuged on a bench-top centrifuge for 3 min and the supernatant separated. The residue is extracted with 5.0 ml of 70% ethanol solution. The precipitate from the ethanol extraction, is dispersed in 2.0 ml of 0.7% acetic acid and stirred for 1/2 hour. Then 5.6 ml of 95% ethanol is added and the suspension stirred for another 1/2 hr. The pH is adjusted to 6.6 with 2N NaOH and glutenin is allowed to precipitate at 0^{o} to 4^{o}C for a few hours. The mixture is centrifuged, supernatant decanted, and precipitate freeze-dried. The freeze-dried precipitate is extracted either with 0.125M Tris-borate buffer, pH 8.9, or with 0.02M sodium phosphate buffer, pH 7.3, containing 1% SDS and 1% β-mercaptoethanol, to give reduced glutenin for SDS-PAGE analysis.

F. SDS-PAGE

Several modifications of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used, as follows:

1. SDS-PAGE was carried out at pH 7.3 according to the method of Weber and Osborn (1969) as modified by Orth and Bushuk (1973b) for the EC470 vertical slab-gel electrophoresis apparatus (E-C Apparatus Corporation, Philadelphia, U.S.A.). Electrophoresis time was increased in certain instances to 5 hr. However, the gel composition, protein solvent, and electrode buffer compositions were identical to those of Orth and Bushuk (1973b). The tracking dye, bromphenol blue, migrated about 8 cm after 3 hr of electrophoresis, while after 5 hour it migrated about 12 cm.

2. SDS-PAGE was also carried out with phosphate buffer at pH 8.0. The buffer consisted of 0.018M Na_2HPO_4, 0.002M $NaH_2PO_4.2H_2O$ containing 0.1% ($^{W}/v$) SDS. All other operations and conditions were identical to those of Orth and Bushuk (1973b).

3. SDS-PAGE was carried out at pH 8.9 according to the method of Koenig *et al.* (1970) as modified by Khan and Bushuk (1977) for the E-C 470 vertical slab-gel electrophoresis apparatus. A 5% gel was prepared by dissolving 10.2 g acrylamide and 0.27 g bisacrylamide in 200 ml of 0.125M Tris-borate buffer, pH 8.9, containing 0.1% SDS ($^{W}/v$). To this solution were added 50 mg sodium sulfite, 0.5 ml dimethylaminopropionitrile, and 4 ml of a 2% ammonium persulfate solution. The solution was deaerated and poured into the electrophoresis apparatus to form a slab approximately 6 mm thick.

For this SDS-PAGE, the glutenin was reduced and complexed at 40°C

for at least 5 hr (or overnight) in 0.125M Tris-borate, pH 8.9, containing 1% ($^{W}/v$) SDS and 1% ($^{V}/v$) β-mercaptoethanol. When reduction was not required the β-mercaptoethanol was omitted from the protein solvent. The proteins were electrophoresed for 3 to 5 hr at 110 volts or 50 mA. Both electrode buffers consisted of 0.125M Tris-borate, pH 8.9, containing 0.1% ($^{W}/v$) SDS. The tracking dye, bromphenol blue, migrated about 7 cm in 3 hr; after 5 hr it migrated approximately 11 cm.

4. SDS-PAGE was also carried out at pH 8.9 with 0.06M and 0.40M Tris-borate buffer containing 0.1% ($^{W}/v$) SDS. All procedures were identical to those of Koenig *et al.* (1970) as outlined above.

G. Staining and Destaining Procedures

Two different staining-destaining procedures were used, as follows:

1. The first procedure was the same as that described by Wasik and Bushuk (1974).

2. In the second procedure SDS-gels were stained and destained as follows: At the conclusion of electrophoresis, the SDS-gel was rinsed in distilled water and soaked in a 10 to 12% trichloroacetic acid (TCA) solution on a shaker for at least 45 min. The TCA was then decanted, the gel rinsed in distilled water and kept in the staining solution overnight with gentle shaking.

The staining solution consisted of 1.6 g Coomassie Brilliant Blue (CBB) R (Sigma) in 800 ml ethanol, 800 ml water, and 176 ml glacial acetic acid. The destaining solution consisted of 400 ml of 10% acetic acid plus 100 ml of methanol. Gels were stored in the destaining solution.

H. Molecular Weight Determination by SDS-PAGE

The procedure for determining the molecular weights for the subunits of glutenin was the same as that outlined by Weber and Osborn (1969). The relative mobility after staining and destaining 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 standard proteins used for calibrating the gels, and their mol wt and source are as follows:

Protein	Mol Wt	Source
β-galactosidase	130,000	Sigma
Bovine Serum Albumin		Calbiochem
- dimer	136,000	
- monomer	68,000	
Pepsin	35,000	Calbiochem
α-Chymotrypsin	21,600	Calbiochem
Cytochrome C	13,000	Calbiochem

I. Reduction and Alkylation of Glutenin

Purified glutenin was reduced with β-mercaptoethanol and its cysteine residues alkylated with 4-vinylpyridine (Aldrich Chem. No. V320-4) according to the method of Friedman *et al.* (1970). The glutenin (1.0 g) was dispersed in 100 ml of buffer solution consisting of 16.11 g Tris, 7.12 g HNO_3, 0.75 g KCl, 484.8 g urea, and 1 mg Na_2EDTA[1], pH 7.5, per liter. Nitrogen was bubbled through this dispersion for 15 min;

[1] Ethylenediaminetetraacetic acid, disodium salt.

1 ml of β-mercaptoethanol (1% $^{v}/_{v}$) was added, and the mixture stirred for at least 8 hr at room temperature. Then 1.5 ml (1.5% $^{v}/_{v}$) of 4-vinylpyridine was added and the solution stirred for 2 hr at room temperature. The pH of the solution was adjusted to 3.0 with glacial acetic acid. The solution was then dialyzed against distilled water for 5 days, and freeze-dried to give the S-(4-pyridylethyl)-L-cysteine derivatized glutenin (PEC glutenin).

J. Gel-filtration Chromatography

Sephadex G-200 was dispersed in distilled water, boiled in a water bath for 5 hr, cooled to room temperature, and finally equilibrated with the 0.1N acetic acid-3M urea solvent. The Sephadex was then poured into a Sephadex column as outlined in the booklet entitled "Gel-filtration in Theory and Practice" supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Bacterial growth was prevented by incorporating sodium azide (0.02%) in the equilibrating buffer. The column was operated by the downward flow technique. The eluant was monitored continuously on a UV recorder and fractions were collected by an automatic fraction collector. Appropriate fractions were pooled, dialyzed against distilled water, and freeze-dried for further analyses as required.

K. Isoelectric Focusing

The procedure for isoelectric focusing was based on the method of Jamieson *et al.* (1972). A 220 ml isoelectric focusing column designed after the LKB 8100-10 column and a gradient mixing device obtained from Kontes Glass Company (Vineland, N.J.) were used. Experiments were performed with ampholine carrier ampholytes (LKB) with the pH ranges 3-10,

5-9, and 6-8. The focusing column was prepared as follows. The dense electrode solution, containing 0.4 ml concentrated sulfuric acid, 24 g sucrose and 28 ml of 6M urea solution, was added to the anode at the bottom of the column. Then sucrose solution containing the carrier ampholytes was slowly introduced into the column using the gradient mixer to form the required density gradient. The dense gradient solution (DGS) contained 56 g sucrose, 3.6 ml ampholine carrier ampholytes, and 84 ml of 6M urea solution. The light gradient solution (LGS) contained 1.2 ml ampholine carrier ampholytes in 120 ml of 6M urea solution. The final concentration of carrier ampholytes in the column gradient was 1% ($^{w}/v$). The protein sample (40 - 50 mg) was dissolved in a small volume of LGS and added to the LGS solution after about 25 ml of solution had entered the column. When the column was filled, the light electrode solution consisting of 10 to 20 ml 1% ($^{w}/v$) NaOH solution was added to the cathode at the top of the column. About 4 to 5 hr were required to fill the column. All procedures were performed at 2°C with water at 2°C circulating through the cooling jackets of the column. When the focusing was completed the valve at the bottom of the column was closed and the column was emptied through the lower exit at a flow rate of about 0.5 ml per min. Fractions of about 1.5 ml were collected with an automatic fraction collector. The pH and the Optical Density (O.D.) at 280 mμ of the fractions were determined. The appropriate fractions were pooled as indicated, dialyzed against distilled water, and freeze-dried for subsequent analyses.

L. Carbohydrate Determination

Carbohydrate content was determined by the Anthrone method according to the procedure of Spiro (1966). Glucose was used as the standard sugar.

M. Elution of the First Two High Molecular Weight Subunits from SDS-Gels

The following three solutions were used to elute the subunits of glutenin as described in the procedure below:

Solution A: .05M NH_4HCO_3 containing 2% ($^{W}/v$) SDS.

Solution B: 6M urea - 0.1M NH_4HCO_3.

Solution C: 6M urea - 0.05M NH_4HCO_3.

The method for eluting the first two high mol wt bands from SDS-gels was based on the procedure of Weber and Kuter (1971). Alkylated glutenin (see Materials and Methods) was electrophoresed according to the method of Orth and Bushuk (1973b). After electrophoresis the slab-gel was soaked in 10% TCA until the glutenin subunits appeared as bright, white bands. The TCA solution was decanted, the gel rinsed with distilled water, and the appropriate bands cut out with a razor blade. The cut pieces of gel were then placed in a 10 ml syringe and squirted into 50 ml of solution A by pressing with a plunger. This suspension was kept on a shaking water bath overnight at 40°C to elute and solubilize the protein. The solubilized protein was collected by filtering the acrylamide suspension through glass wool in a 10 ml

syringe, rinsing with solution A and finally squeezing the solution out of the acrylamide with a plunger. The protein solution was then freeze-dried and dissolved in 10 to 15 ml of solution B. A 1.0 cm x 15 cm glass column was packed with Dowex 1 - X2 (Bio Rad Laboratories) in solution C, the elution buffer. The protein in solution B was applied to the top of the column and allowed to enter the resin. The protein was then eluted with solution C. The eluant was monitored on a UV recorder and the SDS-free protein emerged as a symmetrical peak. The SDS was bound to the Dowex resin. The appropriate fraction was collected, freeze-dried, taken up in about 20 ml of distilled water, dialyzed against distilled water to remove the urea, and freeze-dried again. The freeze-dried sample was used for SDS-PAGE and amino acid composition analyses.

N. Amino Acid Composition Determination

The hydrolysis procedure for amino acid composition analysis of whole meal, flour, and protein fractions was carried out as follows: 4.0 ml of 6N HCl (3 times distilled) containing 1 drop of caprilic acid (anti-foaming agent) was added to the sample containing approximately 5 mg protein and then flushed with nitrogen 5 min. The sample, in a stoppered test tube, was kept in a forced-air draft oven at 110^{o}C for 24 hr for hydrolysis. The test tube was removed from the oven, 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 8.0 ml of sodium citrate buffer, pH 2.2, and centrifuged to remove insoluble material. The supernatant was used for 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.* (1959).

O. N-Terminal Amino Acid Determination

The procedure for determining N-terminal amino acids was that of Zanetta *et al.* (1970). The N-terminal residue of the polypeptide was converted to the dansyl derivative with 1-Dimethylaminonaphthalene-5-sulfonyl chloride (dans-Cl) and then hydrolyzed. Dans-Cl and dans-amino acids were purchased from Pierce Chemical Company. Protein samples ranged from 1 to 2 mg.

One dimensional chromatography was performed on polyamide sheets (5 x 5 cm, Cheng-Chin polyamide or Schleicher and Schuell micropolyamide sheets). The solvents used were benzene:acetic acid (9:1) or formic acid:H_2O (1.5:100).

P. Reagents and Chemicals

Urea solutions were purified by passing through a mixed bed resin (Bio Rad (AG-501-X8(D), 20-50 mesh).

Distillation was used to purify 4-vinylpyridine (Aldrich Chem. No. V320-4) before use in alkylation experiments.

All other chemicals were used as purchased.

IV. RESULTS AND DISCUSSION

A. SDS-PAGE Results

This section deals with the subunit composition of glutenin as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results of these experiments should provide additional information on the physicochemical properties of glutenin. The significance of the results obtained in the chemistry of breadmaking quality of wheat flour will be discussed in this section and also in the General Discussion section of this thesis.

In addition to the presentation and discussion of results obtained in the present study, an attempt will be made to clarify conflicting results published previously on the subunit composition of glutenin.

1. Molecular Weights of Glutenin Subunits[1]

The main objective of these experiments was to clarify the discrepancy between the molecular weight (mol wt) values of subunits obtained by Bietz and Wall (1972) and those obtained by Bushuk and coworkers (Orth and Bushuk, 1973b; Wasik and Bushuk, 1975). The values of the latter workers are approximately 20% higher. It appeared that the calibration curves used by Orth and Bushuk (1973b) and Wasik and Bushuk (1975) might be in error since the mobility of reference proteins were not determined in the presence of excess (over disulfide (S-S) content) reducing agent as is done in the case of glutenin.

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[1] Some of the results in this section appeared in a publication by Khan and Bushuk, 1976.

Fig. 1 shows the calibration curves obtained for reference proteins in the absence and presence of β-mercaptoethanol, a disulfide reducing agent. There was a definite shift in the position of the calibration curve when the reference proteins were not reduced. The shift was more pronounced in the higher mol wt region. The mol wts of the high mol wt subunits of glutenin estimated from the curve for reference proteins without β-mercaptoethanol gave much higher values than those calculated from the curve with β-mercaptoethanol (Table 1).

In addition to the high mol wt subunits, reduced glutenin contains many other subunits when examined by SDS-PAGE at pH 7.3 after 3 hr electrophoresis. Bread wheat glutenin contains approximately 17 subunits (Fig. 2A) while durum wheat glutenin contains approximately 15 subunits (Fig. 2B). The mol wt values for all subunits are listed in Table 2 for Manitou glutenin (representative of bread wheats) and for Stewart 63 glutenin (representative of durum wheats).

The mol wts of the subunits of alkylated [pyridylethylated (PEC)] Manitou and Stewart 63 glutenins were also obtained using PEC reference proteins. There were negligible differences in the mobilities between the reduced standards and the PEC standards (Table 3). Also the mobilities of reduced glutenin and PEC glutenin subunits that can be resolved by SDS-PAGE at pH 7.3 are almost identical. There are only slight differences in resolution of certain subunits in the 47,000 to 30,000 mol wt region between reduced glutenin and PEC glutenin for both Manitou and Stewart 63. Therefore, the mol wt values listed in Table 2 can be used for the subunits of both reduced glutenin and PEC glutenin.

Figure 1. Log of mol wt vs relative mobility for standard (reference) proteins in the presence and absence of β-mercaptoethanol, a disulfide reducing agent.

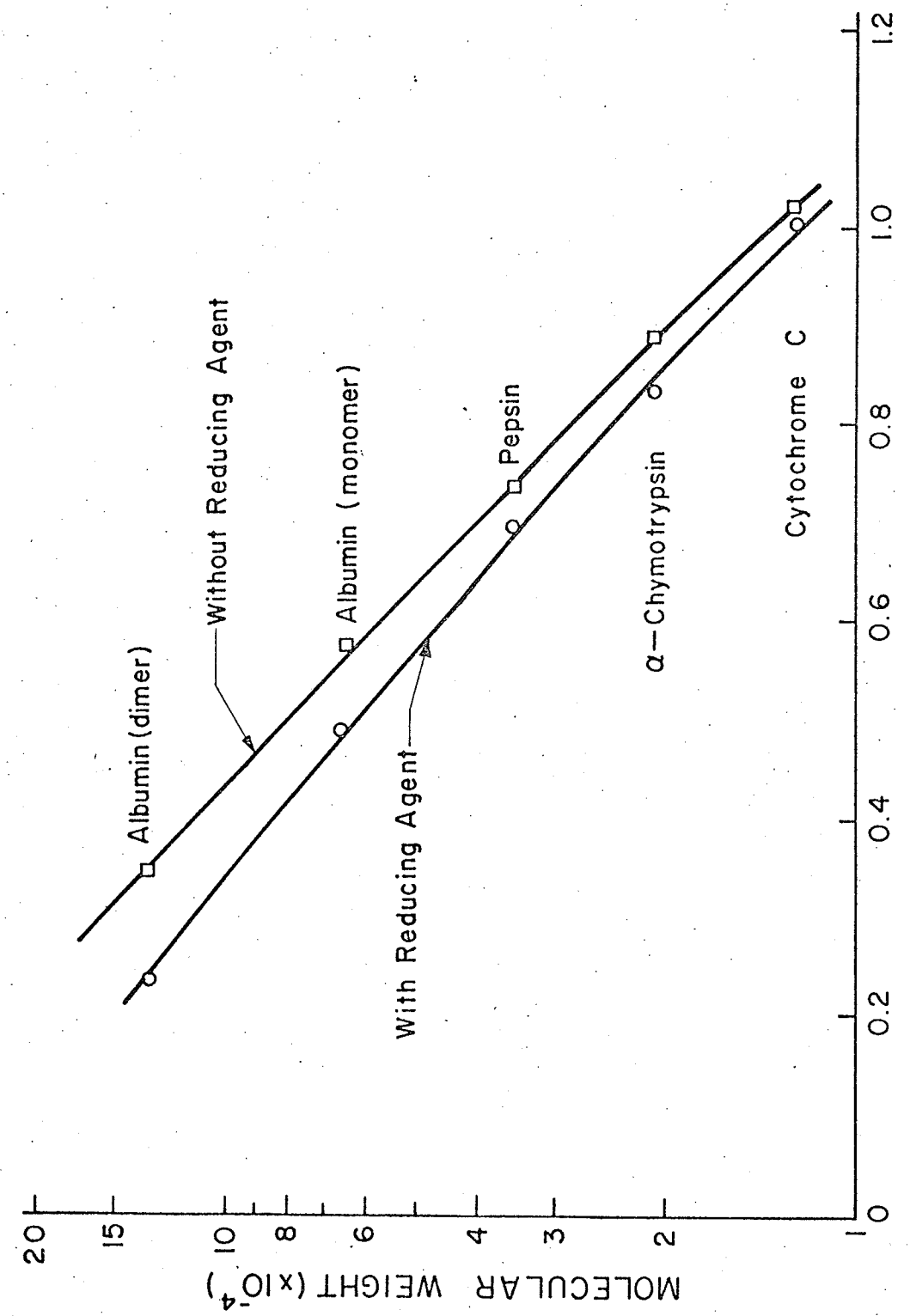

MOLECULAR WEIGHT ($\times 10^{-4}$)
20
15
10
8
6
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
1.2
RELATIVE MOBILITY
Albumin (dimer)
Without Reducing Agent
Albumin (monomer)
With Reducing Agent
Pepsin
α—Chymotrypsin
Cytochrome C

TABLE 1. Molecular Weights of Glutenin Subunits by SDS-PAGE at pH 7.3 from Standard Proteins Calibrated with and without β-Mercaptoethanol (Disulfide Reducing Agent)

Subunits	Molecular weight	
	With reducing agent	Without reducing agent
Bread wheat		
1	134,000	150,000
2	132,000	140,000
3	110,000	120,000
4	98,000	105,000
5	90,000	98,000
Durum wheat		
1	110,000	120,000
2	98,000	105,000

TABLE 2. The Molecular Weights by SDS-PAGE at pH 7.3 of the Subunits of Reduced Manitou and Stewart 63 Glutenins

Subunits	Molecular weight	
	Manitou	Stewart 63
1	134,000	110,000
2	132,000	98,000
3	110,000	88,000
4	98,000	80,000
5	90,000	68,000
6	80,000	60,000
7	68,000	47,000
8	60,000	45,000
9	47,000	43,000
10	40,000	35,000
11	33,000	32,000
12	28,000	27,000
13	25,000	25,000
14	21,000	15,000
15	18,000	12,000
16	15,000	
17	12,000	

TABLE 3. Comparison of Relative Mobilities of Reduced Standard Proteins with Reduced and Alkylated[1] Standard Proteins

Standard protein	Molecular weight	Relative mobility	
		Reduced	Reduced and alkylated
Phosphorylase a	98,000	0.432	0.432
BSA - dimer	136,000	0.310	0.300
BSA - monomer	68,000	0.605	0.598
Pepsin	35,000	0.770	0.770

[1]Standard proteins reduced, and alkylated with 4-vinylpyridine according to the method of Friedman *et al.* (1970).

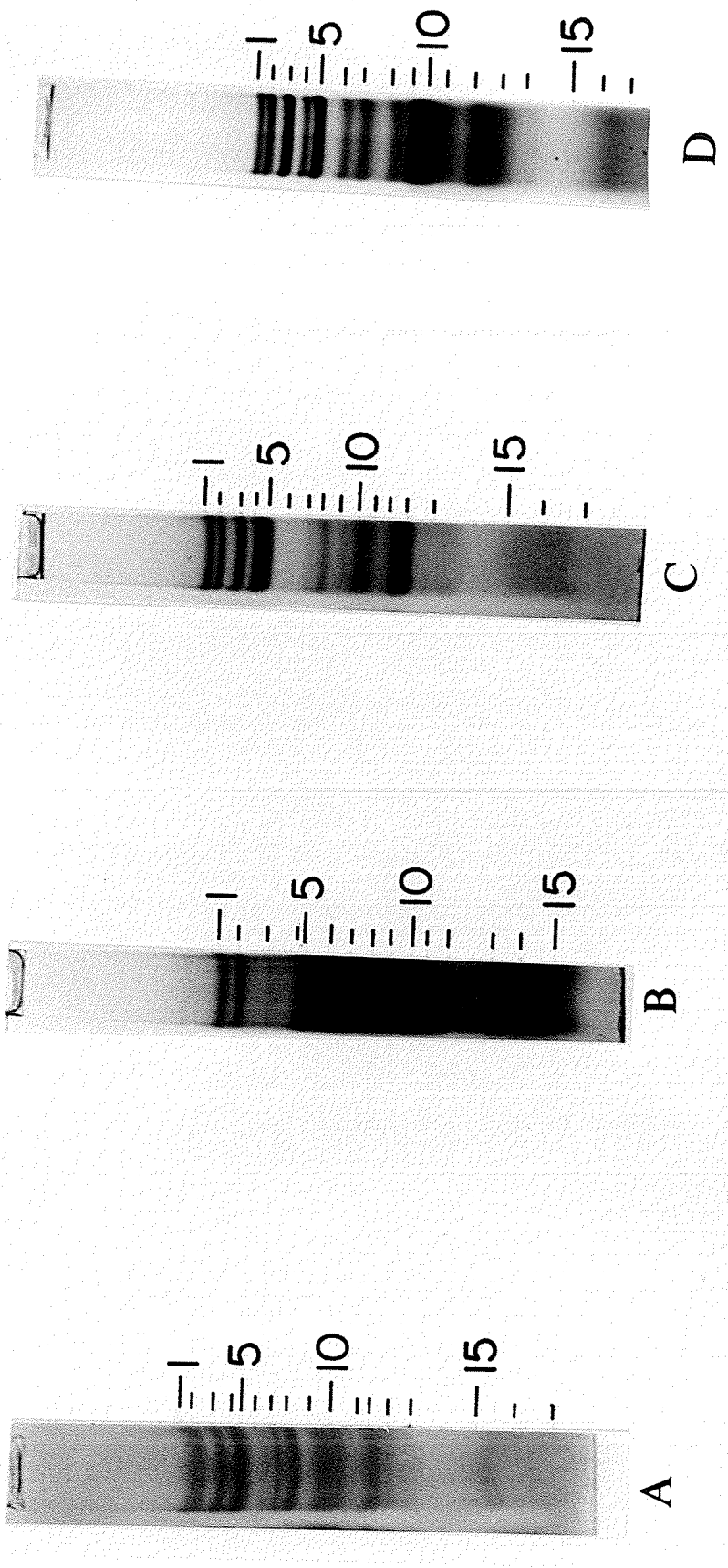
Figure 2. SDS-PAGE electrophoretograms of reduced glutenin isolated from gluten according to the procedure of Orth and Bushuk (1973a):

A - Manitou glutenin electrophoresed at pH 7.3 for 3 hr.

B - Stewart 63 glutenin electrophoresed at pH 7.3 for 3 hr.

C - Prelude glutenin electrophoresed at pH 7.3 for 3 hr.

D - Red River 68 glutenin electrophoresed at pH 8.9 for 3 hr.


1
5
10
15
D
1
5
10
15
C
1
5
10
15
B
1
5
10
15
A

Bietz and Wall (1972) used an SDS-PAGE system at pH 8.9 (Koenig *et al.*, 1970) to determine the mol wt values of the subunits of glutenin. The values obtained at pH 7.3 in this study with reduced reference proteins are in general agreement with the values obtained by Bietz and Wall (1972) at pH 8.9.

The mol wt values given in Table 2 for the subunits of Manitou glutenin are values calculated after 3 hr electrophoresis at pH 7.3. Cultivars that show better resolution of the 5 high mol wt subunits such as Prelude at pH 7.3 (Fig. 2C) and Red River 68 at pH 8.9 (Fig. 2D) can have mol wt values for subunit 2 as low as 128,000 to 130,000 instead of the 132,000 given in Table 2 for the poorly resolved subunit 2 of Manitou glutenin at pH 7.3.

This study, therefore, showed that reduction of reference proteins is necessary for a more accurate estimation of the mol wt values of the subunits of glutenin. It should be noted, however, that mol wt values obtained by SDS-PAGE are accurate only to ±10% when compared to more accurate methods such as sedimentation equilibrium or amino acid sequence analyses.

2. Subunit Composition of Glutenin of Three Hexaploid Wheats and Their Extracted AABB Tetraploids[1]

Orth and Bushuk (1973c) used the SDS-PAGE method at pH 7.3 to examine the effect of the removal of the D genome on the subunit composition of glutenin from the hexaploids Canthatch, Prelude, Rescue, and Thatcher, and their extracted AABB tetraploids. They reported that the highest mol wt subunit was deleted on removal of the D genome in all four cultivars. Bietz *et al.* (1975), using the same cultivars

[1] Some of the results in this section appeared in a publication by Khan and Bushuk, 1977.

with their SDS-PAGE method at pH 8.9, reported the presence of a high mol wt subunit on removal of the D genome. This high mol wt subunit, however, had either higher or lower mobility, depending on the cultivar, than the high mol wt subunit of the hexaploid counterpart. Accordingly, the glutenins from these related cultivars were re-examined by SDS-PAGE both at pH 7.3 and 8.9 in an attempt to resolve the apparent conflict between the two sets of results.

Glutenin from the hexaploids Prelude, Rescue, and Thatcher, and their extracted AABB tetraploids was isolated by the procedure of Chen and Bushuk (1970a; Procedure 3). The durum wheat cultivar Stewart 63, a natural AABB tetraploid, was included in this study for comparison purposes. The hexaploid cultivar Chinese Spring, used extensively in cytogenetic studies, was also included for comparison purposes.

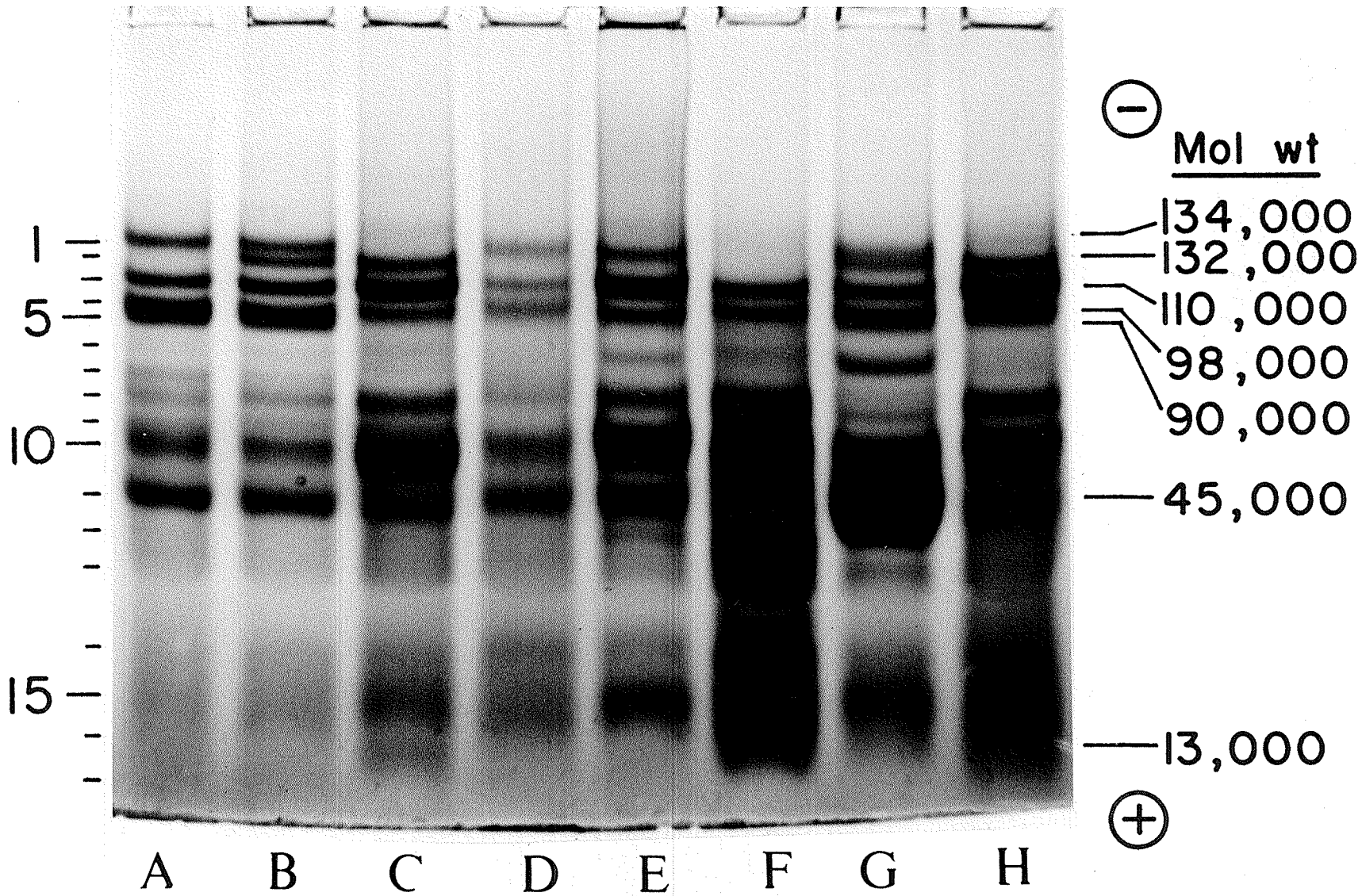
Fig. 3 shows the SDS-PAGE patterns of reduced glutenin at pH 7.3 from the hexaploids Prelude, Rescue, Thatcher, and their extracted AABB tetraploids. Prelude and Thatcher have two subunits, 134,000 and 132,000 daltons. It was previously thought that only one subunit of 133,000 existed in this mol wt region (Bietz *et al.*, 1975). On removal of the D genome these cultivars lost the 134,000 subunit (compare patterns B and C, and G and H). In contrast, the pattern for Rescue (pattern D) did not show two subunits of 134,000 and 132,000 at pH 7.3 but only one subunit of 133,000. Tetrarescue (pattern E) also showed one subunit of 133,000. On the other hand, Chinese Spring (pattern A) showed only one subunit of 134,000, the 132,000 subunit being absent in the pattern of this cultivar.

Removal of the D genome also resulted in deletion of the 90,000

Figure 3. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of reduced glutenin from the procedure of Chen and Bushuk (1973a):

A - Chinese Spring
B - Prelude
C - Tetraprelude
D - Rescue
E - Tetrarescue
F - Stewart 63
G - Thatcher
H - Tetrathatcher

Electrophoresis time was 3 hr. Staining procedure 2 was used.


1
5
10
15
A
B
C
D
E
F
G
H
⊖
Mol wt
134,000
132,000
110,000
98,000
90,000
45,000
13,000
⊕

subunit in all three hexaploids (patterns C, E, and H).

Fig. 4 shows the SDS-PAGE patterns of reduced glutenin at pH 8.9 of the hexaploids Prelude, Rescue, Thatcher, and their extracted AABB tetraploids. These patterns are somewhat different from those at pH 7.3 (Fig. 3). Prelude (pattern F) still had two subunits of 134,000 and 132,000 mol wt but Thatcher (pattern A) showed only one subunit of 133,000 at pH 8.9. Tetraprelude (pattern G) and Tetrathatcher (pattern B) still showed only the 132,000 subunit as at pH 7.3. Rescue (pattern C), however, now showed two subunits of 134,000 and 132,000 in contrast to only one subunit of 133,000 at pH 7.3. Tetrarescue (pattern D) unlike Tetraprelude and Tetrathatcher, lacked the 132,000 subunit but retained the 134,000 subunit. This could clearly be seen at pH 8.9. Chinese Spring (pattern E) had only one subunit of 134,000 at pH 8.9 as found for pH 7.3.

As at pH 7.3, the results at pH 8.9 showed that the 90,000 subunit was deleted on removal of the D genome for all three cultivars.

Stewart 63, a natural tetraploid, lacked the 134,000, 132,000, and 90,000 subunits both at pH 7.3 (Fig. 3, pattern F) and 8.9 (Fig. 4, pattern H).

Subunits 1 and 2 (134,000 and 132,000) were generally better resolved at pH 7.3 while subunits 4 and 5 (98,000 and 90,000) were better resolved at pH 8.9.

The apparent conflict between the results of Orth and Bushuk (1973c) and Bietz *et al.* (1975) arose from the use of different SDS-PAGE methods which resolve the high mol wt subunits of glutenin differently. Apparently there are at least five different high mol

Figure 4. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of reduced glutenin from the procedure of Chen and Bushuk (1970a) (procedure 3):

A - Thatcher
B - Tetrathatcher
C - Rescue
D - Tetrarescue
E - Chinese Spring
F - Prelude
G - Tetraprelude
H - Stewart 63

Electrophoresis time was 3 hr. Staining procedure 2 was used.

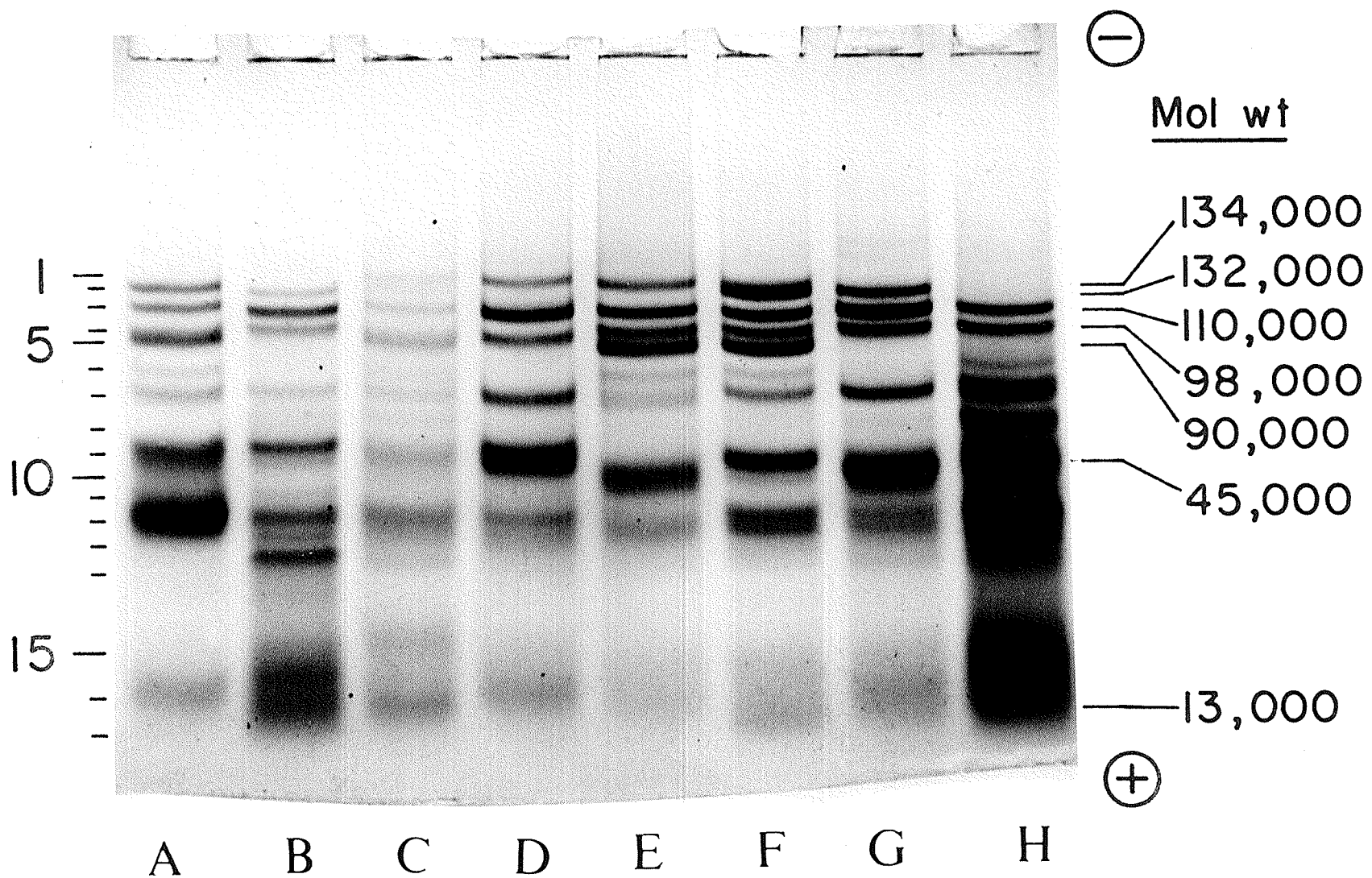
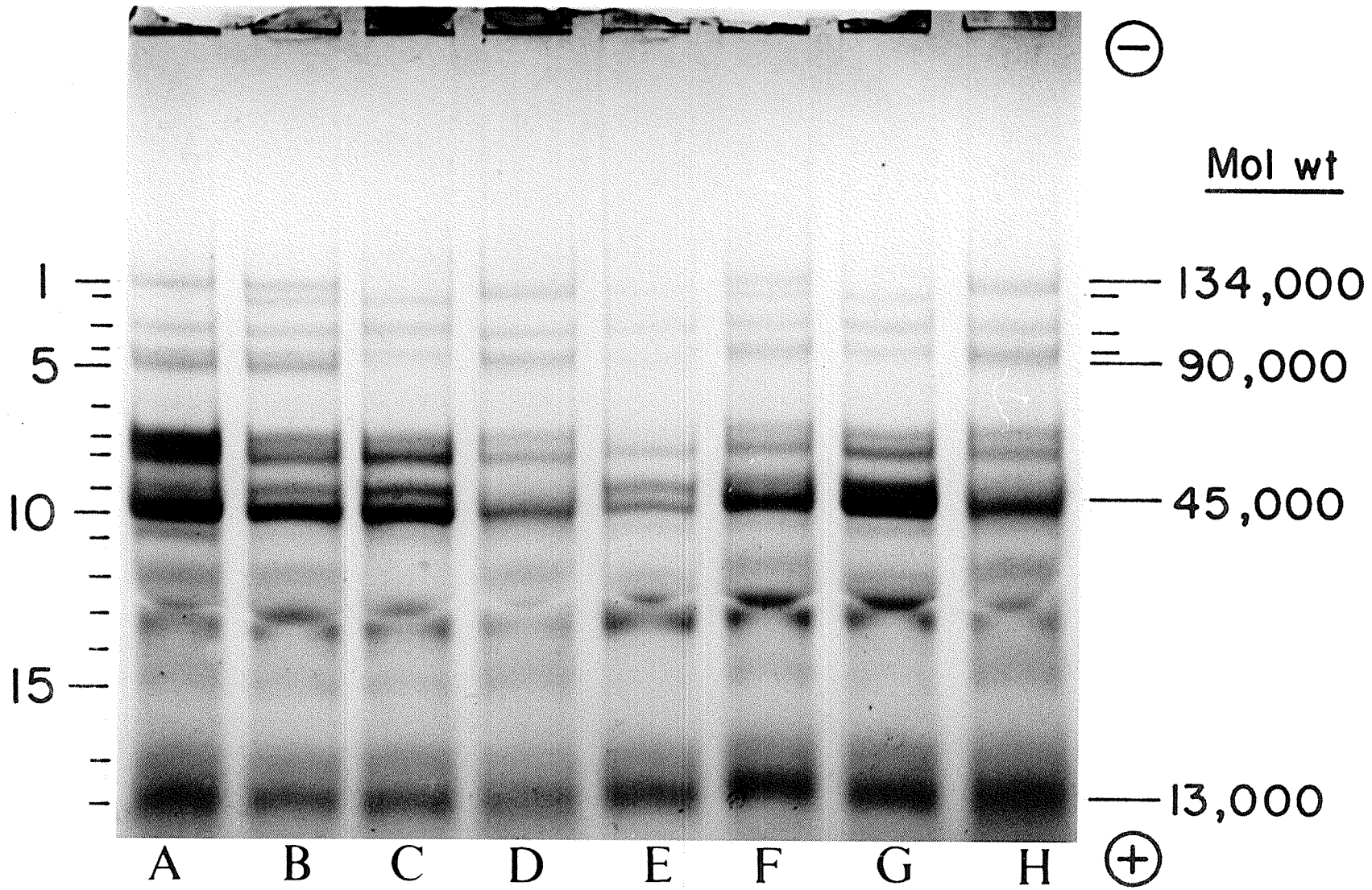

⊖
Mol wt
134,000
132,000
110,000
98,000
90,000
45,000
13,000
⊕
1
5
10
15
A
B
C
D
E
F
G
H

Figure 5. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of reduced glutenin isolated from single kernels (Bietz *et al.*, 1975):

A - Chinese Spring
B - Prelude
C - Tetraprelude
D - Rescue
E - Tetrarescue
F - Thatcher
G - Tetrathatcher

Electrophoresis time was 3 hr. Staining procedure 2 was used.

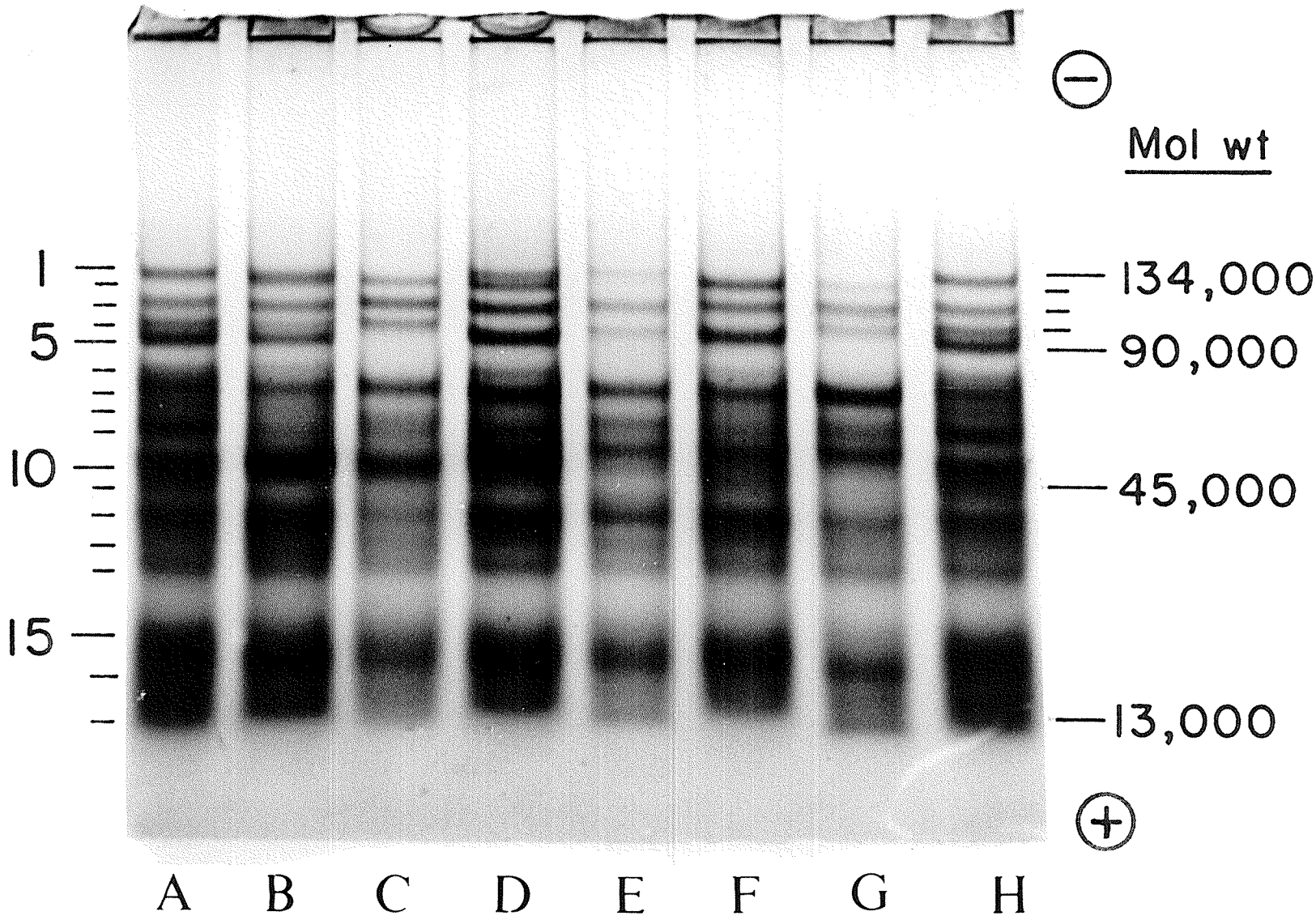


C1

Figure 6. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of reduced glutenin isolated from single kernels (Bietz *et al.*, 1975):

A - Chinese Spring
B - Prelude
C - Tetraprelude
D - Rescue
E - Tetrarescue
F - Thatcher
G - Tetrathatcher
H - Chinese Spring

Electrophoresis time was 3 hr. Staining procedure 2 was used.


1
5
10
15
Mol wt
134,000
90,000
45,000
13,000
A
B
C
D
E
F
G
H

wt subunits (134,000, 132,000, 110,000, 98,000, and 90,000) in the glutenin of the hexaploids Prelude, Rescue, and Thatcher. Orth and Bushuk (1973c) succeeded in resolving subunits 1 and 2 but not subunits 4 and 5 which ran as one broad band at pH 7.3. They, therefore, identified four of the five high mol wt subunits. Bietz *et al.* (1975), on the other hand, resolved subunits 4 and 5 but did not resolve subunits 1 and 2, which ran as one broad band at pH 8.9. They, also, saw four high mol wt subunits. Both groups, however, missed one, but different subunit, of the five high mol wt subunits.

Since Bietz *et al.* (1975) used a single-kernel procedure for isolating their glutenin, it was necessary to try their procedure in order to eliminate any variations in subunit composition arising from different purification procedures. Fig. 5 and Fig. 6 show the SDS-PAGE patterns of the hexaploids and their extracted AABB tetraploids from single kernels at pH 7.3 and pH 8.9, respectively. The patterns are essentially identical to those obtained for glutenin from the procedure of Chen and Bushuk (1970a) at the respective pH's (compare Fig. 3 and 5). The resolution of the subunits in the 68,000 and lower mol wt region is somewhat better resolved for the glutenin from single kernels than from the Osborne procedure of Chen and Bushuk (1970a).

3. Comparison of SDS-PAGE Methods for the Subunits of Glutenin[1]

Since it was observed that the high mol wt subunits of glutenin from the same cultivar gave different SDS-PAGE patterns with the pH 7.3 and pH 8.9 buffers, it was necessary to compare the subunit patterns of glutenin by a number of different SDS-PAGE methods from certain hexaploids to establish the possible reason(s) for these deviations in

[1] Some of the results in this section appeared in a publication by Khan and Bushuk, 1977.

resolution.

The cultivars used were Thatcher, Rescue, Prelude, Chinese Spring, Ponca, Manitou, Red River 68, Stewart 63, and Talbot. All except Stewart 63 are hexaploids. The natural tetraploid Stewart 63 was used in these studies because the first two high mol wt subunits of glutenin from this cultivar can be used as effective markers to locate the first five high mol wt subunits of hexaploid wheats.

Fig. 7 shows the SDS-PAGE patterns of reduced glutenin of the hexaploids on a 5% polyacrylamide SDS-gel electrophoresed for 3 hr at pH 7.3 using 0.02M phosphate buffer (Orth and Bushuk, 1973b). Subunits 1 and 2 are resolved for Thatcher (pattern A), Prelude (pattern C), Ponca (pattern E), and Manitou (pattern G), but not for Chinese Spring, Rescue, Red River 68 and Talbot. Subunits 4 and 5, which are clearly resolved only for Prelude and Chinese Spring and poorly resolved for Rescue, appear as a thick, darkly-stained band at pH 7.3 for the other cultivars.

Fig. 8 shows the patterns of the same cultivars electrophoresed at the same conditions for 5 hr instead of 3 hr. The first 2 subunits were better resolved after 5 hr of electrophoresis. Subunits 1 and 2 are more clearly resolved for all the cultivars except Rescue, Chinese Spring, and Red River 68. Subunits 4 and 5 show the same resolution after 5 hr and 3 hr except that the Manitou pattern showed two poorly resolved subunits.

In a subsequent experiment the pH of the 0.02M phosphate buffer system was changed from 7.3 to 8.0 to test whether pH affected the resolution of the high mol wt subunits. Fig. 9 shows the SDS-PAGE

Figure 7. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of reduced glutenin isolated according to the procedure of Chen and Bushuk (1970a) from the following hexaploid cultivars:

A - Thatcher
B - Rescue
C - Prelude
D - Chinese Spring
E - Ponca
F - Red River 68
G - Manitou
H - Talbot

Electrophoresis time was 3 hr. Staining procedure 2 was used.

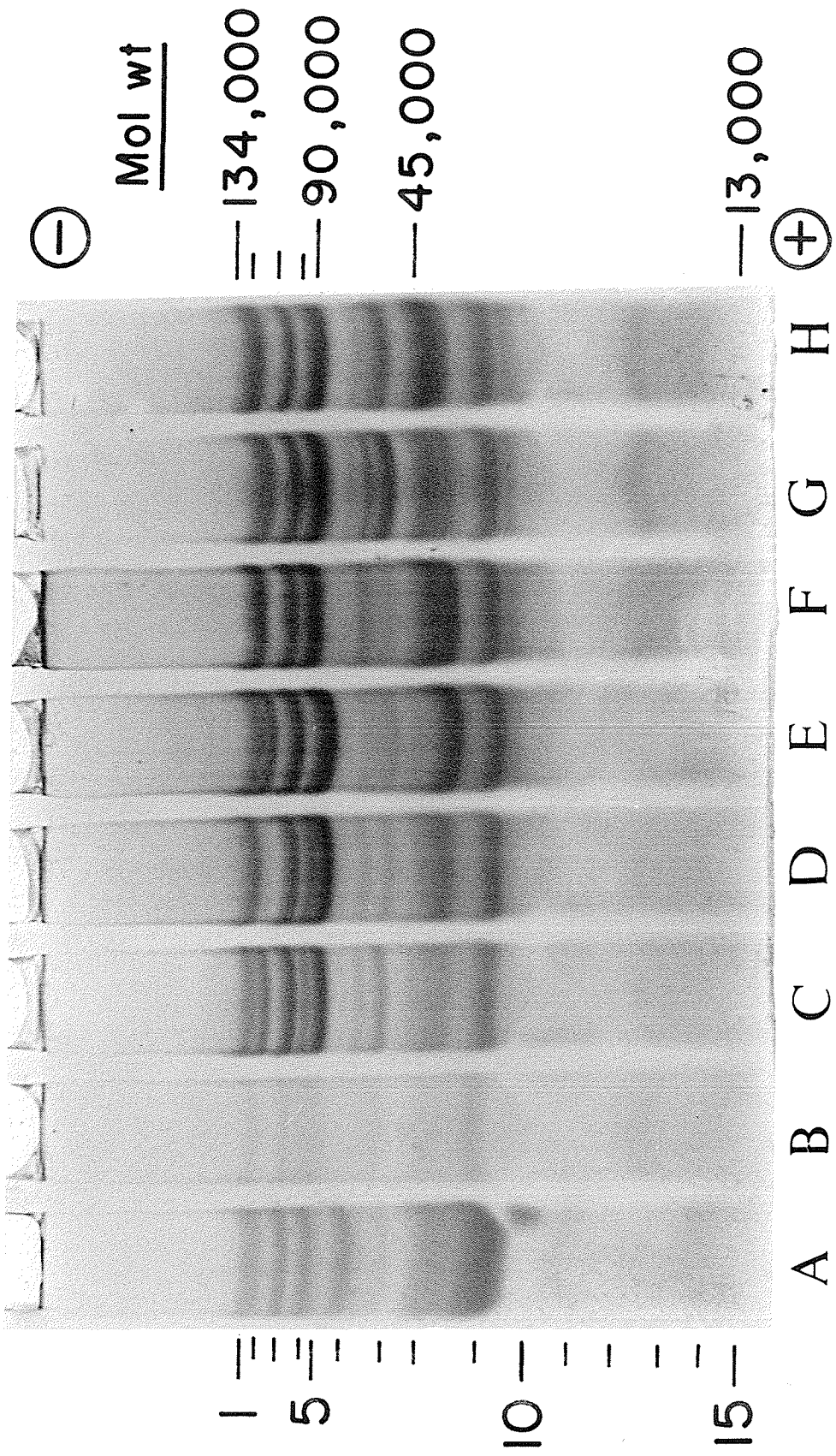

Mol wt
134,000
90,000
45,000
13,000
A
B
C
D
E
F
G
H
1
5
10
15

Figure 8. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of reduced glutenin from the procedure of Chen and Bushuk (1970a) from the following hexaploid cultivars:

A - Thatcher
B - Rescue
C - Prelude
D - Chinese Spring
E - Ponca
F - Red River 68
G - Manitou
H - Talbot

Electrophoresis time was 5 hr. Staining procedure 2 was used.

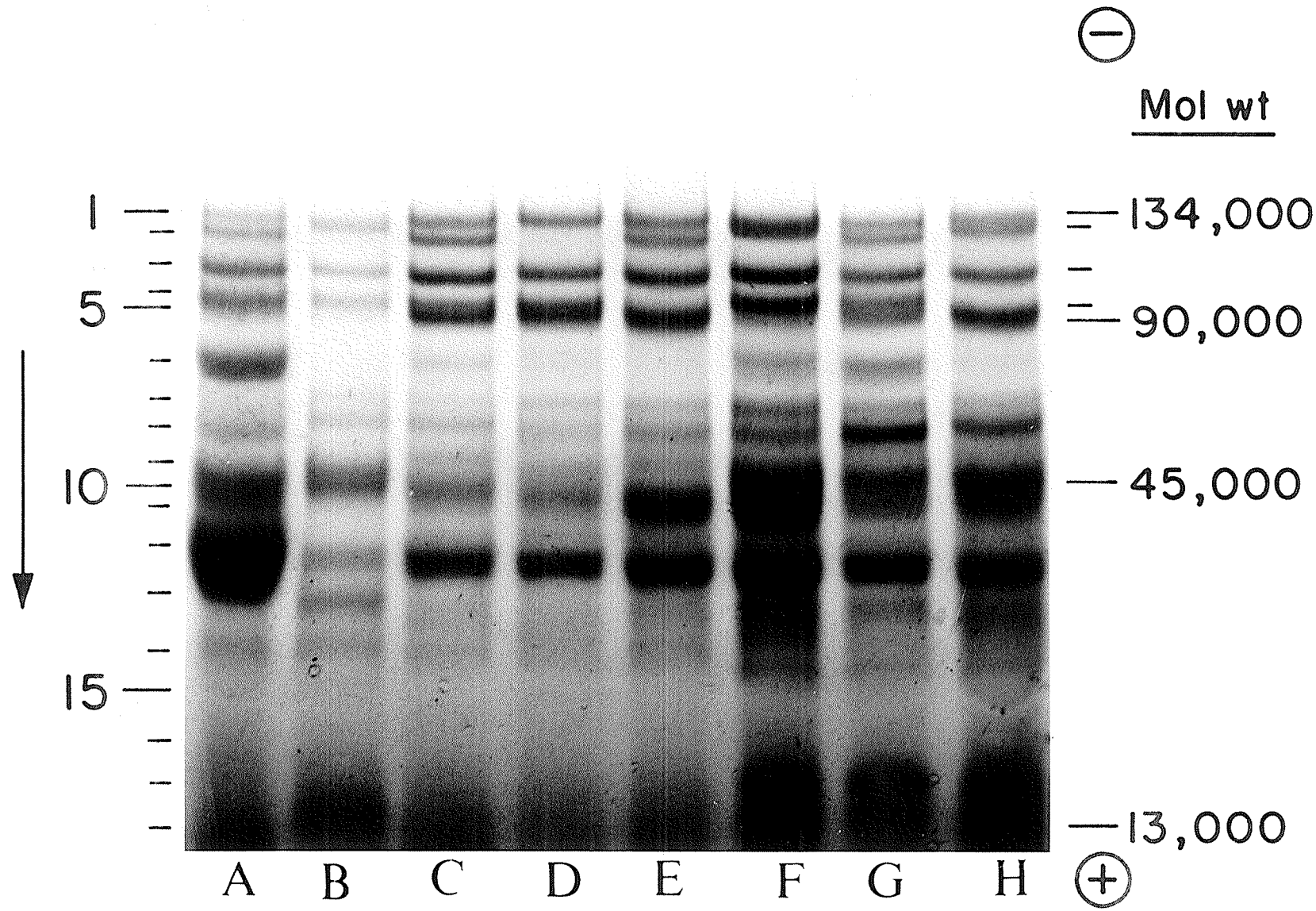
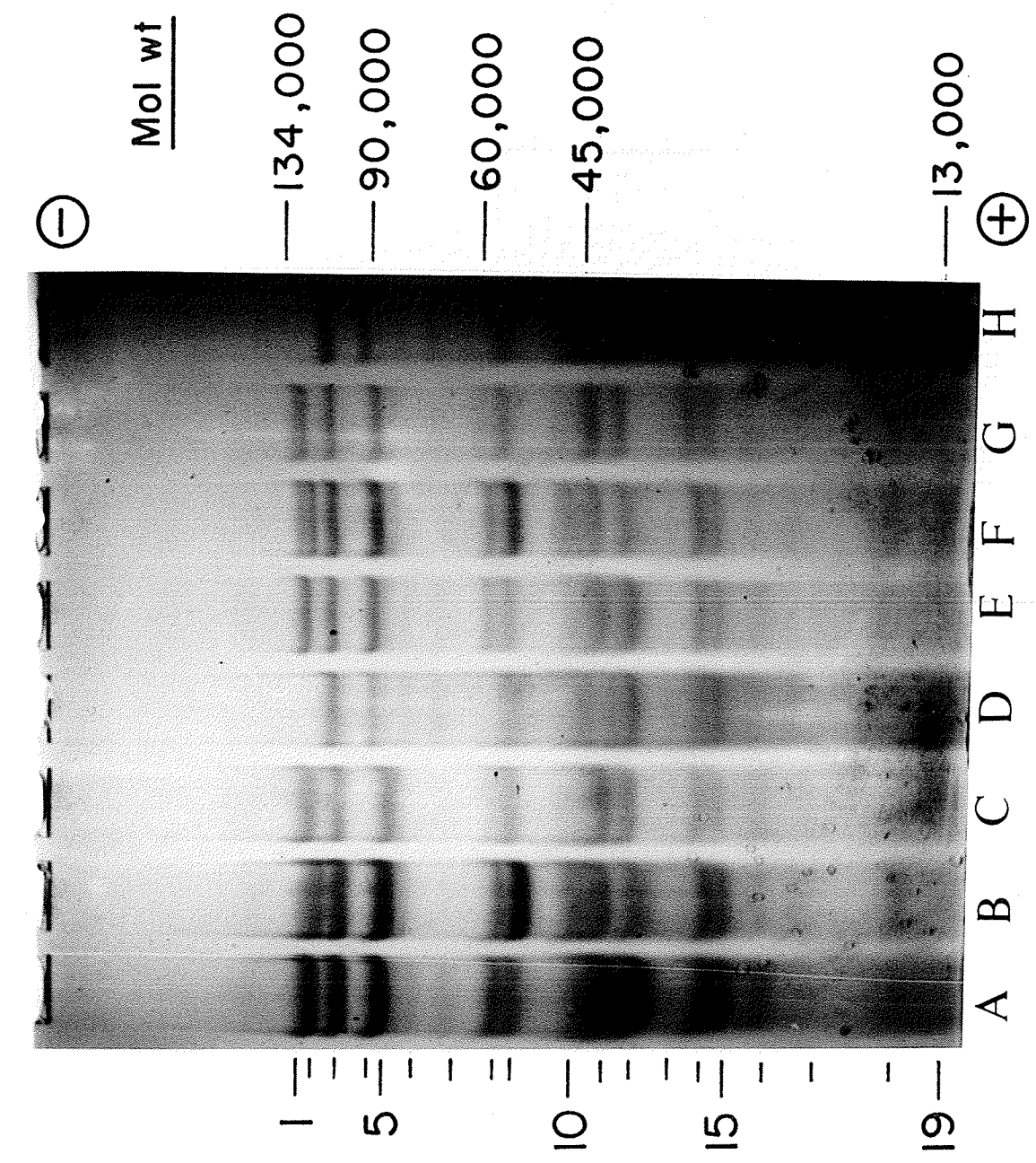

⊖
Mol wt
1
5
10
15
134,000
90,000
45,000
13,000
A
B
C
D
E
F
G
H
⊕

Figure 9. SDS-PAGE electrophoretograms at pH 8.0 (0.02M phosphate buffer) of reduced glutenin from the pH precipitation procedure of Orth and Bushuk (1973a):

A - Red River 68 (50 µl)
B - Manitou (50 µl)
C - Talbot (50 µl)
D - Stewart 63 (50 µl)
E - Red River 68 (20 µl)
F - Manitou (20 µl)
G - Talbot (20 µl)
H - Stewart 63 (20 µl)

Samples were applied in duplicate from a 10 mg/ml concentration to test the effect of the amount of protein on the resolution of subunits. Electrophoresis time was 4 1/2 hr. Staining procedure 2 was used.


Mol wt
134,000
90,000
60,000
45,000
13,000
A
B
C
D
E
F
G
H
1
5
10
15
19

patterns at pH 8.0 after 4 1/2 hr electrophoresis. The resolution of the high mol wt subunits and the subunits with mol wts of 68,000 and lower is similar to that at pH 7.3. Therefore, the pH does not affect the resolution of the subunits of glutenin from these cultivars.

The ionic strength of the phosphate buffer system was increased from 0.02M to 0.05M at pH 7.3 to test whether ionic strength affected the resolution of the high mol wt subunits. Fig. 10 shows that the first two high mol wt subunits are resolved as at 0.02M ionic strength, after 4 1/2 hr electrophoresis, for the cultivars Manitou and Talbot. Subunits 1 and 2 of Red River 68, however, are still not resolved at the higher ionic strength. On the other hand, the resolution of subunits 4 and 5 is slightly improved. Of the three hexaploid cultivars, the best resolution of subunits 4 and 5 was for Red River 68. It seems that a higher ionic strength with the phosphate buffer system at pH 7.3 improves the resolution of subunits 4 and 5.

The effect of the type of buffer system on subunit resolution was examined by comparing results obtained with phosphate and Tris-borate buffers.

Fig. 11 shows the glutenin subunit patterns of hexaploid wheats electrophoresed for 3 hr at pH 8.9 using 0.125M Tris-borate buffer. The first two high mol wt subunits are clearly resolved in the patterns for Rescue (pattern B) and Red River 68 (pattern F) and somewhat less clearly resolved for Prelude (pattern C). Chinese Spring (pattern D) shows one subunit of 134,000 daltons. The other cultivars show one brightly-stained subunit of about 133,000.

Fig. 12 shows the patterns of the same (as Fig. 11) cultivars

Figure 10. SDS-PAGE electrophoretograms at pH 7.3 (0.05M phosphate buffer) of reduced glutenin from the procedure of Orth and Bushuk (1973a):

A - Red River 68 (50 µl)
B - Red River 68 (20 µl)
C - Manitou (50 µl)
D - Manitou (20 µl)
E - Talbot (50 µl)
F - Talbot (20 µl)
G - Stewart 63 (50 µl)
H - Stewart 63 (20 µl)

Samples were applied in duplicate as in Fig. 9. Electrophoresis time was 4 1/2 hr. Staining procedure 2 was used.

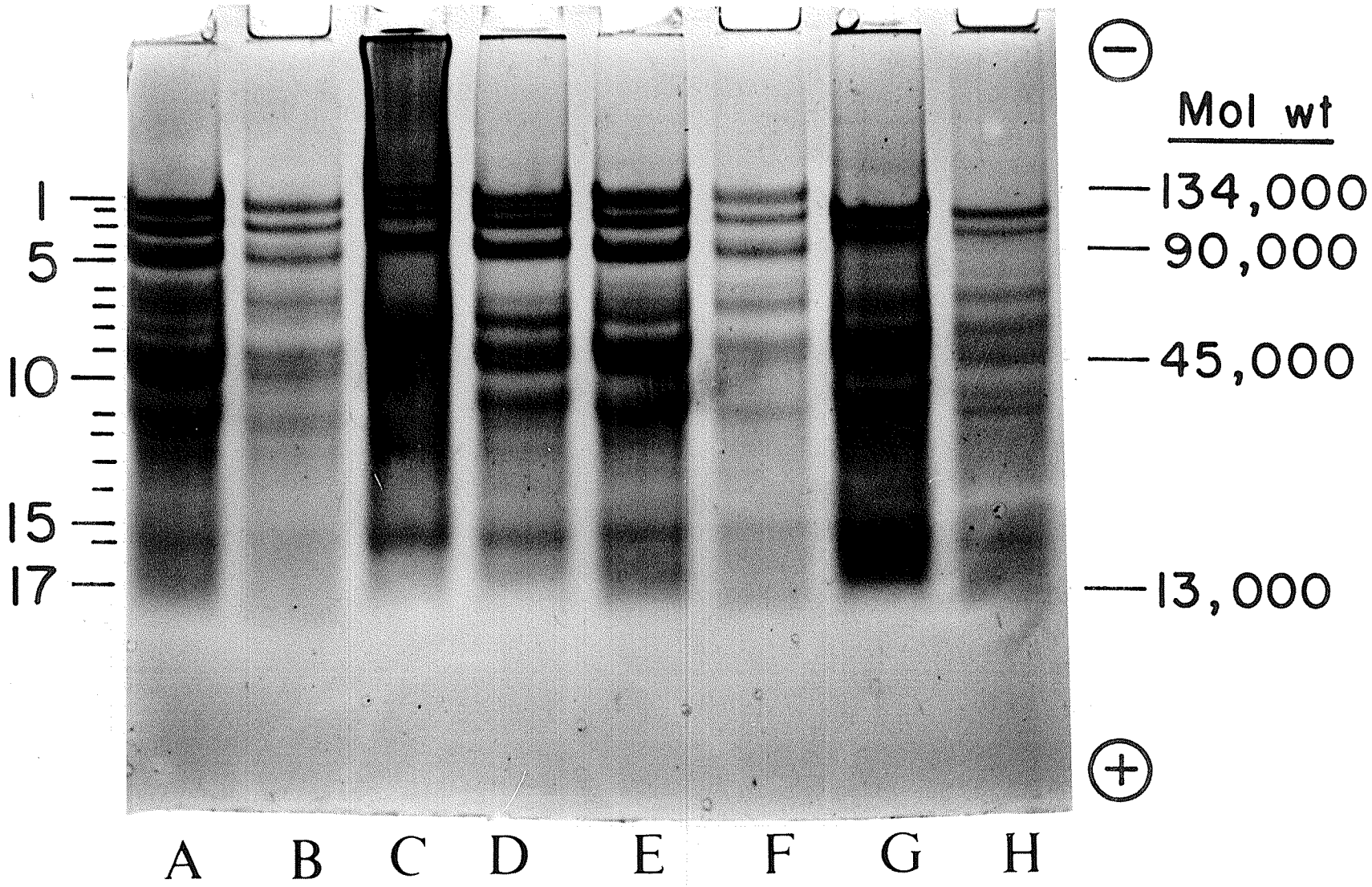

1
5
10
15
17
⊖
Mol wt
134,000
90,000
45,000
13,000
⊕
A
B
C
D
E
F
G
H

Figure 11. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of reduced glutenin from the procedure of Chen and Bushuk (1970a) from the following hexaploids:

A - Thatcher
B - Rescue
C - Prelude
D - Chinese Spring
E - Ponca
F - Red River 68
G - Manitou
H - Talbot

Electrophoresis time was 3 hr. Staining procedure 2 was used.

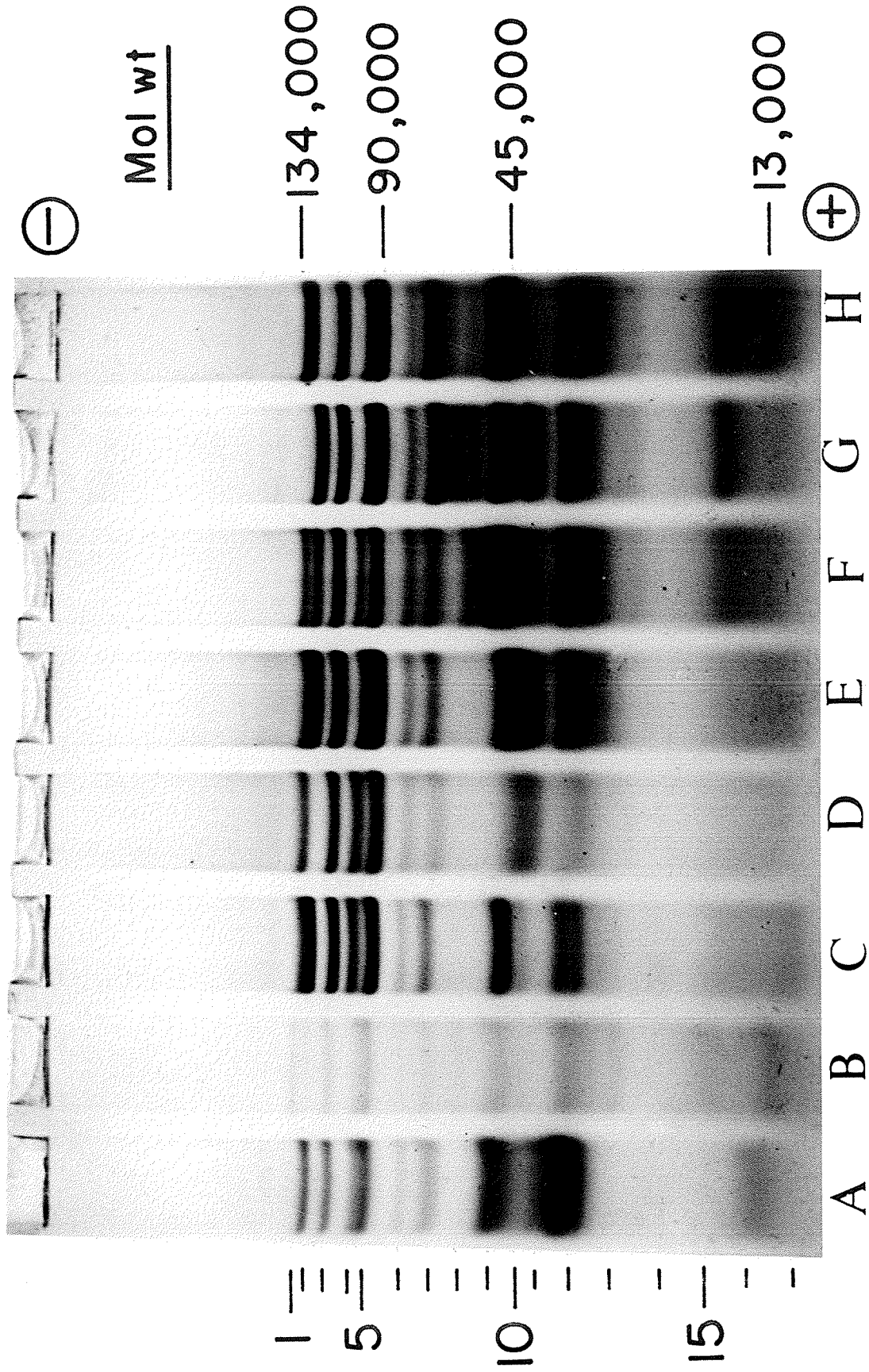

Mol wt
134,000
90,000
45,000
13,000
⊖
⊕
A
B
C
D
E
F
G
H
1
5
10
15

Figure 12. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of reduced glutenin from the following hexaploid cultivars (isolation procedure in brackets):

A - Thatcher (Chen and Bushuk, 1970a)

B - Rescue (Bietz *et al.*, 1975)

C - Prelude (Chen and Bushuk, 1970a)

D - Chinese Spring (Chen and Bushuk, 1970a)

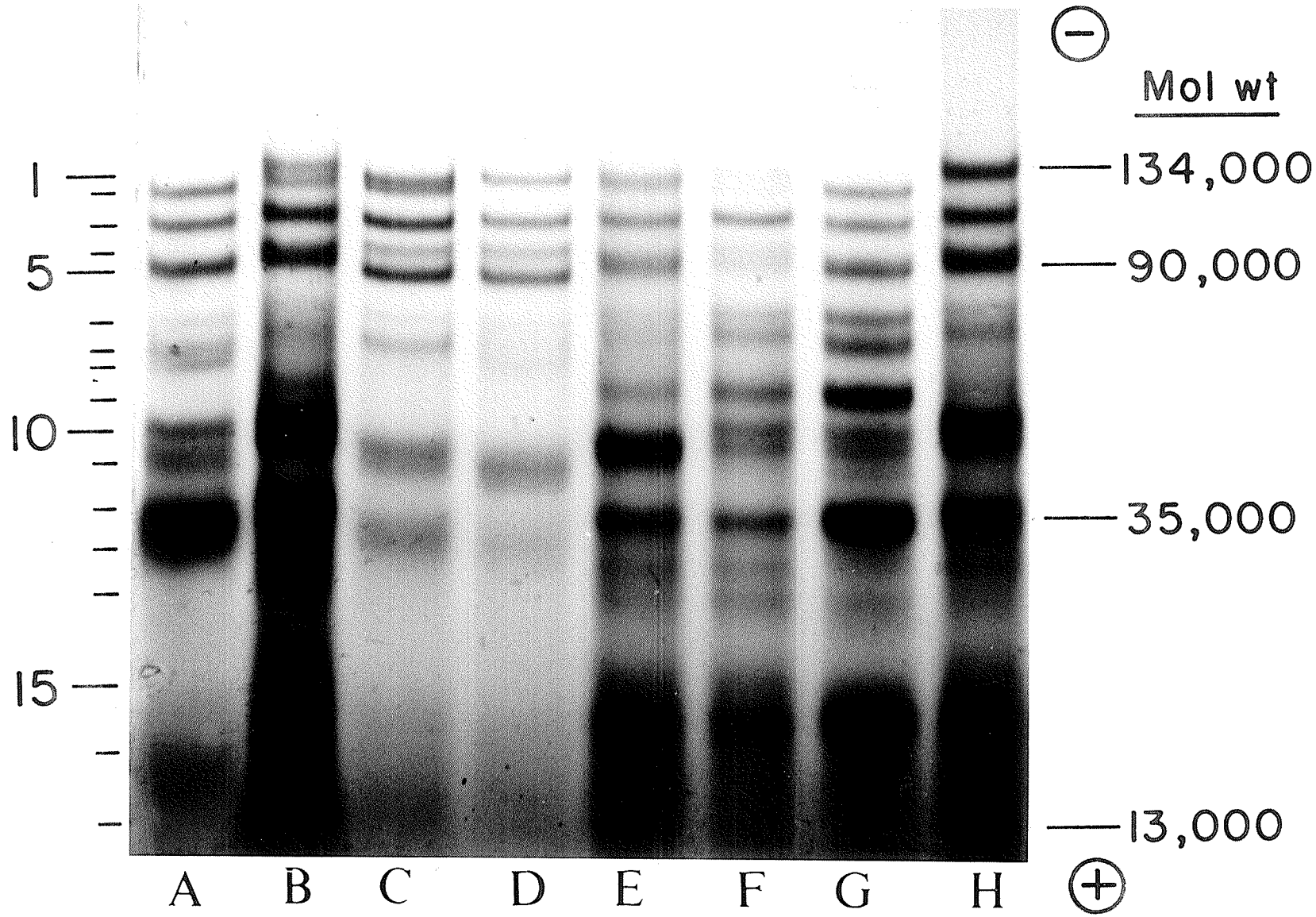
E - Ponca (pH precipitation procedure of Jones *et al.*, 1959)

F - Red River 68 (" " " ")

G - Manitou (" " " ")

H - Talbot (" " " ")

Electrophoresis time was 4 1/2 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
35,000
13,000
1
5
10
15
A
B
C
D
E
F
G
H
⊕

electrophoresed for 4 1/2 hr instead of 3 hr. The resolution of the first two high mol wt subunits is the same after 4 1/2 hr as after 3 hr. Subunits 4 and 5, which generally are well resolved at pH 8.9, were only slightly better resolved after 4 1/2 hr. Subunits 4 and 5 were best resolved in the patterns of Red River 68, Prelude and Chinese Spring of all the cultivars examined.

The 0.125M Tris-borate, pH 8.9, buffer was diluted to 0.06M, pH 8.9, to test whether ionic strength at the higher pH had any effect on the resolution of the high mol wt subunits. Fig. 13 shows the SDS-PAGE patterns that were obtained. The resolution of subunits 4 and 5 improved considerably (compare Figs. 13 and 12). Subunits 1 and 2 are still not resolved at the lower ionic strength except for Red River 68, which has all its five high mol wt subunits resolved at both buffer concentrations.

In a further experiment on the effect of ionic strength, the concentration of the Tris-borate, pH 8.9, buffer was increased to 0.40M. Fig. 14 shows that the high mol wt subunits of Red River 68 (patterns A and B) are not as clearly resolved as at the lower ionic strengths. The first two high mol wt subunits are still not resolved for the other hexaploids examined. Subunits 4 and 5 of Manitou and Talbot showed similar resolution as at 0.125M ionic strength but not as good as at 0.06M.

Thus, it seems that the resolution of the high mol wt subunits of glutenin is affected more by ionic strength than by the pH of the buffer solution.

The resolution of the subunits with mol wts of 68,000 and lower is also affected by changes in ionic strength. However, due to the heterogeneity of the subunits in this mol wt region it is difficult to identify

Figure 13. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris-borate buffer) of reduced glutenin from the procedure of Orth and Bushuk (1973a).

A - Red River 68 (50 µl)
B - Manitou (50 µl)
C - Alkylated Manitou (50 µl)
D - Talbot (50 µl)
E - Stewart 63 (50 µl)
F - Manitou (30 µl)
G - Manitou (20 µl)
H - Talbot (20 µl)

Samples applied for electrphoresis were from a concentration of 10 mg/ml. Electrophoresis time was 4 hr. Staining procedure 2 was used.

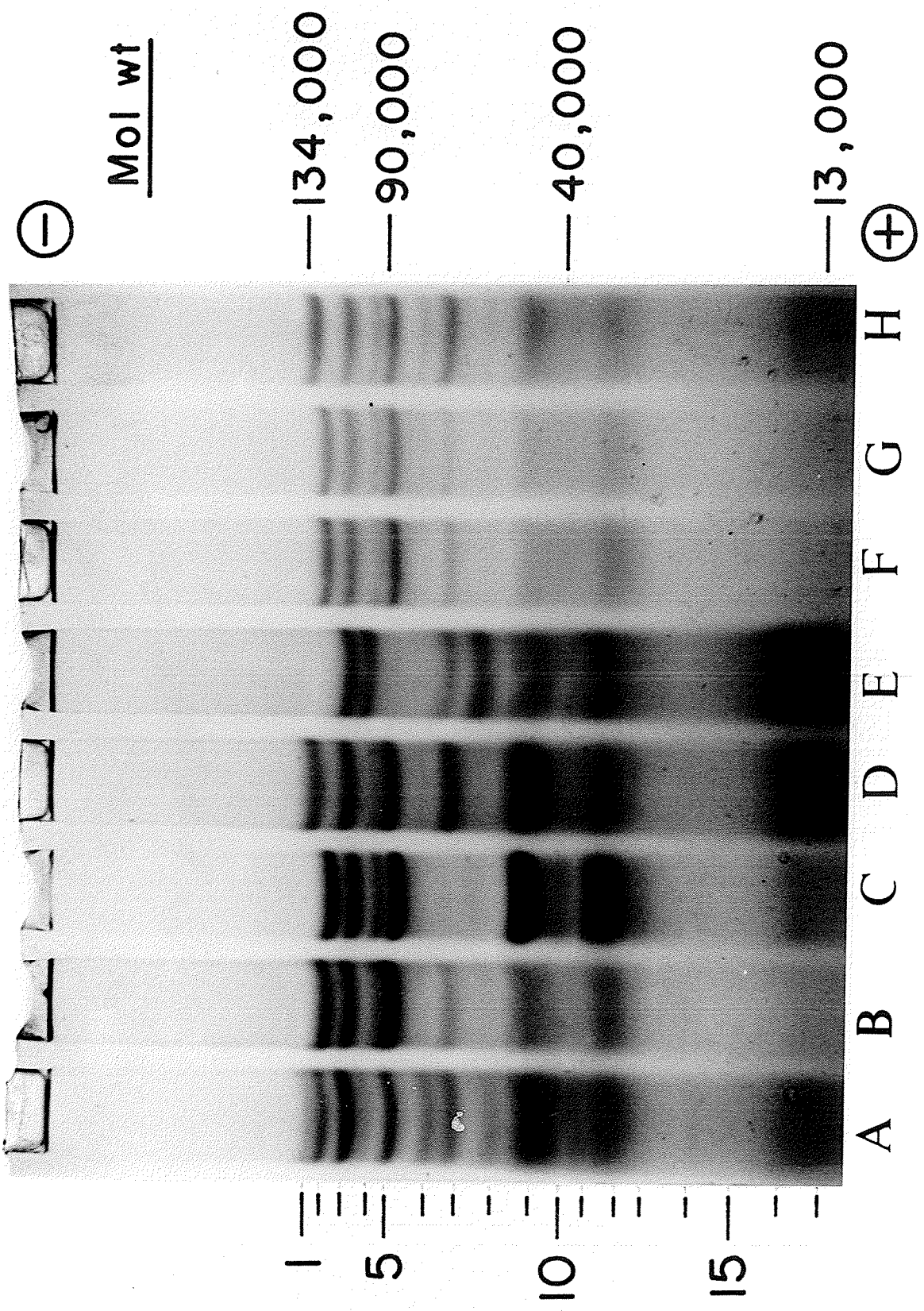
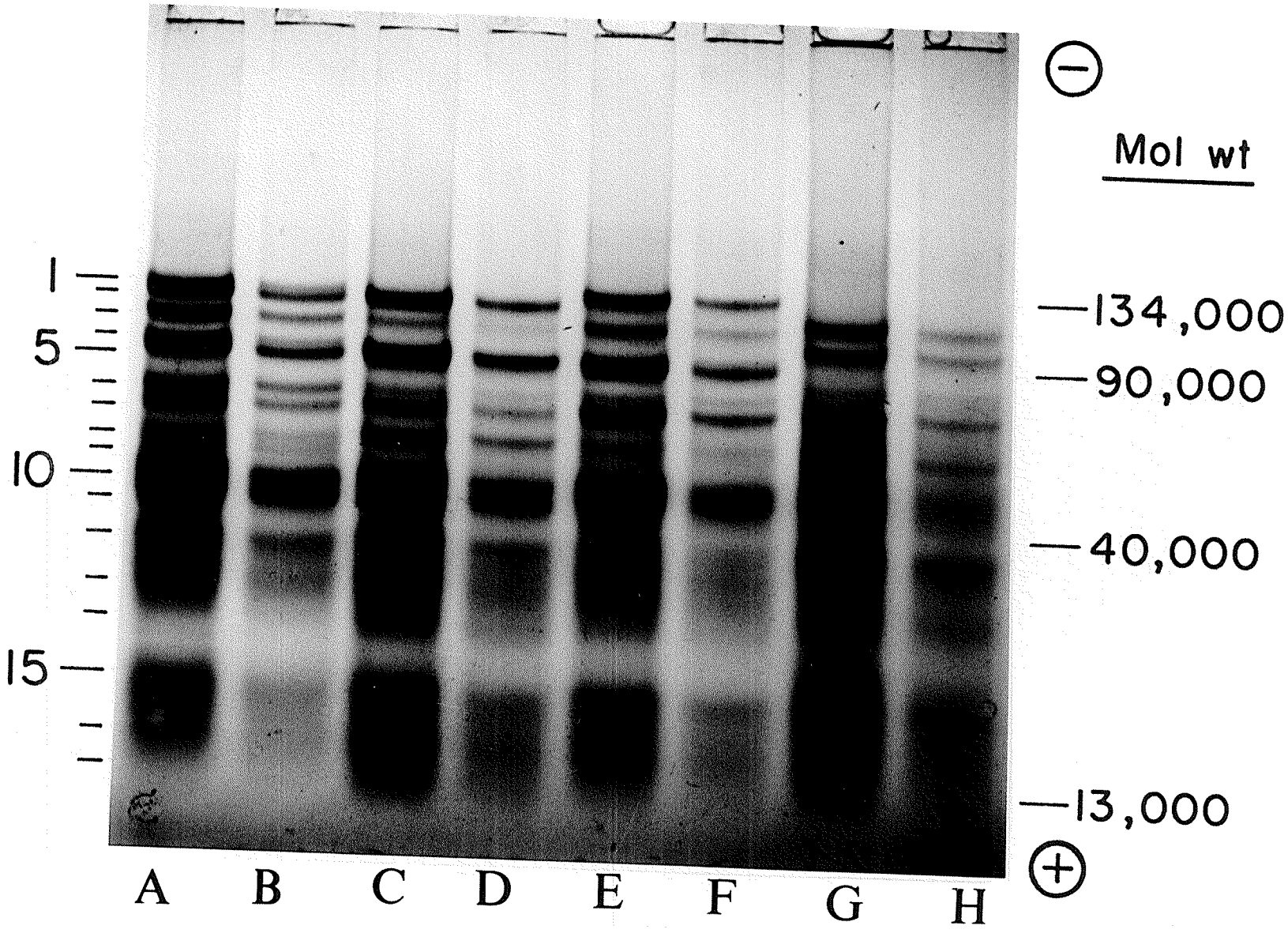

Mol wt
134,000
90,000
40,000
13,000
⊖
⊕
A
B
C
D
E
F
G
H
1
5
10
15

Figure 14. SDS-PAGE electrophoretograms at pH 8.9 (0.40M Tris-borate buffer) of reduced glutenin from the procedure of Orth and Bushuk (1973a):

A - Red River 68 (50 µl)
B - Red River 68 (20 µl)
C - Manitou (50 µl)
D - Manitou (20 µl)
E - Talbot (50 µl)
F - Talbot (20 µl)
G - Stewart 63 (50 µl)
H - Talbot (20 µl)

Samples were applied in duplicate from a 10 mg/ml concentration. Electrophoresis time was 4 hr. Staining procedure 2 was used.


⊖
Mol wt
1
5
10
15
134,000
90,000
40,000
13,000
⊕
A
B
C
D
E
F
G
H

individual subunits. In contrast, the high mol wt subunits of hexaploids are very easily identified especially when compared with the subunit pattern of the natural tetraploid, Stewart 63, as already mentioned.

4. Extraction Procedures for Glutenin

During the course of the investigation on the subunit composition of glutenin at different stages of grain maturity (Khan and Bushuk, 1976), it was observed that the subunit compositions of glutenin from the modified Osborne procedure (procedure 3) of Chen and Bushuk (1970a) and that prepared from gluten by the pH precipitation procedure (procedure 1) of Orth and Bushuk (1973a) showed certain qualitative and quantitative differences. This observation prompted a more thorough investigation of the subunit composition of glutenins prepared by a number of different procedures. The results from these experiments are presented in this section.

Before the results are presented, however, the two terms, "qualitative" and "quantitative", used in discussing differences in gel patterns, will be explained. The term "qualitative" difference is used to indicate absence of a particular band that is present in the gel used in the comparison. The term "quantitative" difference is used to indicate that a certain stained protein band in one SDS-gel pattern is more intensely stained than the band of the same mobility in another SDS-gel. These differences are based solely on visual estimation of the stained bands immediately after destaining.

a. Subunit Composition of Glutenin Prepared by Different Procedures

The subunit compositions of reduced Manitou glutenin, isolated by different extraction procedures, were compared by SDS-PAGE at pH 7.3 and 8.9. The patterns for different preparations (Fig. 15) at pH 7.3 show both qualitative and quantitative differences in subunit composition. The subunits from the single-kernel procedure (procedure 5) (Bietz *et al.*, 1975) and the pH precipitation procedure (procedure 2) (Jones *et al.*, 1959) stained more brightly, especially bands 14 to 20 (patterns A and B). The intensity of bands 14 to 20 in the other patterns are much fainter even though the staining intensity of the first 5 high mol wt subunits seems to be the same in all the patterns except in that of the β-mercaptoethanol extract. Glutenin purified by the pH precipitation procedure of Orth and Bushuk (1973a) shows fewer and fainter bands in the 68,000 and lower mol wt region than the other preparations. Glutenin from the modified Osborne procedure of Chen and Bushuk (1970a) (pattern C) seems to be deficient in subunits 6 and 9.

Figure 16 shows the subunit patterns of glutenins from different extraction procedures electrophoresed at pH 8.9. Bands 14 to 20 are more intense in patterns B and C (just as in patterns A and B at pH 7.3) of reduced glutenin from the single-kernel procedure and glutenin from the pH precipitation procedure of Jones *et al.* (1959). Subunits 6 and 9 also seem to be absent from the pattern of glutenin from the modified Osborne procedure of Chen and Bushuk (1970a), as found for pH 7.3 (pattern D). The absence or presence of subunit 9 is more clearly seen in the pH 8.9 patterns than in those at pH 7.3.

Figure 15. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of reduced glutenin prepared by different procedures:

A - Glutenin from single-kernel procedure of Bietz *et al.* (1975)

B - Glutenin from the pH precipitation procedure of Jones *et al.* (1959)

C - Glutenin from the modified Osborne procedure of Chen and Bushuk (1970a)

D - Mercuric chloride-soluble glutenin from the Osborne procedure of Bietz and Wall (1975)

E - β-mercaptoethanol-soluble glutenin from procedure of Bietz and Wall (1975)

F - Glutenin from the pH precipitation procedure of Orth and Bushuk (1973a)

Electrophoresis time was 5 hr. Staining procedure 2 was used.

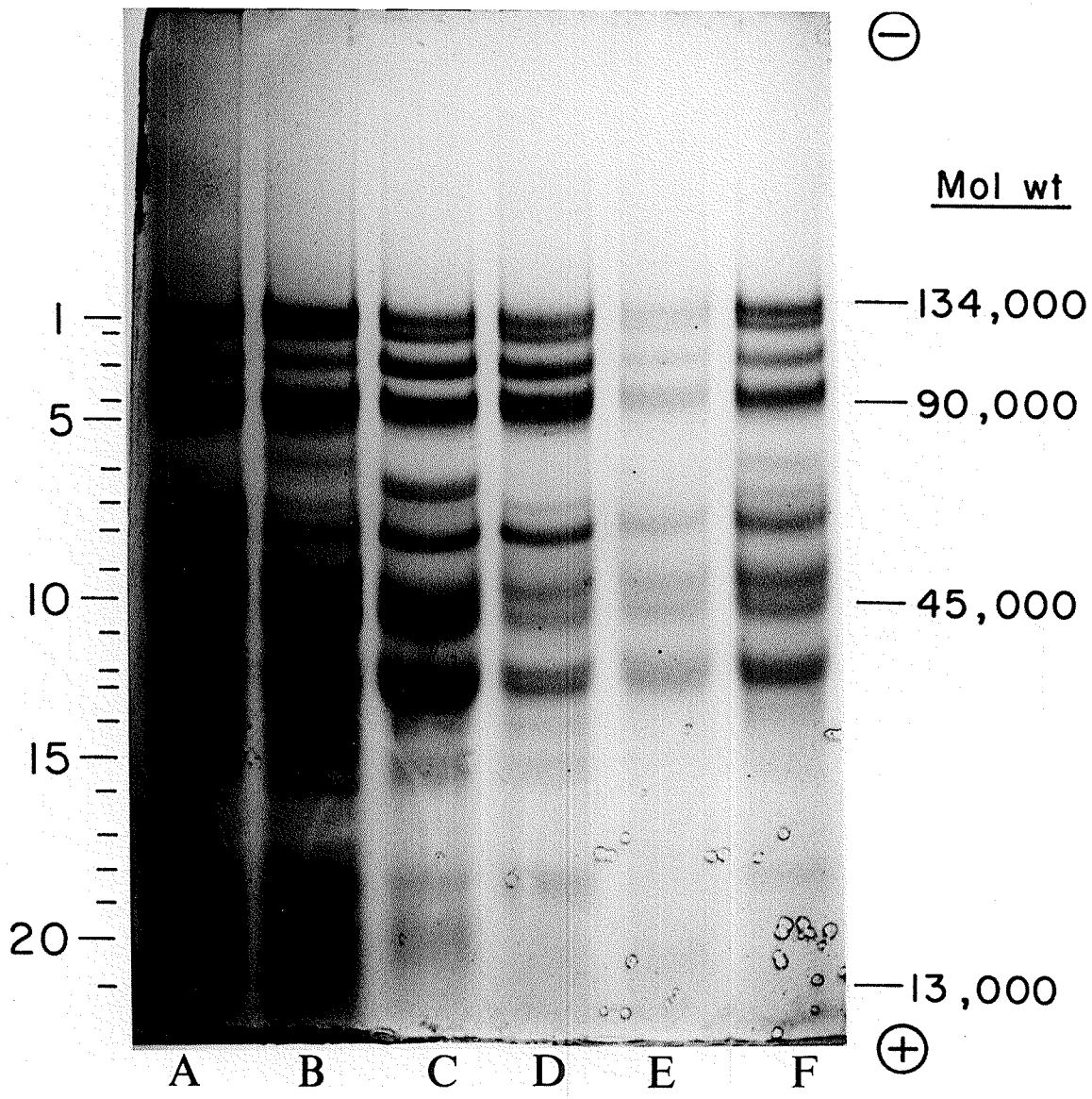

⊖
Mol wt
1
5
10
15
20
134,000
90,000
45,000
13,000
⊕
A
B
C
D
E
F

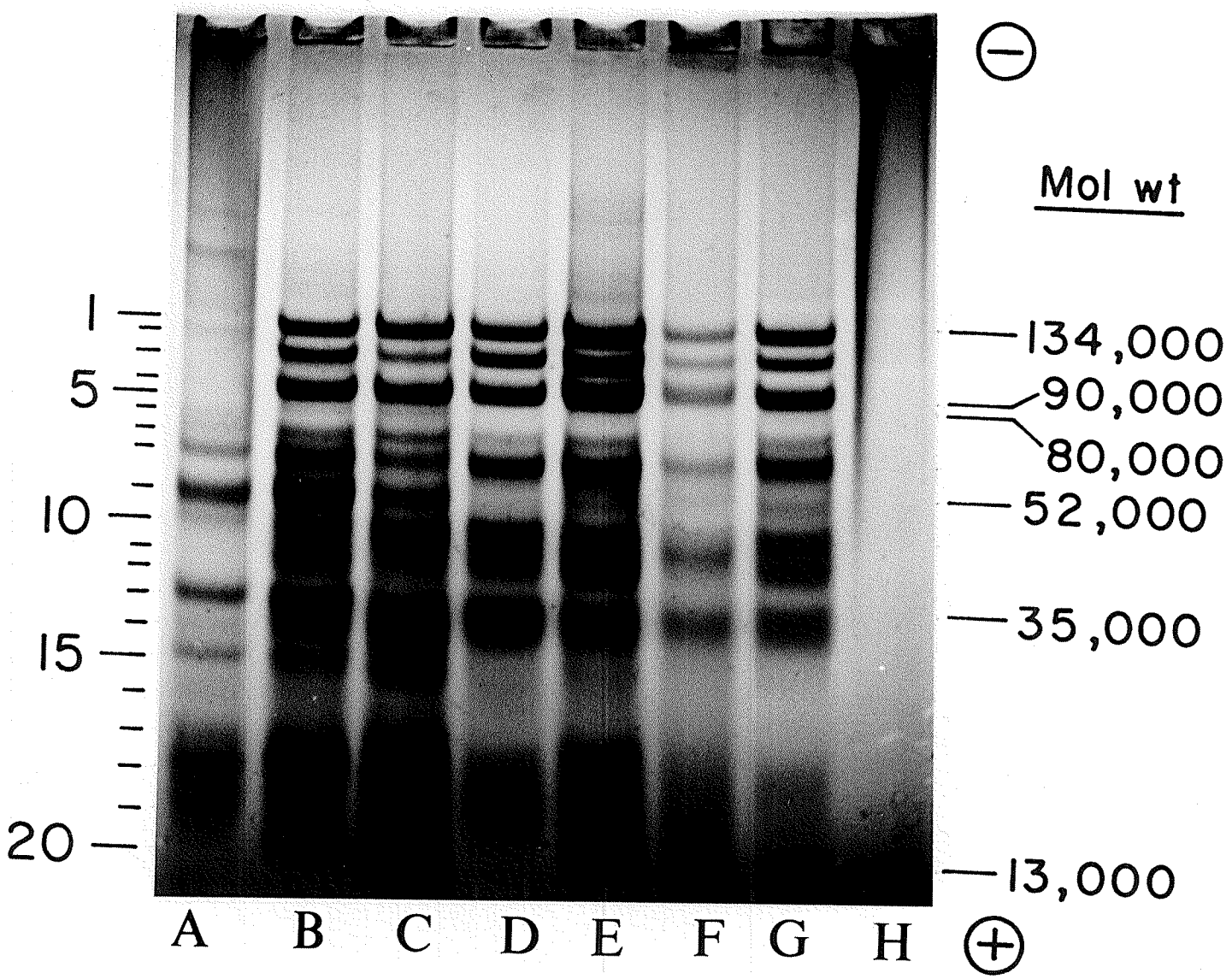
Figure 16. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of unreduced glutenin (patterns A and H) and reduced glutenin (patterns B to G) prepared by different procedures;

A - Unreduced glutenin from the single-kernel procedure of Bietz *et al.* (1975).

B → G - Sample description same as in Figure 15.

H - Unreduced glutenin from the pH precipitation procedure of Orth and Bushuk (1973a).

Electrphoresis time was 4 1/2 hr. Staining procedure 2 was used.


⊖
Mol wt
1
5
10
15
20
134,000
90,000
80,000
52,000
35,000
13,000
A B C D E F G H
⊕

Bietz and Wall (1975) also observed qualitative and quantitative differences in the subunit composition of glutenin prepared by different procedures. However, they observed the qualitative difference for subunit 9 (their subunit 8) in reduced glutenin isolated from gluten according to the procedure of Jones *et al.* (1959).

Included with the reduced proteins in Fig. 16, for comparison purposes, are the patterns of unreduced glutenin from the single-kernel procedure (Bietz *et al.*, 1975) (pattern A) and the pH precipitation procedure (pattern H) of Orth and Bushuk (1973a). Many prominent protein bands are seen on the SDS-gel from unreduced glutenin from the single-kernel procedure whereas there are fewer and fainter protein bands seen on the gel from glutenin from the procedure of Orth and Bushuk (1973a). It should be noted that pattern H is for glutenin that was purified three times with sulfoethyl-Sephadex after pH precipitation according to the procedure of Orth and Bushuk (1973a).

b. Glutenin from the Single-Kernel Procedure

Since many prominent protein components entered the SDS-gel from unreduced glutenin from the single-kernel procedure of Bietz *et al.* (1975) (Fig. 16, pattern A), it was assumed that this glutenin was not thoroughly purified. Therefore, the following purification procedure was developed to attempt to remove the "contaminating" proteins that enter SDS-gels when unreduced glutenin from the single-kernel procedure is subjected to SDS-PAGE.

The single-kernel procedure was chosen for these experiments since it requires very little material, is rapid, and the SDS-PAGE pattern of reduced glutenin from this procedure is generally better resolved than

that of reduced glutenin prepared by other procedures.

The original procedure of Bietz *et al.* (1975) was modified as follows. To improve the extraction of the albumins and the globulins, the volume of the NaCl solution was increased to 10 ml from 5 ml and the extraction made 3 times instead of 2, each time for 1/2 hr. For better extraction of the gliadins, the volume of the 70% ethanol solution was increased to 10 ml from 5 ml and the extraction made 4 times, each time for 1/2 hr. The residue remaining after ethanol extraction was dispersed in 4.0 ml, instead of 2.0 ml, of 0.7% acetic acid, and stirred for 1 hr, instead of 1/2 hr. Then 11.2 ml of 95% ethanol was added to the suspension to make it 70% in ethanol. The acetic acid-ethanol suspension was stirred for 1/2 hr. The pH of the suspension was then adjusted to 6.6 with 2N NaOH and was left for 5 hr at 0 to 4^{o}C to allow the soluble glutenin to precipitate. The preparation was then centrifuged and the supernatant containing "contaminating" proteins, was discarded. The precipitate was re-dispersed in 4.0 ml of 0.7% acetic acid and the pH precipitation procedure repeated. The twice precipitated "purified" glutenin was freeze-dried.

Purified glutenin was solubilized overnight in 0.3 ml of 0.06M Tris-borate buffer, pH 8.9, containing 1% (^{w}/v) SDS. The preparation was centrifuged on a bench-top centrifuge for 3 min, and the supernatant retained separately for electrophoresis. This extraction with SDS was repeated 5 times, and each supernatant was retained separately. The precipitate remaining after the 5 extractions was treated with 0.1 ml of 0.06M Tris-borate buffer, pH 8.9, containing 1% SDS and 1% β-mercaptoethanol. The precipitate was centrifuged, and the supernatant,

containing reduced glutenin, was retained for electrophoresis.

The pattern of unreduced "purified" glutenin (Fig. 17) shows that this glutenin contains proteins that enter the SDS-gel (patterns A to E). Pattern A represents the first extraction with Tris-borate-SDS buffer, while pattern E represents the fifth extraction. As can be seen, proteins, although present in trace amounts, still enter the SDS-gel even after the fifth extraction. Many of these protein bands (from unreduced glutenin) correspond in mobility to many of the subunits of reduced glutenin (compare with pattern F). However, the first 5 high mol wt subunits (pattern F), which are clearly resolved and prominent in the pattern for reduced glutenin, were not obtained on SDS-gels in the patterns of the unreduced samples.

The observation here, of the appearance of these low mol wt protein bands on SDS-gels from glutenin that was extensively purified, may be analogous to the observations of Dalek-Zawistowska *et al.* (1975) by gel-filtration of glutenin. These workers reported that rechromatography of glutenin (isolated from gluten by gel-filtration chromatography on Sephadex) on Sephadex G-200, produced two protein peaks, the second peak eluting at the position of gliadin proteins. They, did not analyze their protein fractions by SDS-PAGE. From their results, they postulated an equilibrium dissociation of glutenin into high and low mol wt glutenins to account for the appearance of peak II proteins after rechromatography of glutenin (peak I).

c. Glutenin from a Modified Osborne Procedure

Many studies on the role of glutenin (acetic acid-soluble and residue proteins) in functional (breadmaking) properties have been

Figure 17. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris-borate buffer) of glutenin from the single-kernel procedure of Bietz *et al.* (1975) from the cultivar Manitou:

A - First SDS-buffer extract without reducing agent

B - Second " " " " " "

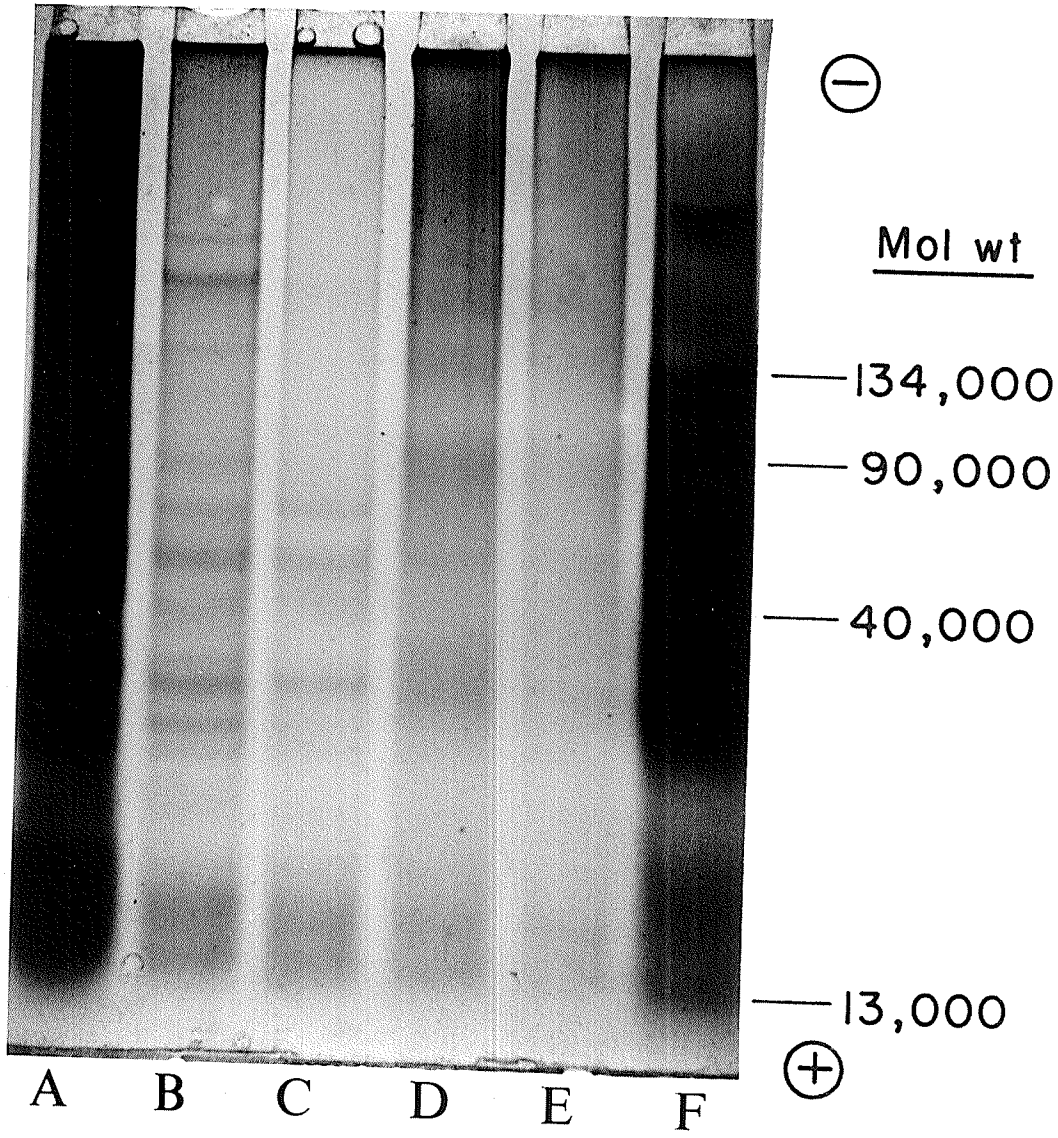
C - Third " " " " " "

D - Fourth " " " " " "

E - Fifth " " " " " "

F - Reduced glutenin from precipitate left after the fifth extraction.

Electrophoresis time was 4 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
40,000
13,000
⊕
A
B
C
D
E
F

carried out using mixing and baking techniques (Tanaka and Bushuk, 1973a, b,c; Orth and Bushuk, 1972; Hoseney *et al.*, 1969; Shogren *et al.*, 1969). However, there have been no SDS-PAGE studies that compared unreduced and reduced glutenins from cultivars of widely different mixing and baking properties. Orth and Bushuk (1973b) and Bietz and Wall (1972; 1975) compared only the reduced glutenins from various cultivars. In the present study, the modified Osborne procedure of Bietz and Wall (1975) was used to isolate glutenin from different cultivars. The glutenins, both unreduced and reduced, were analyzed by SDS-PAGE at pH 8.9 to examine if there were any qualitative and quantitative differences that might be related to functional differences among the cultivars.

Acetic Acid-Soluble Glutenin. Acetic acid-soluble glutenin was isolated from the hexaploid cultivars Red River 68, Manitou, Ponca and Talbot. Fig. 18 shows the SDS-PAGE patterns for both the unreduced and reduced glutenins of the different cultivars. In all cases, much material still remains at the point of sample application on the gels. Many prominent protein bands were seen on the SDS-gel of unreduced glutenins (patterns A, C, E, and G). These patterns show both qualitative and quantitative differences. For example, the unreduced glutenin of Red River 68 contains more protein bands in the region above 134,000 mol wt. The cultivar Talbot (pattern G) seems to contain the least amount of protein in this mol wt region.

The SDS-PAGE patterns of reduced glutenins were qualitatively identical for all the cultivars (patterns B, D, F, H) examined.

Figure 19 compares SDS-PAGE patterns of the various unreduced protein fractions from the modified Osborne procedure of Bietz and Wall

Figure 18. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of the acetic acid-soluble glutenin from the procedure of Bietz and Wall (1975):

A - Unreduced Red River 68 glutenin
B - Reduced " " " "
C - Unreduced Manitou glutenin
D - Reduced " "
E - Unreduced Ponca glutenin
F - Reduced " "
G - Unreduced Talbot glutenin
H - Reduced " "

Electrophoresis time was 3 1/2 hr. Staining procedure 2 was used.

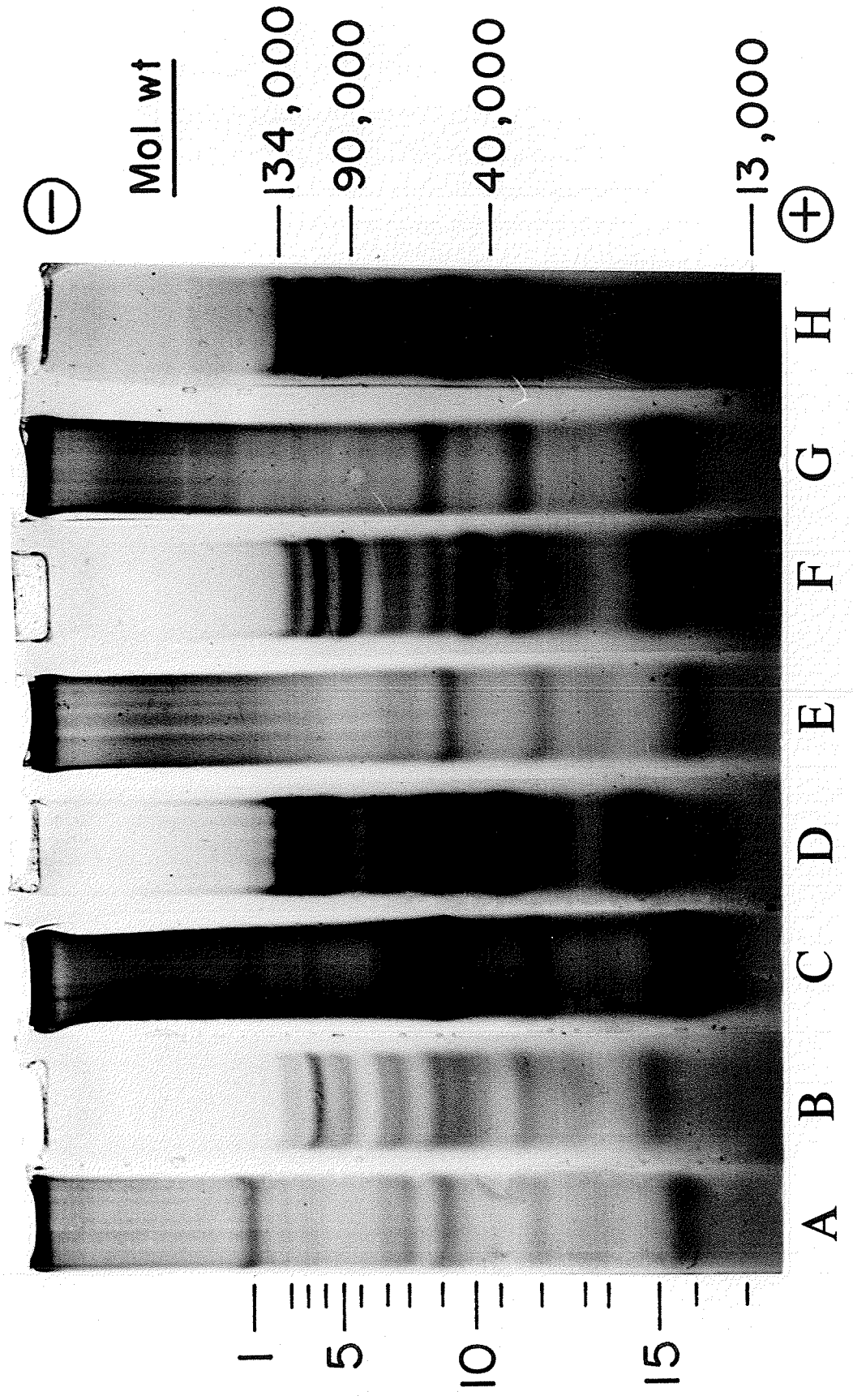

Mol wt
134,000
90,000
40,000
13,000
A
B
C
D
E
F
G
H
1
5
10
15

Figure 19. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris-borate buffer) of the unreduced protein fractions from the modified Osborne procedure of Bietz and Wall (1975) from the cultivar Manitou:

A - Albumins

B - Globulins

C - Gliadins

D - Mercuric chloride-soluble glutenin

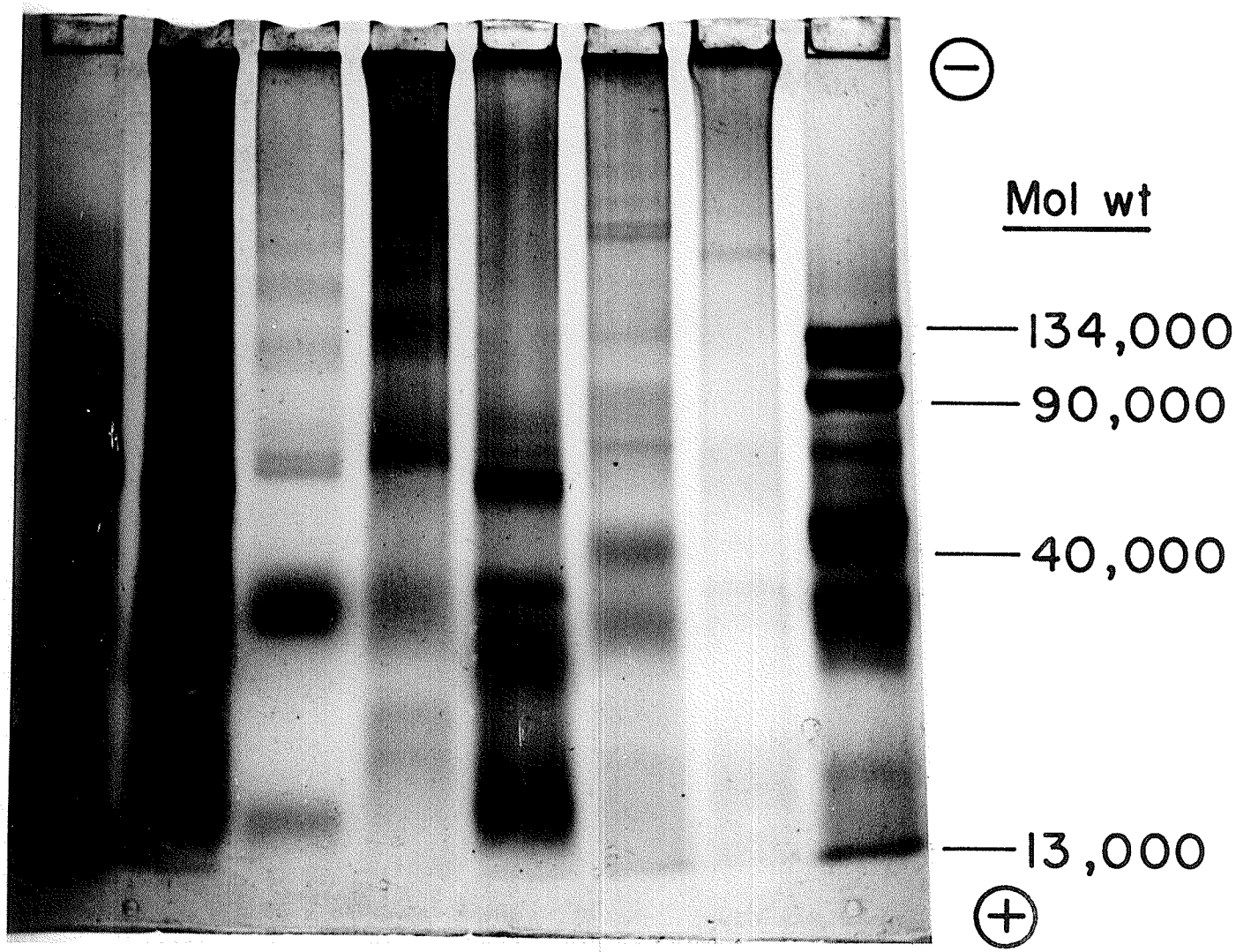
E - Acetic acid-soluble glutenin

F - β-mercaptoethanol-soluble glutenin

G - Glutenin purified according to the pH precipitation procedure of Orth and Bushuk (1973a)

H - Reduced acetic acid-soluble glutenin

Electrophoresis time was 3 1/2 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
40,000
13,000
⊕
A B C D E F G H

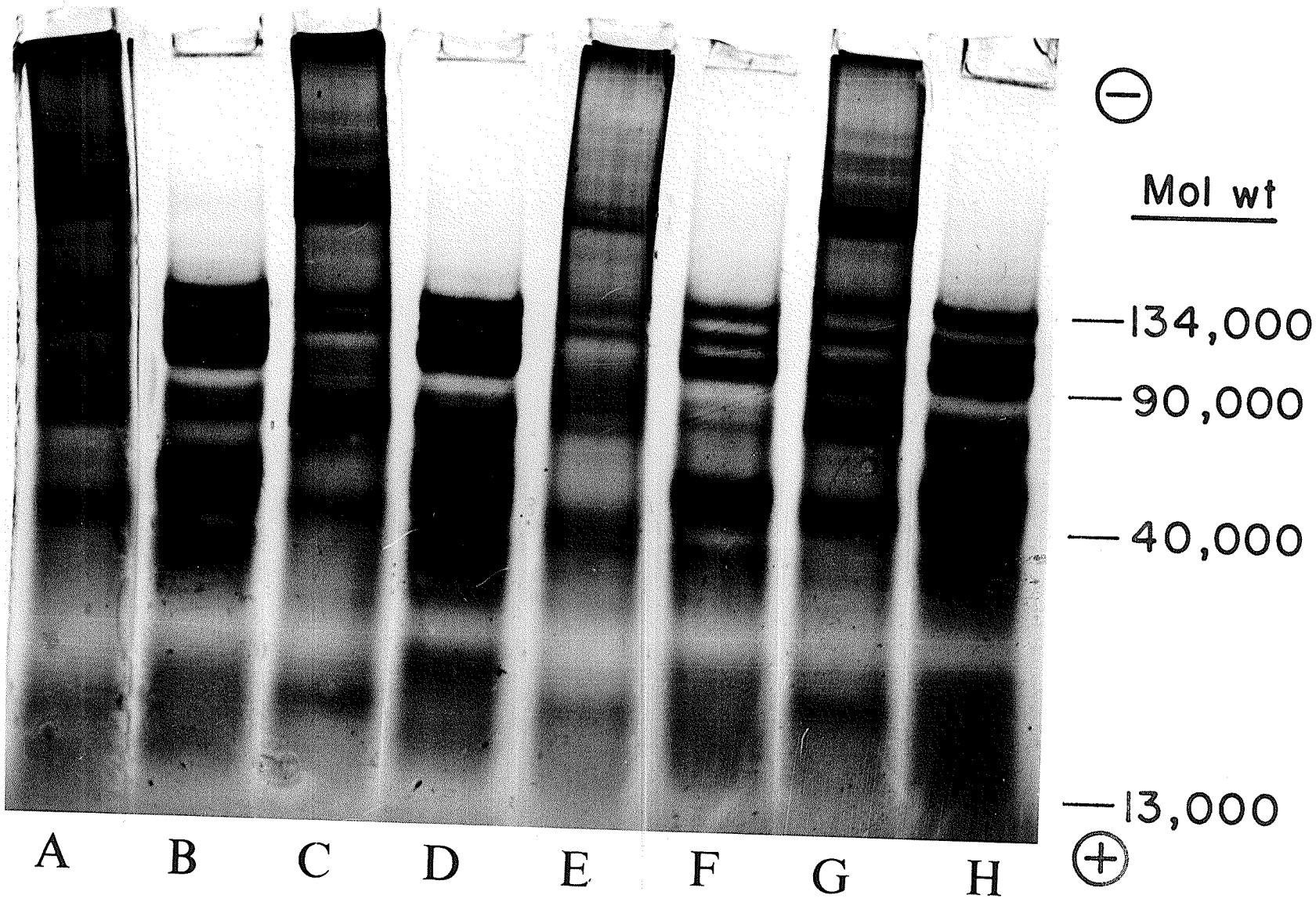
(1975) (patterns A to F) with those of unreduced and reduced glutenins. As can be seen there are many protein bands in the pattern of albumin (pattern A), globulins (pattern B), and gliadins (pattern C) that correspond in mobility to many of the protein bands that enter the SDS-gel from the unreduced acetic acid-soluble glutenin (pattern E). The bands from unreduced glutenin could, therefore, be components of other protein fractions present as impurities. Pattern G represents unreduced glutenin from the pH precipitation procedure of Orth and Bushuk (1973a). This glutenin was purified 3 times with sulfoethyl-Sephadex. However, there are still faint protein bands in the pattern for this extensively purified glutenin. Obviously, it is very difficult to remove the "contaminating" proteins from glutenin. The strength of association and the quantity of proteins that are considered to be glutenin "contaminants" may be important in determining the functional properties of a flour.

Mercuric Chloride-Soluble Glutenin. The residue (remaining after acetic acid extraction of flour) was extracted with 2.0×10^{-4}M mercuric chloride ($HgCl_2$) in 0.1N acetic acid (Bietz and Wall, 1975). Figure 20 shows the SDS-PAGE patterns for both the unreduced and reduced $HgCl_2$-soluble proteins. The unreduced samples (patterns A, C, E, and G) show many protein bands that enter the SDS-gel. Both qualitative and quantitative differences are evident in these patterns. Many of these bands correspond in mobility to subunits of reduced glutenin. Unlike the patterns of the unreduced acetic acid-soluble glutenins, in which the bands of mol wt 68,000 and lower were more prominent, the pattern of the unreduced $HgCl_2$-soluble glutenin contains more prominent bands in the higher mol wt region. There are some proteins with mol wts higher

Figure 20. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of the mercuric chloride-soluble glutenin from the procedure of Bietz and Wall (1975):

A - Unreduced Red River 68 glutenin
B - Reduced " " " "
C - Unreduced Manitou glutenin
D - Reduced " "
E - Unreduced Ponca glutenin
F - Reduced " "
G - Unreduced Talbot glutenin
H - Reduced " "

Eletrophoresis time was 3 1/2 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
40,000
13,000
⊕
A
B
C
D
E
F
G
H

than the highest mol wt subunit of reduced glutenin. When these glutenins were reduced, the SDS-PAGE patterns of all the cultivars examined were qualitatively identical (patterns B, D, F, and H) in subunit composition, similar to those of the reduced acetic acid-soluble glutenin.

Reduction of Glutenin with Mercuric Chloride. Danno *et al.* (1975) reported that 1.0×10^{-3}M mercuric chloride ($HgCl_2$) can reduce the disulfide bonds of glutenin. To confirm (or refute) this possibility, it was decided to subject the acetic acid-soluble glutenin from the procedure of Bietz and Wall (1975) to treatment with 1.0×10^{-3}M $HgCl_2$ in 0.125M Tris-borate, pH 8.9, SDS buffer and examine the resulting products by SDS-PAGE. The resulting pattern was compared to the subunit pattern of the acetic acid-soluble glutenin reduced with β-mercaptoethanol.

Figure 21 shows that glutenin treated with 1.0×10^{-3}M $HgCl_2$ (pattern C) gave the same SDS-PAGE pattern as glutenin treated with 1% ($^{v}/v$) β-mercaptoethanol (pattern A). However, subunits 4 and 5 in the $HgCl_2$-treated glutenin are not as clearly resolved as in the β-mercaptoethanol-reduced glutenin.

Included in Figure 21, for comparison purposes, is the pattern of gliadin (the alcohol-soluble flour proteins) obtained from the procedure of Bietz and Wall (1975). Gliadin treated with 1.0×10^{-3}M $HgCl_2$ (pattern F) shows the same pattern as gliadin treated with β-mercaptoethanol (pattern H). Unreduced gliadin (pattern G) is qualitatively different from the $HgCl_2$- and β-mercaptoethanol-treated gliadins. Unreduced gliadin shows a number of subunits, ranging in mol wt from

Figure 21. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of Manitou glutenin treated as follows:

A - β-Mercaptoethanol-reduced acetic acid-soluble glutenin.

B - Unreduced acetic-acid soluble glutenin.

C - Acetic acid-soluble glutenin treated with 1.0×10^{-3}M $HgCl_2$ (1 mM)

D - Unreduced $HgCl_2$-soluble glutenin

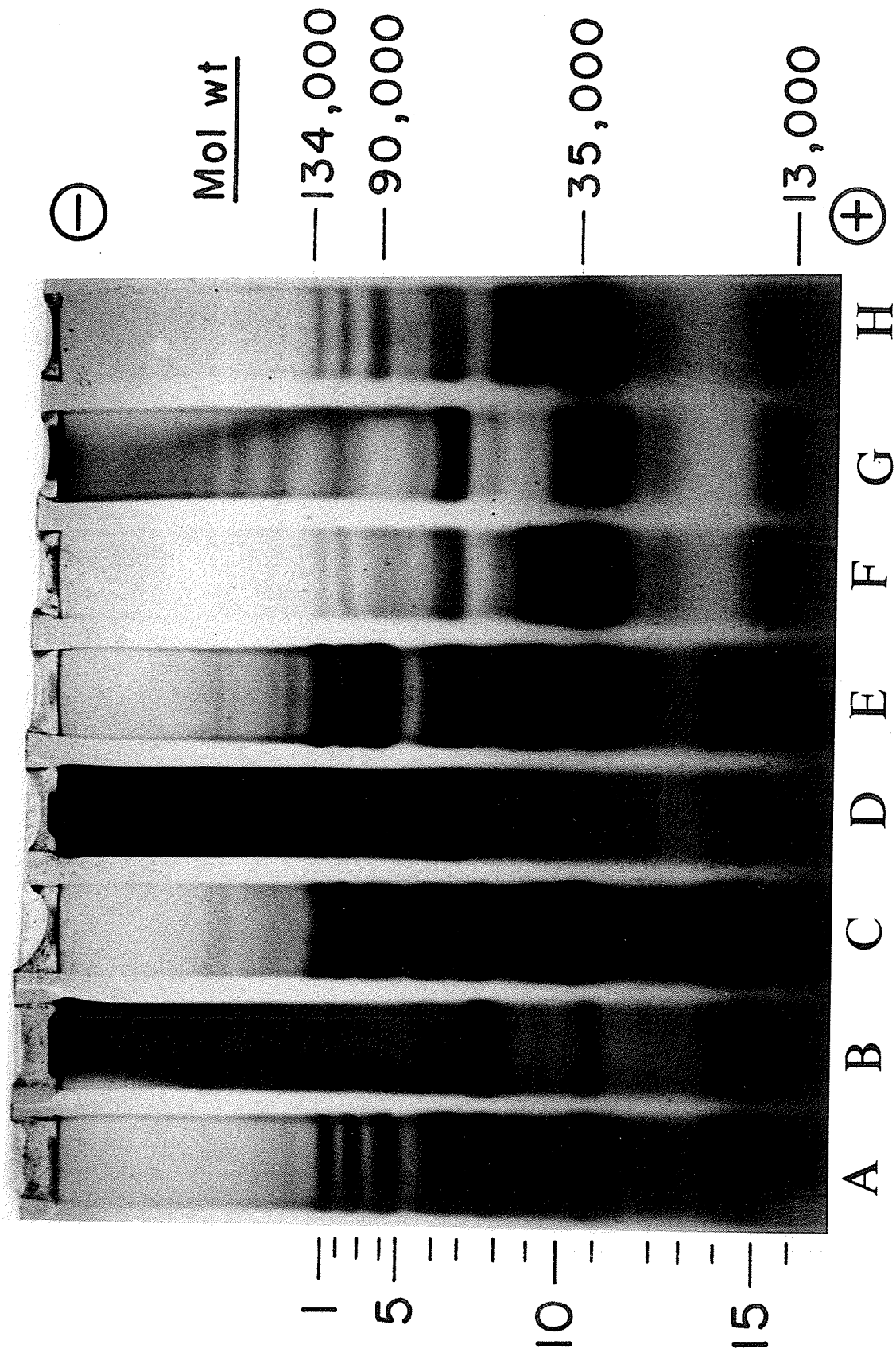
E - β-Mercaptoethanol-reduced $HgCl_2$-soluble glutenin

F - Gliadin treated with 1.0×10^{-3}M $HgCl_2$

G - Unreduced gliadin

H - β-Mercaptoethanol-reduced gliadin

Electrophoresis time was 3 1/2 hr. Staining procedure 2 was used.


Mol wt
134,000
90,000
35,000
13,000
⊖
⊕
A
B
C
D
E
F
G
H
1
5
10
15

approximately 160,000 and lower, with some material remaining at the origin of the gel. Upon treatment of the gliadin with $HgCl_2$ and β-mercaptoethanol, both the high mol wt subunits and the material at the origin of the gel disappear giving rise to 3 additional protein bands that have the same mobility as the high mol wt subunits of reduced glutenin. The material remaining at the origin seems to be glutenin that did not enter the SDS-gel because of its large size.

Glutenin Isolated from the Gliadin Fraction of Bietz and Wall (1975). Gliadin from the procedure of Bietz and Wall (1975) was subjected to the pH precipitation procedure of Jones *et al.* (1959) to isolate the glutenin from the alcohol-soluble fraction. The precipitated material amounted to approximately 10% (by weight) of the total gliadin fraction. Figure 22 shows the SDS-PAGE patterns of the total gliadin fraction, the precipitated material, and the supernatant fraction after precipitation, both in their reduced and unreduced states. As can be seen, there is some protein at the origin of the pattern of the unreduced total gliadin (pattern A) but not at the origin of the pattern of reduced total gliadin (pattern B). Also, there is no protein at the origin of both the unreduced (pattern C) and reduced (pattern D) supernatant gliadin fractions after the pH precipitation step. The pattern of the unreduced glutenin (pattern E) isolated from gliadin shows considerable protein at the origin and only very faint protein bands in the SDS-gel. The reduced glutenin (pattern F) isolated from gliadin shows fewer protein bands than the reduced acetic acid-soluble glutenin (Fig. 21, pattern A). Protein bands of mol wt 30,000 and lower are almost absent in the reduced glutenin

Figure 22. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris-borate buffer) of glutenin isolated from the gliadin fraction (Bietz and Wall, 1975) from cv. Manitou according to the pH precipitation procedure of Jones *et al.* (1959):

A - Unreduced gliadin

B - Reduced gliadin

C - Unreduced supernatant proteins from pH precipitation of gliadin

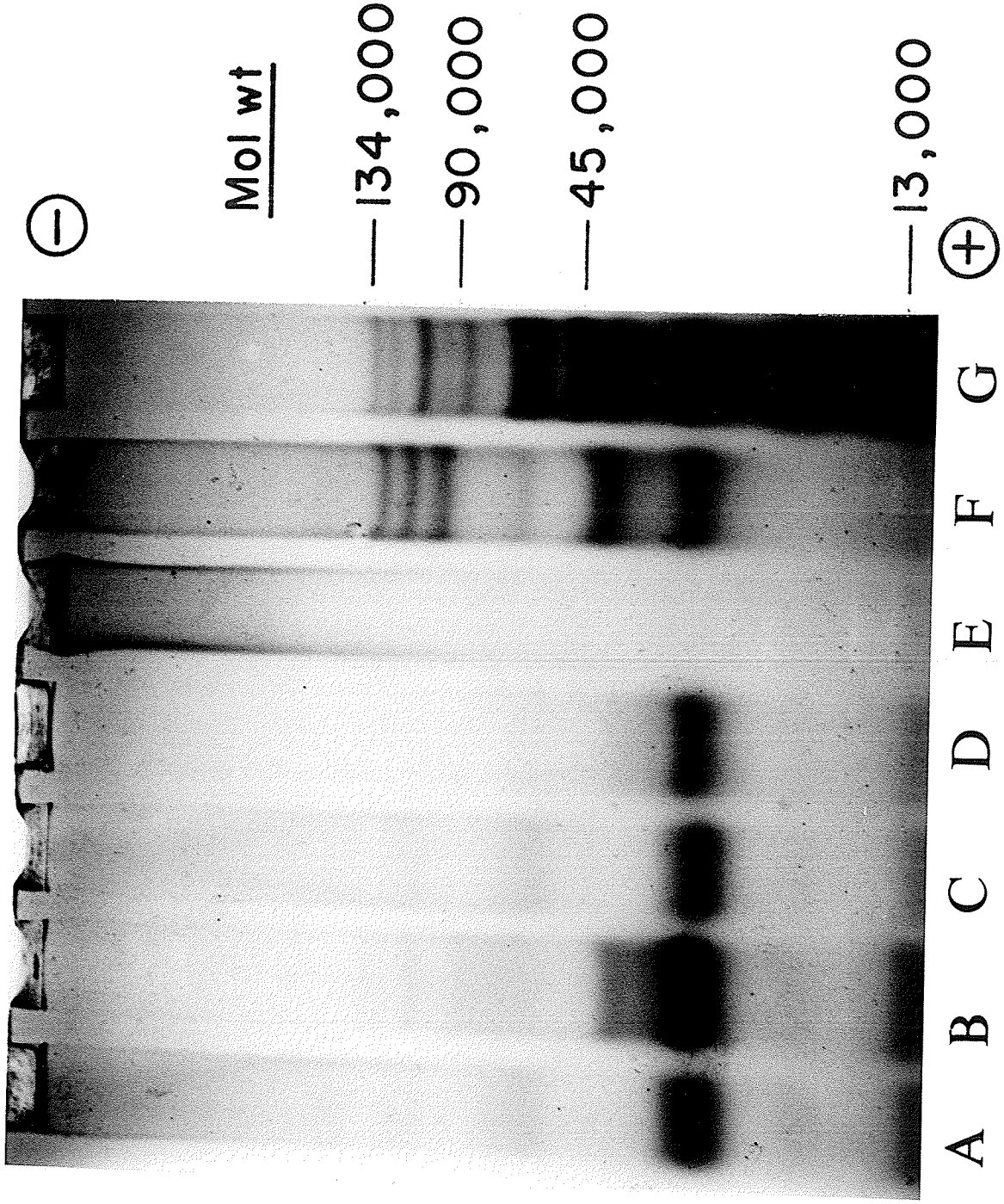
D - Reduced supernatant

E - Unreduced glutenin isolated from gliadin

F - Reduced glutenin isolated from gliadin

G - Reduced Red River 68 acetic acid-soluble glutenin from the procedure of Bietz and Wall (1975)

Electrophoresis time was 3 1/2 hr. Staining procedure 2 was used.


Mol wt
134,000
90,000
45,000
13,000
A B C D E F G

isolated from gliadin.

Mercuric Chloride-Soluble Glutenin Treated with EDTA[1]. It was decided to treat the 0.2 mM $HgCl_2$-soluble glutenin of Bietz and Wall (1975) with ethylenediamine tetraacetic acid (EDTA) (a known chelating agent) to determine whether EDTA could reverse the action of mercuric chloride.

The $HgCl_2$-soluble glutenin was dissolved in 0.06M Tris-borate, pH 8.9, containing 1% SDS and 1.0×10^{-2}M EDTA. The acetic acid-soluble (HoAc-soluble) glutenin from the procedure of Bietz and Wall (1975) was treated similarly for comparison purposes.

Figure 23 shows that the HoAc-soluble glutenin treated with 1.0×10^{-3}M $HgCl_2$ plus 1.0×10^{-2}M EDTA (pattern B) gave the same pattern as the unreduced HoAc-soluble glutenin (pattern A). Mercuric chloride, which was shown to breakdown glutenin into subunits (Fig. 21, pattern C), did not show this effect in the presence of EDTA. This is more clearly seen by comparing pattern B with pattern C which represents the HoAc-soluble glutenin treated with 1.0×10^{-3}M $HgCl_2$ without EDTA. Pattern C is similar to pattern D, the pattern of the β-mercaptoethanol-reduced HoAc-soluble glutenin. There is considerably more protein at the origins of patterns A and B and almost no material at the origins of patterns C and D. Pattern E, on the other hand, represents the $HgCl_2$-soluble glutenin from the procedure of Bietz and Wall (1975) while pattern F represents this same $HgCl_2$-soluble glutenin treated with 1.0×10^{-2}M EDTA. As can be seen, many of the prominent protein bands visible in pattern E are very faint in the patterns of the sample that was treated with EDTA. Also, there appears to be much

[1] Ethylenediaminetetraacetic acid, disodium salt.

Figure 23. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris borate buffer) of Red River 68 acetic acid-soluble glutenin (HOAc-soluble) and mercuric choride-soluble ($HgCl_2$-soluble) glutenin from the procedure of Bietz and Wall (1975) treated as follows:

A - Unreduced HoAc-soluble glutenin

B - HOAc-soluble glutenin treated with 1.0×10^{-3}M $HgCl_2$ and $1.0\ 10^{-2}$M EDTA

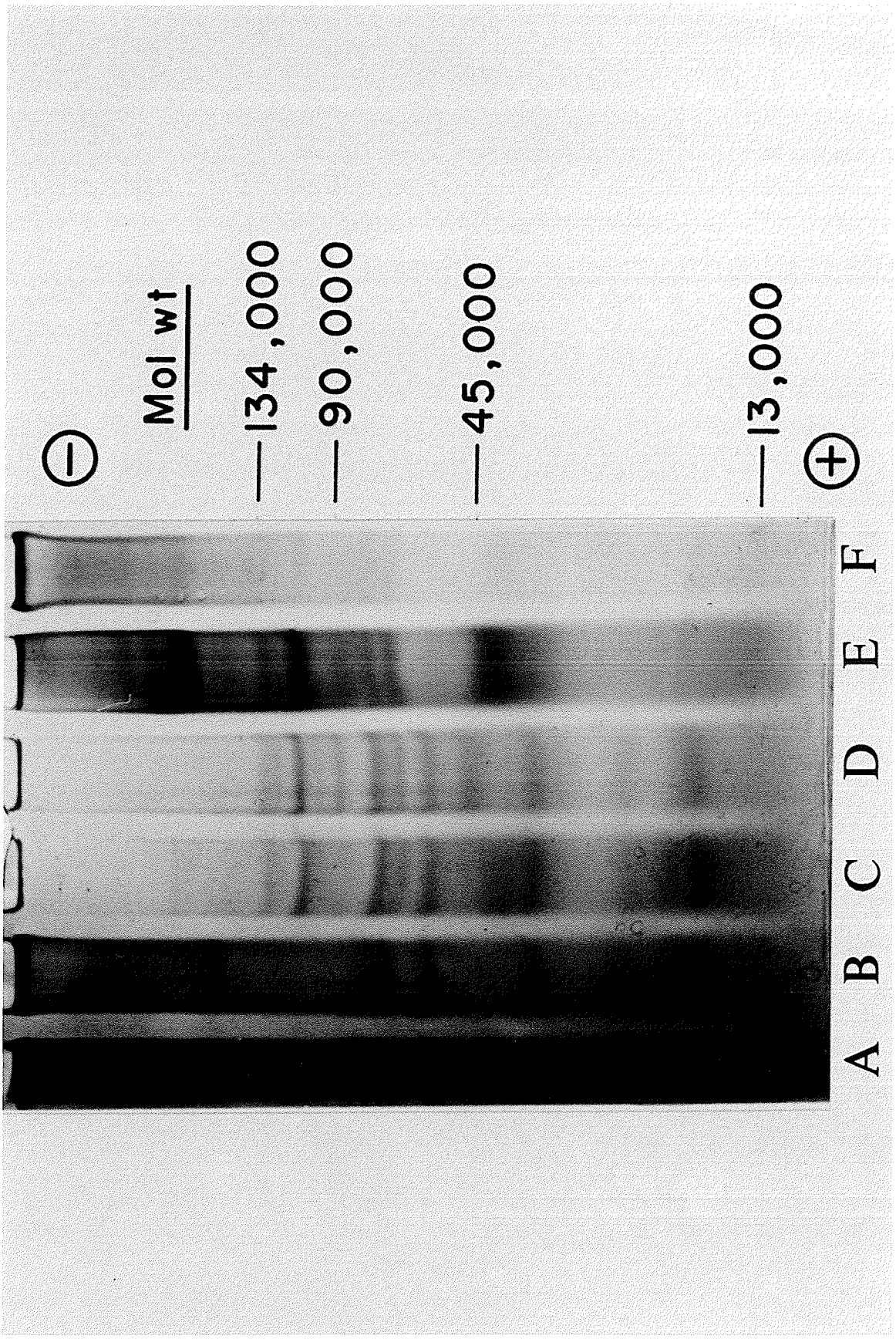
C - HOAc-soluble glutenin treated with 1.0×10^{-3}M $HgCl_2$

D - HOAc-soluble glutenin treated with 1% ($^{v}/v$) β-mercaptoethanol

E - $HgCl_2$-soluble glutenin

F - $HgCl_2$-soluble glutenin treated with 1.0×10^{-2}M EDTA

Electrophoresis time was 4 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
45,000
13,000
⊕
A B C D E F

less protein in pattern E than in pattern F. Therefore, it seems that EDTA chelated the mercuric ion from its binding site on glutenin's subunits which then reverted either to an aggregated state, or to an oxidized state by reformation of intra- and inter-polypeptide disulfide bonds to form large molecules which could not enter the 5% polyacrylamide SDS-gel but remained at the origin as in pattern F, Figure 23.

Since glutenin treated with 0.2 mM $HgCl_2$ in acetic acid (Fig. 23, pattern E) is not as extensively reduced as glutenin treated with 1.0×10^{-3}M $HgCl_2$, it was thought that this partial reduction with 0.2 mM $HgCl_2$ might have contributed to the reversibility observed with EDTA. To test whether incompleteness of reduction by 0.2 mM $HgCl_2$ (of glutenin) contributed to the apparent reversibility caused by EDTA, glutenin was reduced with 1.0 mM $HgCl_2$ (in acetic acid) and freeze-dried. This glutenin was then treated with EDTA (1.0×10^{-2}M) in Tris-borate-SDS buffer at pH 8.9. The same glutenin was also treated with both EDTA and 8M urea to observe the effect of the reversibility in the presence of a dissociating agent.

Figure 24 shows that the SDS-PAGE patterns of glutenin treated with 1.0×10^{-3}M $HgCl_2$ (patterns B and D) and glutenin treated with both $HgCl_2$ and EDTA (patterns C and E) are indeed different. Patterns B and D show a subunit pattern that is the same as that obtained for glutenin reduced with β-mercaptoethanol (pattern I). Patterns C and E, however, show a subunit pattern as that obtained with unreduced glutenin (patterns A and H) with much protein material remaining at the origin of the gel. Accordingly, EDTA has the same reversible effect on treated glutenin whether it is treated with 0.2 mM $HgCl_2$ or

Figure 24. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris-borate buffer) of Talbot HoAc-soluble glutenin and glutenin from the pH precipitation procedure of Orth and Bushuk (1973a) treated as follows:

A - Unreduced HoAc-soluble glutenin

B - HoAc-soluble glutenin treated with 1.0×10^{-3}M $HgCl_2$

C - HoAc-soluble glutenin treated with 1.0×10^{-3}M $HgCl_2$ and 1.0×10^{-2}M EDTA

D - Glutenin from the pH precipitation procedure of Orth and Bushuk (1973a) treated with 1.0×10^{-3}M $HgCl_2$

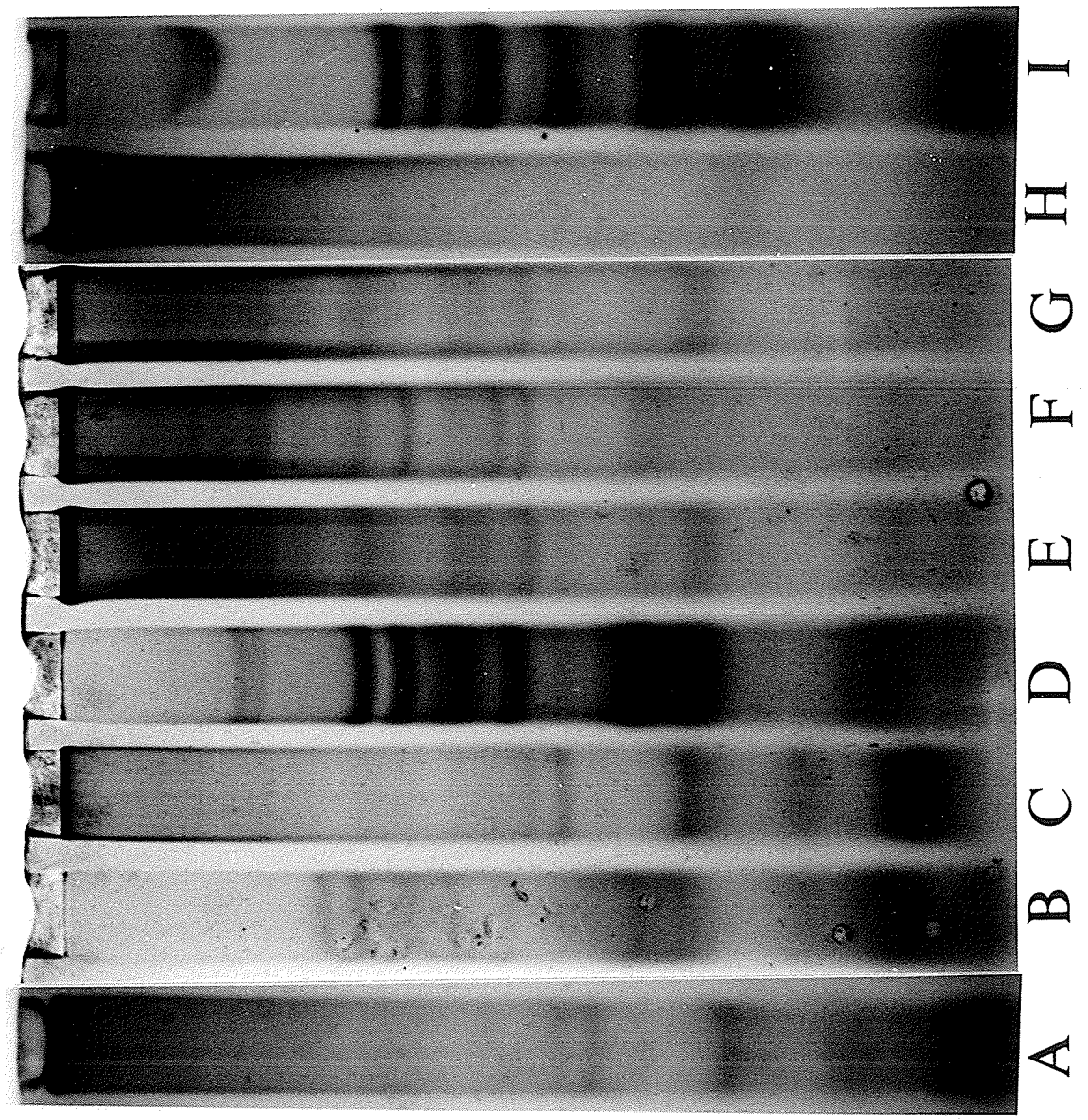
E - pH precipitation glutenin treated with 1.0×10^{-3}M $HgCl_2$ and 1.0×10^{-2}M EDTA

F, G - pH precipitation glutenin treated with 1.0×10^{-3}M $HgCl_2$, 1.0×10^{-2}M EDTA, and 8M urea

H - Unreduced pH precipitation glutenin

I - pH precipitation glutenin reduced with β-mercaptoethanol

Electrophoresis time was 4 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
45,000
13,000
⊕
A B C D E F G H I

with 1.0 mM $HgCl_2$. The urea (8M) (patterns F and G) did not inhibit the reversibility of the reaction since the SDS-PAGE patterns with urea are the same as the pattern without urea (pattern E).

The fact that this reversible reaction occurred in the presence of both SDS and urea, both strong protein dissociating agents, gives credence to the hypothesis that the subunits of glutenin are held by interpolypeptide disulfide bonds. It seems unlikely that the subunits would aggregate in the presence of those denaturing agents.

One possible mechanism for the action of heavy metals on disulfide bonds of protein molecules is as follows, (where "R" represents "protein chains" and "M" represents "metal ions"):

$$RSSR + H_2O \rightleftharpoons RSOH + RSH \qquad (1)$$

$$RSH + M^+ \rightarrow RSM + H^+ \qquad (2)$$

It is assumed in this mechanism that the initial process is a hydrolysis of the disulfide bond to give a thiol and sulphenic acid. The thiol formed reacts with the metal ions.

A second possible mechanism is as follows:

$$RSSR + M^+ \rightarrow \begin{matrix} RSSR \\ | \\ M \end{matrix} \rightarrow RSM + RS^+ \qquad (3)$$

where the heavy metal attacks the disulfide bond directly in the initial reaction.

It was shown that EDTA reverses the action of the mercuric (Hg) ion on glutenin. Accordingly, the second mechanism would seem more likely since re-oxidation to disulfide, after the chelation of the

mercuric ion by EDTA, would be more favourable. The conditions for reformation of interpolypeptide disulfide bonds would become even more favourable by the reformation of the intrapolypeptide disulfide bonds, since the reformation of the latter would orient the polypeptide chains to assume the conformation that existed before reduction. This proper orientation would make it more favourable for the formation of interpolypeptide disulfide bonds according to the second mechanism.

β-Mercaptoethanol-Soluble Glutenin. The residue remaining after $HgCl_2$ extraction of flour (Bietz and Wall, 1975) was extracted with 0.1N acetic acid containing 0.1% ($^{v}/v$) β-mercaptoethanol and freeze-dried. The freeze-dried sample was used for SDS-PAGE analysis.

Figure 25 shows the SDS-PAGE patterns of the β-metcaptoethanol-soluble glutenin. The patterns closely resemble those of glutenin obtained by extracting the residue (after acetic acid extraction) proteins (glutenin) with 0.2 mM $HgCl_2$. Like the glutenin from the 0.2 mM $HgCl_2$ extract, the glutenin from the 0.1% ($^{v}/v$) β-mercaptoethanol extract is only partially reduced. When the glutenin extracted with 0.1% ($^{v}/v$) β-mercaptoethanol is reduced with 1% ($^{v}/v$) β-mercaptoethanol, the SDS-PAGE pattern is qualitatively similar to that obtained from the reduced acetic acid- and mercuric chloride-soluble glutenins.

AUC- and Guanidine Hydrochloride-Soluble Glutenins. In a previous section, it was shown that mercuric chloride modified the structure of the glutenin (residue) protein fraction. If the proteins from the residue (after acetic acid extraction) are to be recovered in their native state for comparison with the acetic acid-soluble glutenin, then another solvent other than acetic acid-mercuric chloride, that does not

Figure 25. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of the unreduced β-mercaptoethanol soluble proteins from the procedure of Bietz and Wall (1975):

A - Red River 68

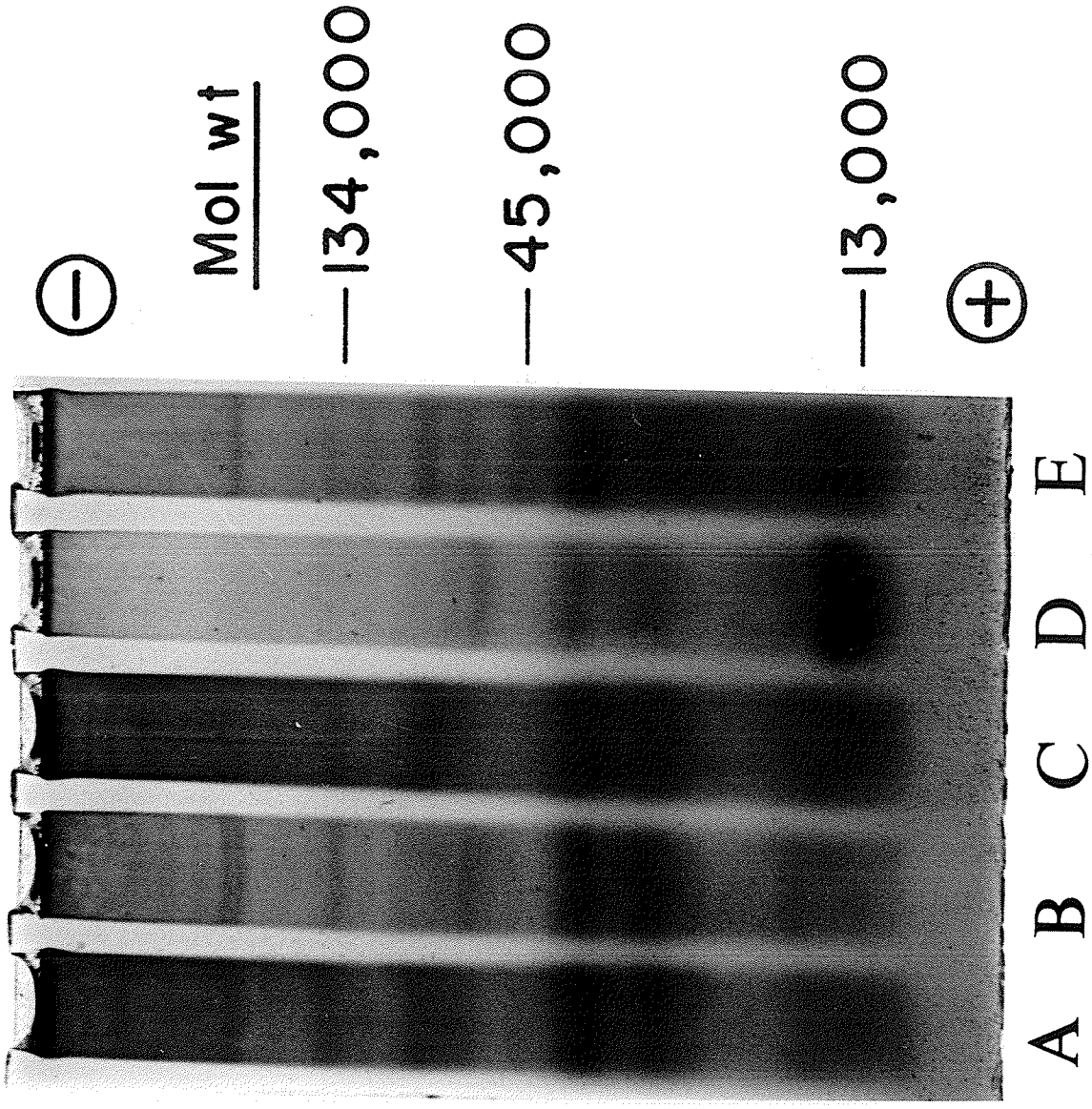
B - Manitou

C - Ponca

D - Talbot

E - Stewart 63

Electrophoresis time was 3 hr. Staining procedure 2 was used.


⊖
Mol wt
—134,000
—45,000
—13,000
⊕
A B C D E

modify the structure of the residue proteins, should be used for the extraction of the residue (glutenin) proteins.

Two composite dissociating solvents, AUC and 0.1N acetic acid containing 6M guanidine hydrochloride (GuHCl) were used to extract the residue proteins from the cultivars Red River 68 and Talbot. The AUC and GuHCl extracts were dialyzed against distilled water for 5 days and then freeze-dried. The freeze-dried samples were used for SDS-PAGE analysis.

Figure 26 shows the SDS-PAGE patterns of the unreduced AUC- and GuHCl-soluble glutenins, together with the unreduced acetic acid-soluble glutenin. As can be seen, the unreduced AUC and GuHCl-soluble glutenins contain many protein components that entered the SDS-gel; proteins that correspond in mobility to those that enter the SDS-gel from unreduced acetic acid-soluble glutenin. There is also considerable protein that remains at the origin. The patterns of the AUC and GuHCl-soluble glutenins are different from the mercuric chloride-solubilized glutenin (compare patterns A to E of Fig. 26 with patterns A, C, E, F, of Fig. 20). The AUC and GuHCl extracts contain protein components in the mol wt region of 68,000 and lower whereas the $HgCl_2$-extracted glutenin contains more protein components in the mol wt region above 68,000 daltons. Therefore, the structure of the glutenin that is extracted with AUC and GuHCl is probably not modified the way it is when the glutenin is extracted with mercuric chloride solution.

Treatment of Glutenin with Different Reducing Agents. The acetic acid-soluble glutenin from the cultivar Manitou isolated by the procedure of Bietz and Wall (1975) and purified by the pH precipitation

Figure 26. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris-borate buffer) of glutenin, as follows:

A - Unreduced acetic acid-soluble glutenin of Red River 68

B - Unreduced AUC extract of residue proteins of Red River 68

C - Unreduced AUC extract of residue proteins of Talbot

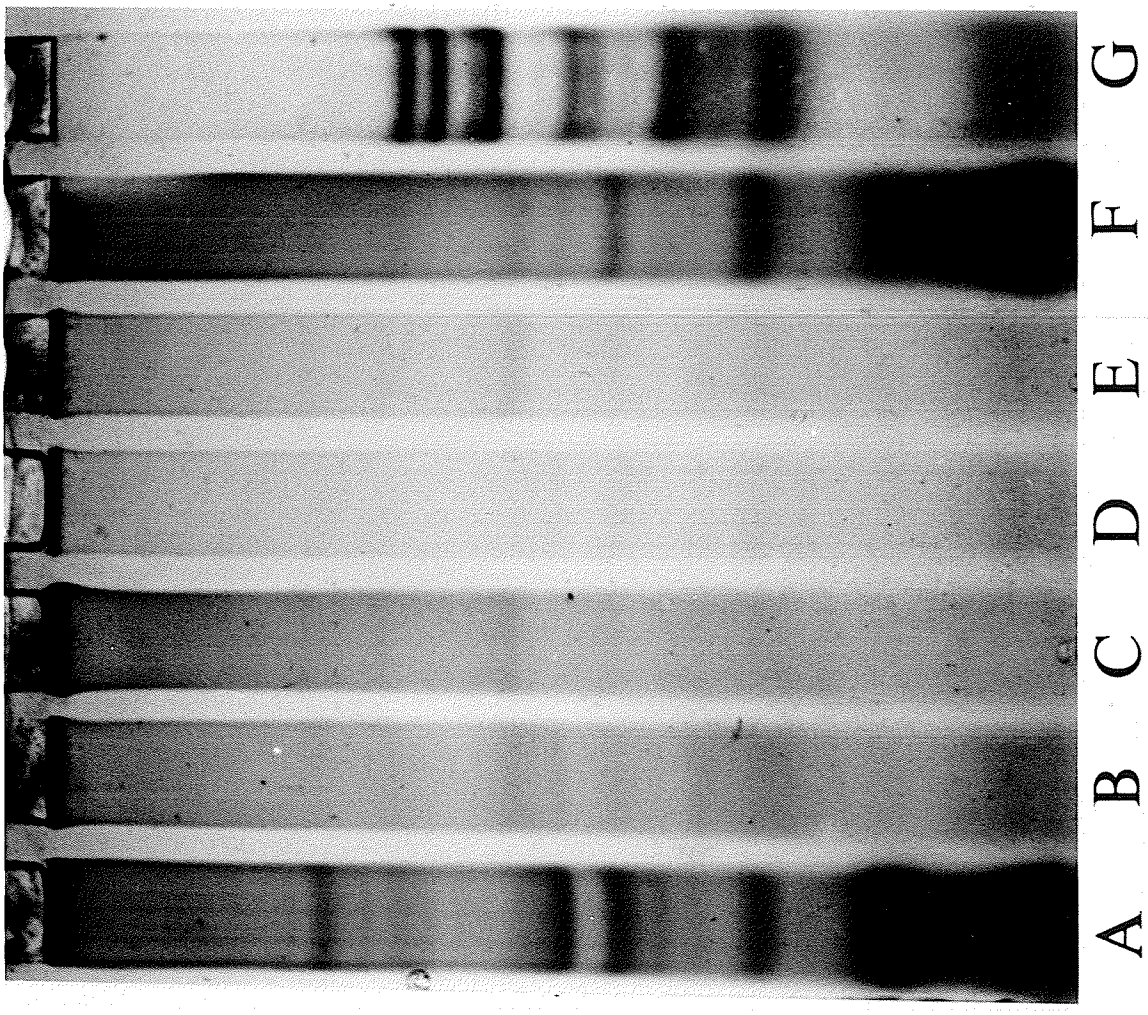
D - Unreduced guanidine hydrochloride extract of residue proteins of Talbot

E - Unreduced guanidine hydrochloride extract of residue proteins of Red River 68

F - Unreduced acetic acid-soluble glutenin from Talbot

G - Reduced acetic acid-soluble glutenin of Talbot

Electrophoresis time was 3 1/2 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
45,000
13,000
⊕
A B C D E F G

procedure of Jones *et al.* (1959), was treated with a number of reducing agents (1% concentration) in 0.125M Tris borate, pH 8.9, buffer containing 1% ($^{w}/v$) SDS. The purpose of this experiment was to examine if the nature of the reducing agent had any effect on the subunit SDS-PAGE pattern.

The glutenin subunit patterns are identical (Fig. 27) for the glutenin treated with β-mercaptoethanol (patterns A, H), dithiothreitol (Cleland's reagent) (pattern B), L-cysteine (pattern C), or sodium sulfite (pattern G). Glutenin treated with N-ethylmaleimide (NEMI) (pattern D) contained very faint low mol wt bands that entered the SDS-gel but a considerable amount of protein remained at the origin. NEMI does not act as an -S-S- reducing agent for glutenin under the conditions used in this study. The pattern for $HgCl_2$ (pattern E) shows much streaking with faint low mol wt bands. The $MgCl_2$ pattern (pattern F) shows a very faint glutenin subunit pattern, with the high mol wt subunits being extremely faint. The $HgCl_2$ and $MgCl_2$ patterns do not show any protein at the origin because most of the glutenin was not soluble in the solution containing these two salts at a 1% ($^{w}/v$) concentration (removed in clarification of the electrophoresis solution).

Since sodium sulfite readily reduces glutenin, it was necessary to check whether the sodium sulfite used as the polymerization catalyst in the pH 8.9 SDS-gel system modified the unreduced glutenin that was applied to the SDS-gel for electrophoresis (see Fig. 18, patterns A, C, E, and G). The pH 7.3 SDS-PAGE system of Orth and Bushuk (1973b), which does not use sodium sulfite as a catalyst, was used to analyze unreduced glutenin. Figure 28 shows that the SDS-PAGE patterns of pH

Figure 27. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of the acetic acid-soluble glutenin from the cultivar Manitou treated with different chemical agents at a 1% concentration:

A - β-Mercaptoethanol

B - Dithiothreitol (Cleland's reagent)

C - L-Cysteine

D - N-Ethylmaleimide

E - Mercuric chloride

F - Magnesium chloride

G - Sodium sulfite

H - β-Mercaptoethanol

Electrophoresis time was 3 hr. Staining procedure 2 was used.

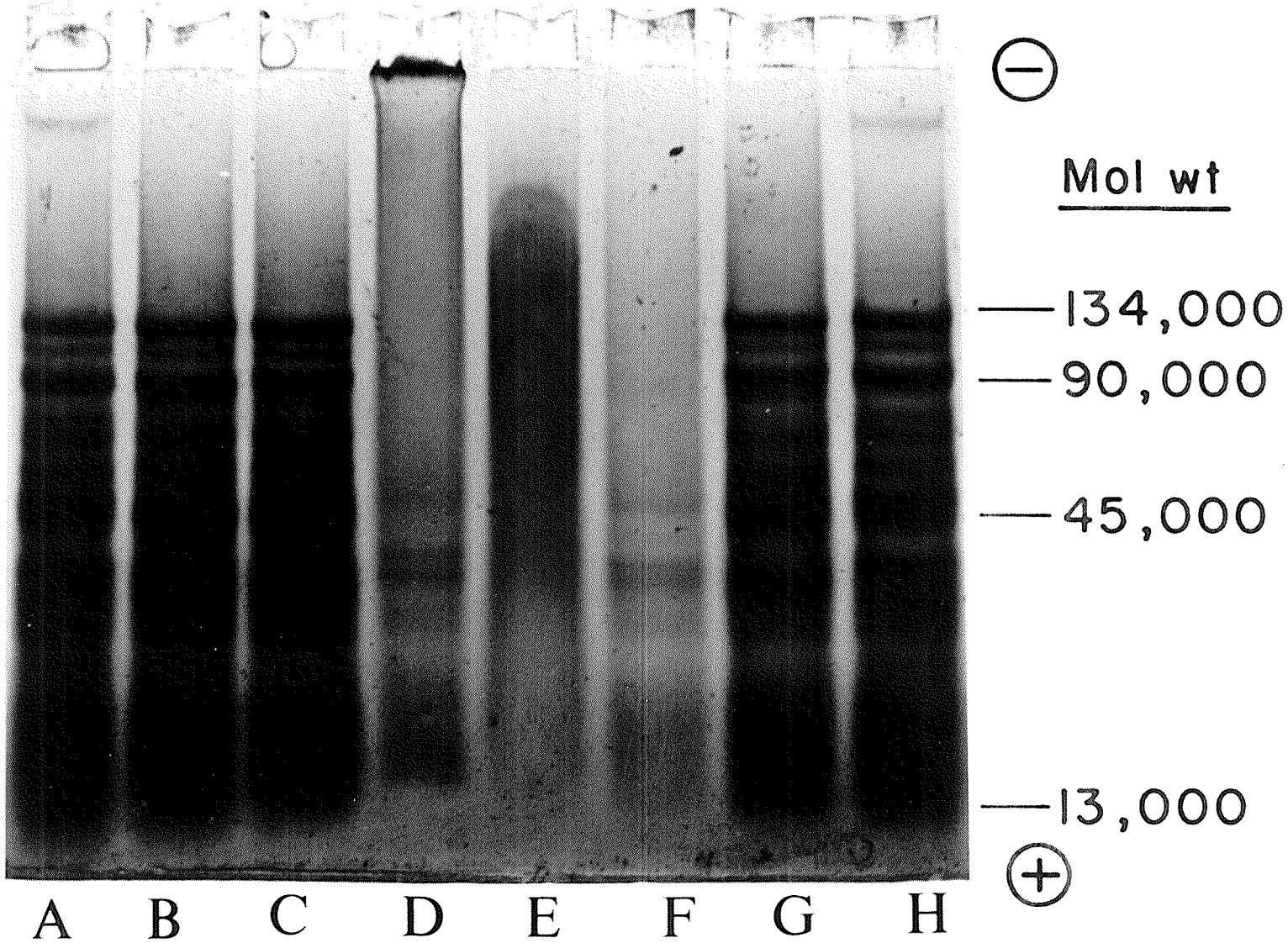

⊖
Mol wt
134,000
90,000
45,000
13,000
⊕
A B C D E F G H

Figure 28. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of unreduced (non-purified) glutenin from the procedure of Bietz and Wall (1975):

A - Red River 68 acetic acid-soluble glutenin

B - Manitou "

C - Ponca "

D - Talbot "

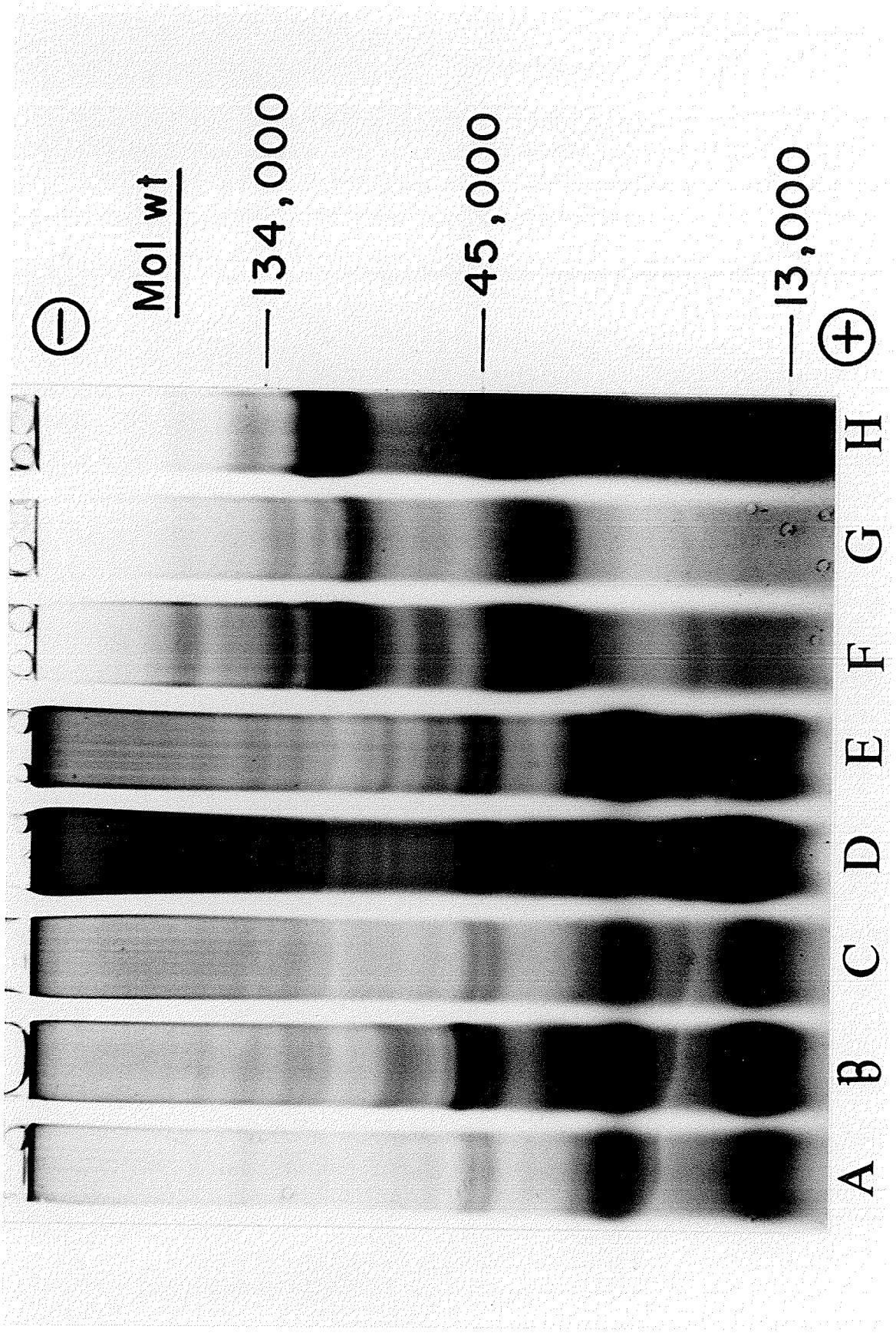
E - Stewart 63 "

F - Red River 68 mercuric chloride-soluble glutenin

G - Talbot "

H - Stewart 63 "

Electrophoresis time was 4 hrs. Staining procedure 2 was used.


⊖
Mol wt
134,000
45,000
13,000
⊕
A B C D E F G H

7.3 are very similar to the patterns at pH 8.9 (Fig. 18) for unreduced glutenin. Accordingly, the sodium sulfite used as catalyst in the pH 8.9 SDS-PAGE system does not modify (reduce) the unreduced glutenin that is applied to the SDS-gel for electrophoresis.

d. Glutenin Supplied by J. A. Bietz, U.S.D.A., Peoria, Illinois

Since it was observed in the previous section that many protein components entered the SDS-gel from unreduced glutenin, it was thought that these preparations were not properly purified. Even after extensive purification with sulfoethyl-Sephadex, faint protein bands were seen on SDS-gels in the pattern of unreduced glutenin. These observations prompted a comparison of SDS-PAGE, at pH 8.9, of glutenins prepared in the present study with those prepared by the Peoria workers (Bietz and Wall, 1975). The purpose of this experiment was to ascertain if the glutenin prepared in a different laboratory was any "purer" than those prepared for this study.

Figure 29 shows the SDS-PAGE patterns at pH 8.9 of unreduced glutenin prepared by Bietz and Wall (1975). As can be seen, there are many protein components that enter the SDS-gel. Those components are similar in mobility to those of the glutenins prepared for the present study (compare Figure 29 with patterns A, C, E, and G of Fig. 18 and Fig. 19). Accordingly, it appears that the glutenin prepared by Bietz and Wall (1975) is similar, in regard to "impurities", to those prepared by the author.

One objection that may be raised against testing for purity by SDS-PAGE at pH 8.9 is that disulfide bonds may be hydrolyzed at this high pH. In order to test this possibility, SDS-PAGE was also carried

Figure 29. SDS-PAGE electrophoretograms at pH 8.9 (0.06M Tris-borate buffer) of unreduced glutenin, donated by J. A. Bietz (Northern Regional Research Laboratory, U.S.D.A., Peoria, Illinois), from different extraction procedures:

A - Chinese Spring acetic acid-soluble glutenin

B - Chinese Spring mercuric chloride-soluble glutenin

C - Ponca acetic acid-soluble glutenin

D - Ponca mercuric chloride-soluble glutenin

E - Ponca twice precipitated glutenin (Jones et al., 1959)

F - Unreduced gliadin (Bietz and Wall, 1975) prepared by the author

Electrophoresis time was 4 hr. Staining procedure 2 was used.

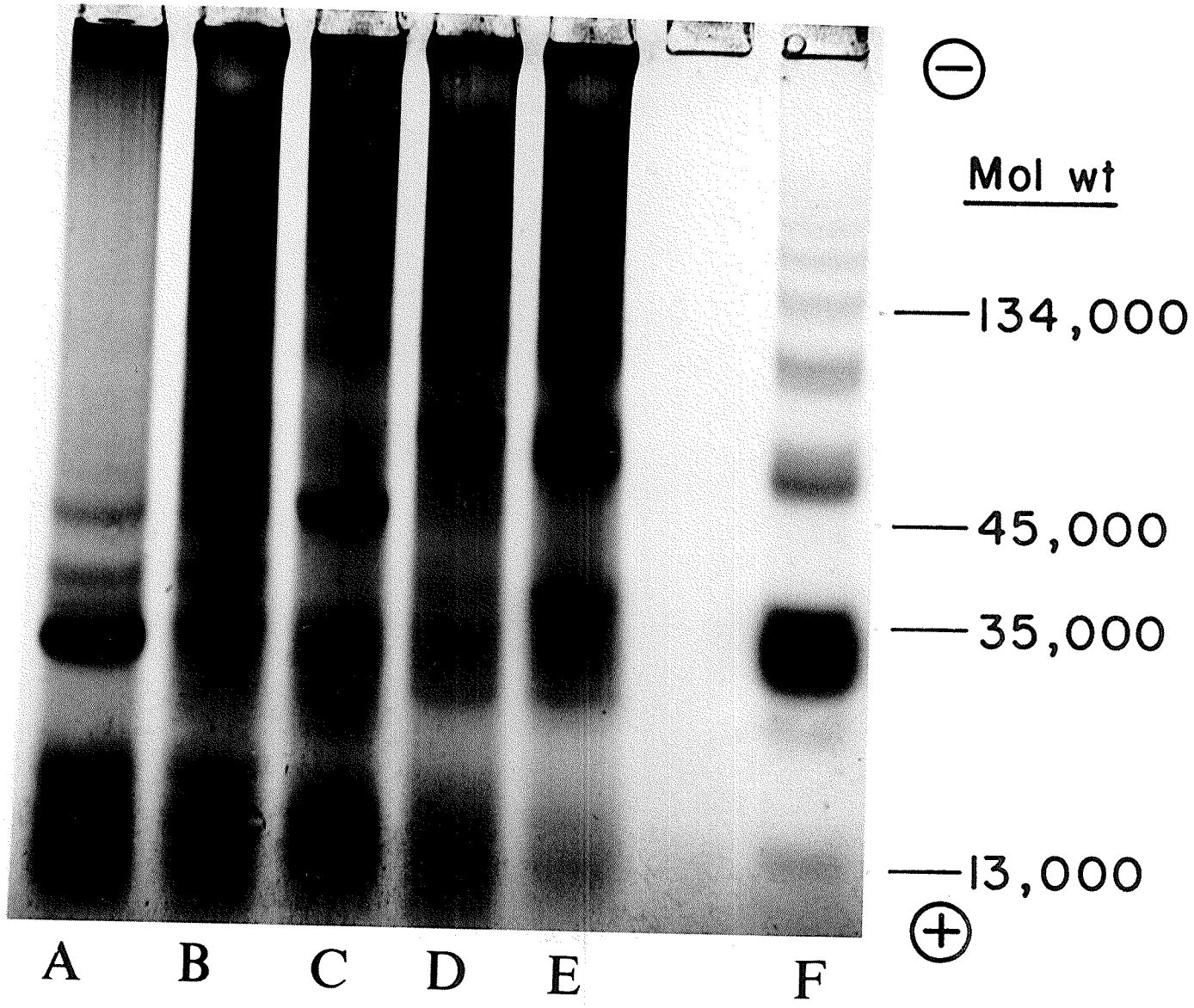

⊖
Mol wt
134,000
45,000
35,000
13,000
⊕
A
B
C
D
E
F

Figure 30. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of glutenin prepared by the author (patterns A, C, and E) and glutenin donated by J. A. Bietz, U.S.D.A., Peoria, Illinois (patterns B, D, and F):

A - Ponca HoAc-soluble glutenin

B - Ponca HoAc-soluble glutenin donated by Bietz

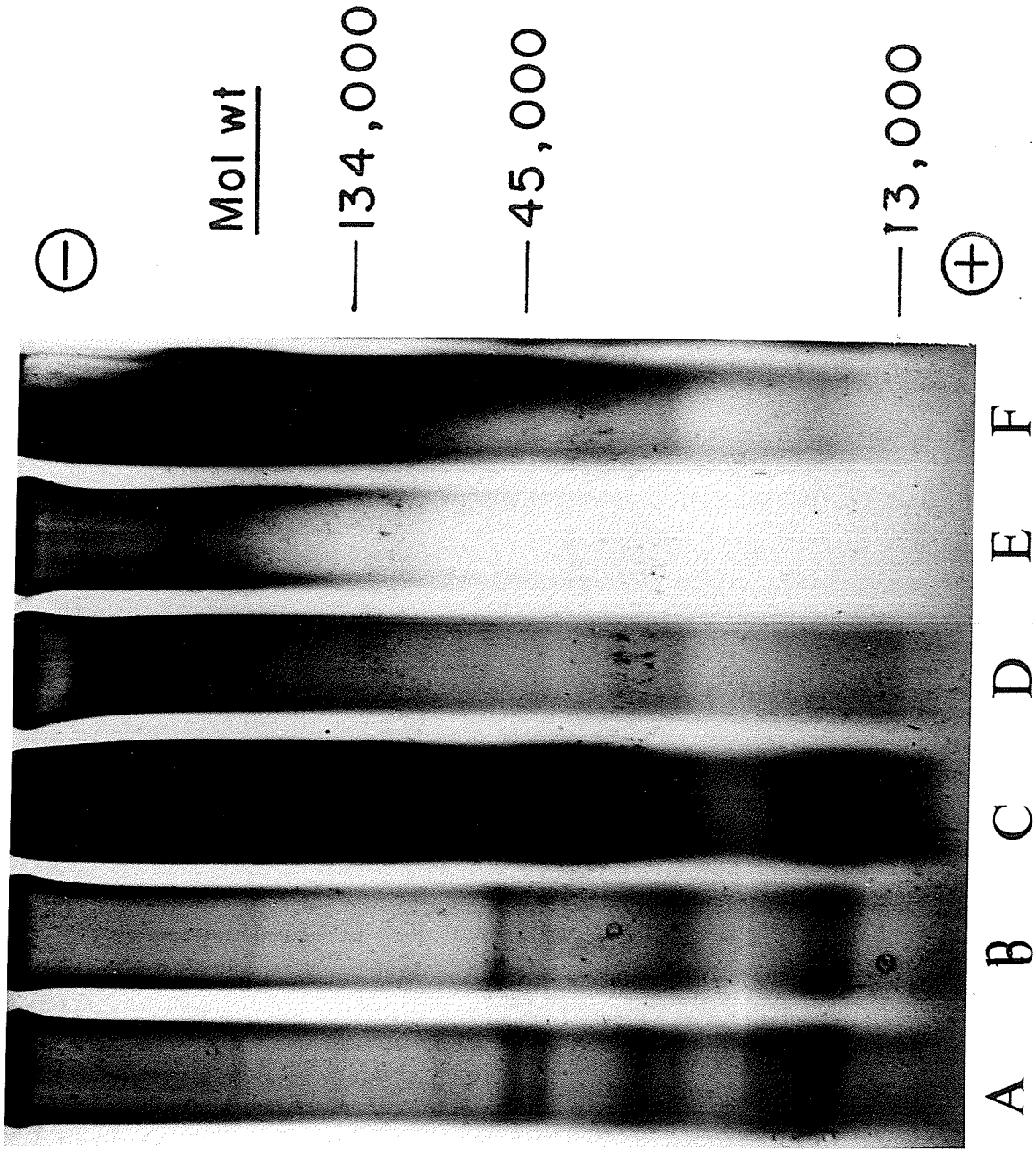
C - Ponca $HgCl_2$-soluble glutenin

D - Ponca $HgCl_2$-soluble glutenin donated by Bietz

E - Manitou glutenin purified with sulfoethyl-Sephadex according to Orth and Bushuk (1973a)

F - Twice precipitated Ponca glutenin donated by Bietz

Electrophoresis time was 3 1/2 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
45,000
13,000
⊕
A B C D E F

out at the lower pH of 7.3 using the method of Orth and Bushuk (1973b). Figure 30 shows the SDS-PAGE patterns of unreduced glutenins at pH 7.3. As can be seen, there are many protein bands on the SDS-gel just as at pH 8.9 (Fig. 29). Accordingly, it does not appear that disulfide bonds are cleaved during electrophoresis at the higher pH (8.9).

It can be concluded that the glutenins prepared for this study and that prepared by Bietz and Wall (1975) are similar on the basis of SDS-PAGE (of unreduced preparations). If the components that enter SDS-gels from the unreduced glutenins are indeed "contaminants", then the glutenins that have been used by previous researchers must be considered relatively impure. However, the results presented here do not rule out the possibility that the apparent "contaminants" are an integral part of the functional glutenin.

e. SDS-PAGE of Supernatant Fraction from pH Precipitation Procedure

The acetic acid- (HoAc) and $HgCl_2$-soluble glutenins from the extraction procedure of Bietz and Wall (1975) were purified by the pH precipitation procedure of Jones *et al.* (1959). The ethanol supernatants from these purifications were retained from the cultivars Red River 68, Manitou, Ponca, and Talbot for further study. The ethanol was evaporated on a rotary evaporator and the proteins were dissolved in 0.01N acetic acid and freeze-dried. The freeze-dried proteins were analyzed by SDS-PAGE, using the 0.125M Tris-borate-SDS system at pH 8.9. Their patterns were compared with those of reduced glutenin.

Figure 31 shows the SDS-PAGE patterns of the unreduced ethanol supernatants of the preparation of HoAc-soluble glutenin. Patterns A to C represent the cultivars Red River 68, Manitou, and Ponca,

respectively. The patterns are qualitatively identical showing predominantly 36,000, 25,000 and 15,000 mol wt components. There are also a number of fainter bands of higher mol wts.

Patterns E to H represent the ethanol supernatants from the preparation of the $HgCl_2$-soluble glutenin of Red River 68, Manitou, Ponca, and Talbot. These patterns show both qualitative and quantitative intercultivar differences. Red River 68 (E) and Talbot (H) contain equal amounts of the 45,000 and 36,000 mol wt components. Manitou (pattern F) and Ponca (pattern G) either lack or are highly deficient in the 45,000 mol wt subunit. Bietz and Wall (1975) observed similar intercultivar differences. They noted that the cultivar Chinese Spring contained equal amounts of the 45,000 and 36,000 mol wt components whereas Ponca contained primarily the 44,000 mol wt component.

Qualitative and quantitative differences were observed between the ethanol supernatants from the HoAc- and $HgCl_2$-soluble preparations (Fig. 31). The HoAc preparation supernatant appears to lack the 80,000 mol wt component whereas the $HgCl_2$-preparation supernatant seems to lack the 25,000 mol wt component. Quantitative differences between the two supernatants are obvious in the 80,000, 45,000, 36,000 and 15,000 mol wt components.

Pattern D (Fig. 31) represents reduced HoAc-soluble glutenin from the cultivar Manitou. As can be seen, many of the subunits of reduced glutenin correspond in mobility to many of the components in the ethanol supernatants of the HoAc- and $HgCl_2$-soluble glutenin preparations.

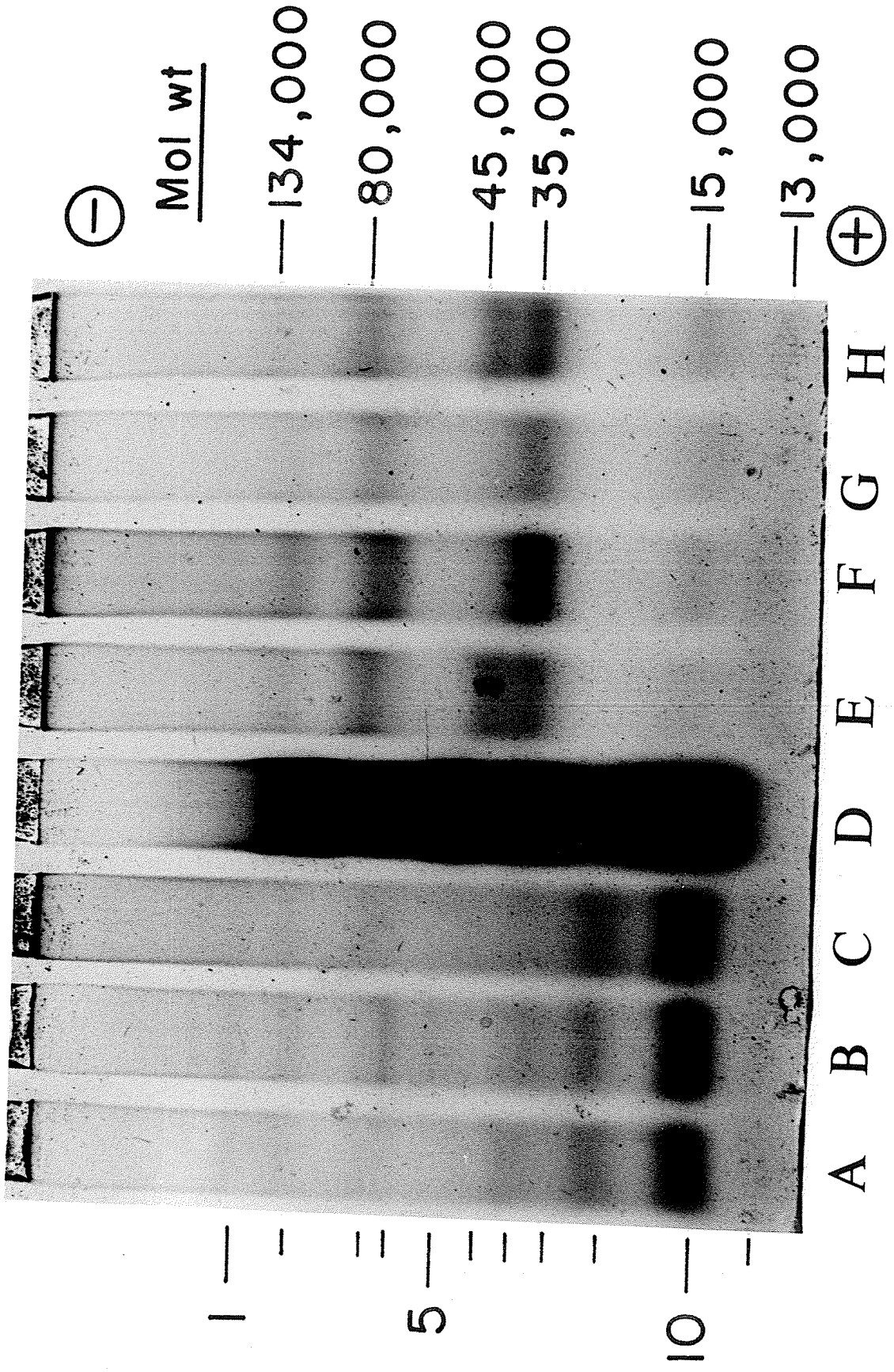
Figure 31. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris borate buffer) of the ethanol-soluble proteins from purification (Jones *et al.*, 1959) of the acetic acid- and mercuric chloride-soluble glutenins from the procedure of Bietz and Wall (1975):

A, B, C - Unreduced ethanol-soluble proteins from the acetic acid-soluble glutenin of Red River 68, Manitou, and Ponca, respectively.

D - Reduced acetic acid-soluble Manitou Glutenin

E, F, G, H - Unreduced ethanol-soluble proteins from the mercuric chloride-soluble glutenin of Red River 68, Manitou, Ponca and Talbot, respectively.

Electrophoresis time was 3 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
80,000
45,000
35,000
15,000
13,000
⊕
A
B
C
D
E
F
G
H
1
5
10

B. Purification and Isolation of the High Molecular Weight Subunits of Glutenin

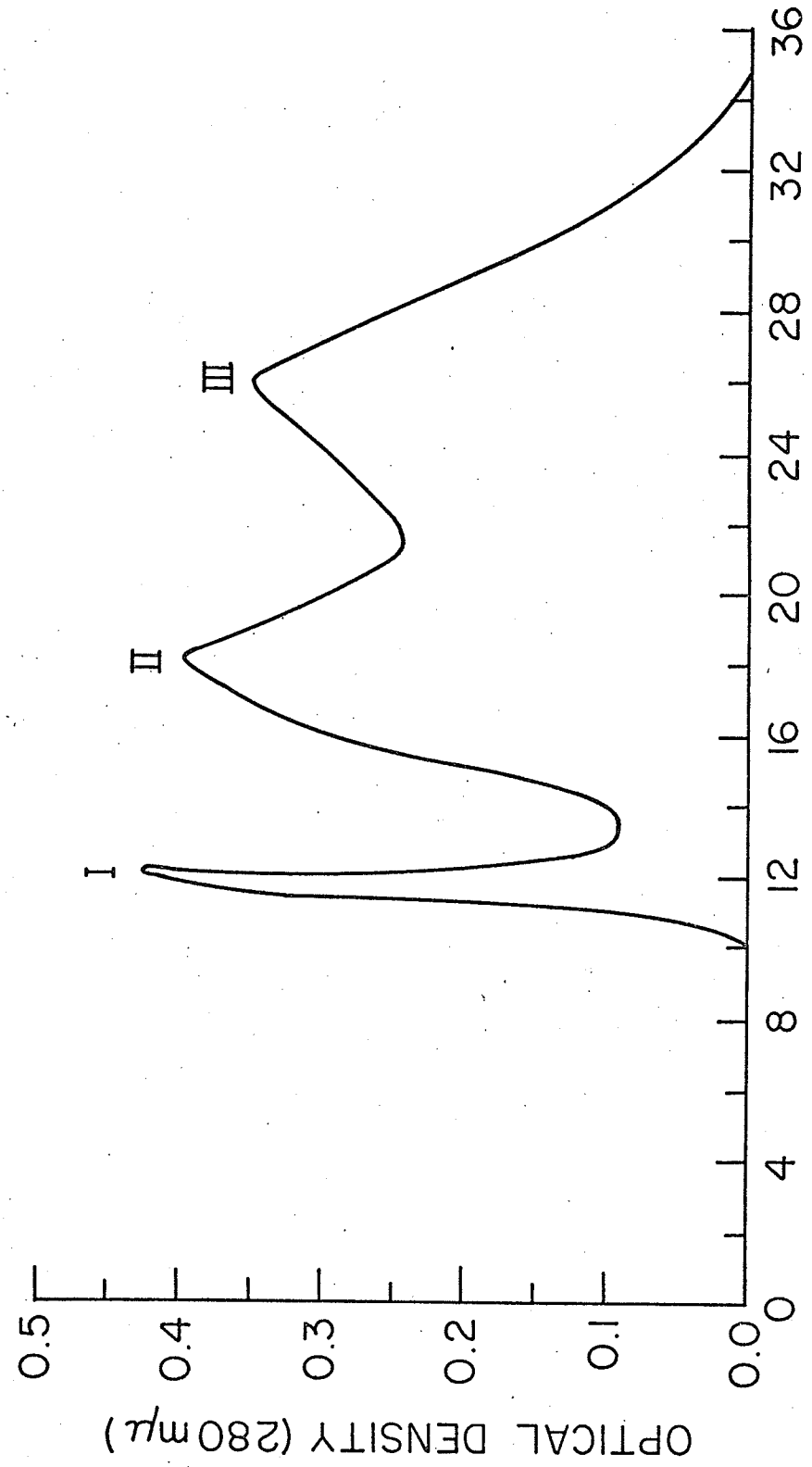
In this section a purification procedure involving Sephadex gel-filtration chromatography and isoelectric focusing is described for isolating some of the high mol wt subunits of glutenin from the cultivar Manitou. The high mol wt subunits were chosen for this study since they appear to be involved in the functional (breadmaking) properties of glutenin (Orth and Bushuk, 1973c). The procedure described here could also be applied to isolate other glutenin subunits. This section will include some additional information on the physicochemical properties of glutenin that was obtained in course of the experiments on subunit isolation and purification.

1. Gel-filtration Chromatography

a. Pyridylethylcysteinyl (PEC) Glutenin of Manitou Wheat

Glutenin from the cultivar Manitou was purified according to the procedure of Orth and Bushuk (1973a). Purified glutenin was then alkylated with 4-vinylpyridine according to the procedure of Friedman *et al.* (1970) to give pyridylethylcysteinyl (PEC) glutenin. Alkylation of glutenin is necessary in order to block the cysteine residues from being re-oxidized to disulfides. The alkylated glutenin was subjected to gel-filtration chromatography on Sephadex G-200 in 0.1N acetic acid in a 2.5 x 50 cm column. The elution profile showed 3 peaks (Fig. 32): peak I, which eluted with the void volume, represented proteins with mol wts higher than 200,000; peak II comprised proteins of mol wt between 130,000 to 60,000; peak III comprised proteins of mol wt between

Figure 32. Elution profile from Sephadex G-200 gel-filtration chromatography of alkylated (pyridylethylated) Manitou glutenin in 0.01N acetic acid-3M urea solvent. Column size was 2.5 x 50 cm. A sample of 35 mg was applied to a 35 cm bed of Sephadex. The column was operated at a flow rate of 20 ml/hr and 4.5 ml fractions were collected per tube.


OPTICAL DENSITY (280mμ)
0.0
0.1
0.2
0.3
0.4
0.5
0
4
8
12
16
20
24
28
32
36
FRACTION NUMBER
I
II
III

60,000 and 30,000. SDS-PAGE of peak I showed subunits ranging in mol wts from 68,000 to 12,000; peak II, on the other hand, consisted of the largest subunits of glutenin ranging in mol wts from 68,000 to 134,000; peak III contained mainly subunits of 45,000 and 35,000 mol wts with trace amount of 60,000 mol wt subunits. Huebner and Wall (1974) found similar results.

Since peak I proteins consisted of lower mol wt proteins than peak II, it appears that some type of association of aggregation occurs during gel-filtration to form peak I proteins from the smaller subunits as indicated by SDS-PAGE. This characteristic aggregation is worthy of further investigation. A second solvent, 0.1N acetic acid-3M urea, incorporating a dissociating agent, was therefore, used as eluant to try to prevent (or disrupt) the association of peak I proteins. However, 3 peaks were again obtained, eluting at the same positions and showing the same subunit distributions as obtained with the first solvent (0.1N acetic acid).

A third, strongly dissociating solvent, incorporating acetic acid, and both urea and the cationic detergent, hexadecyltrimethylammonium bromide (AUC), was also used in an attempt to dissociate the peak I proteins. Again 3 peaks were obtained, each peak showing the same subunit distribution as for the first two solvents.

b. Carbohydrate Content of Fractions from Gel-filtration Chromatography

An explanation was sought for the abnormal behaviour of peak I proteins on the Sephadex column. It had been shown previously that glycoproteins exhibit abnormal behavior by gel-filtration chromatography (Andrews, 1965). Proteins that showed carbohydrate contents

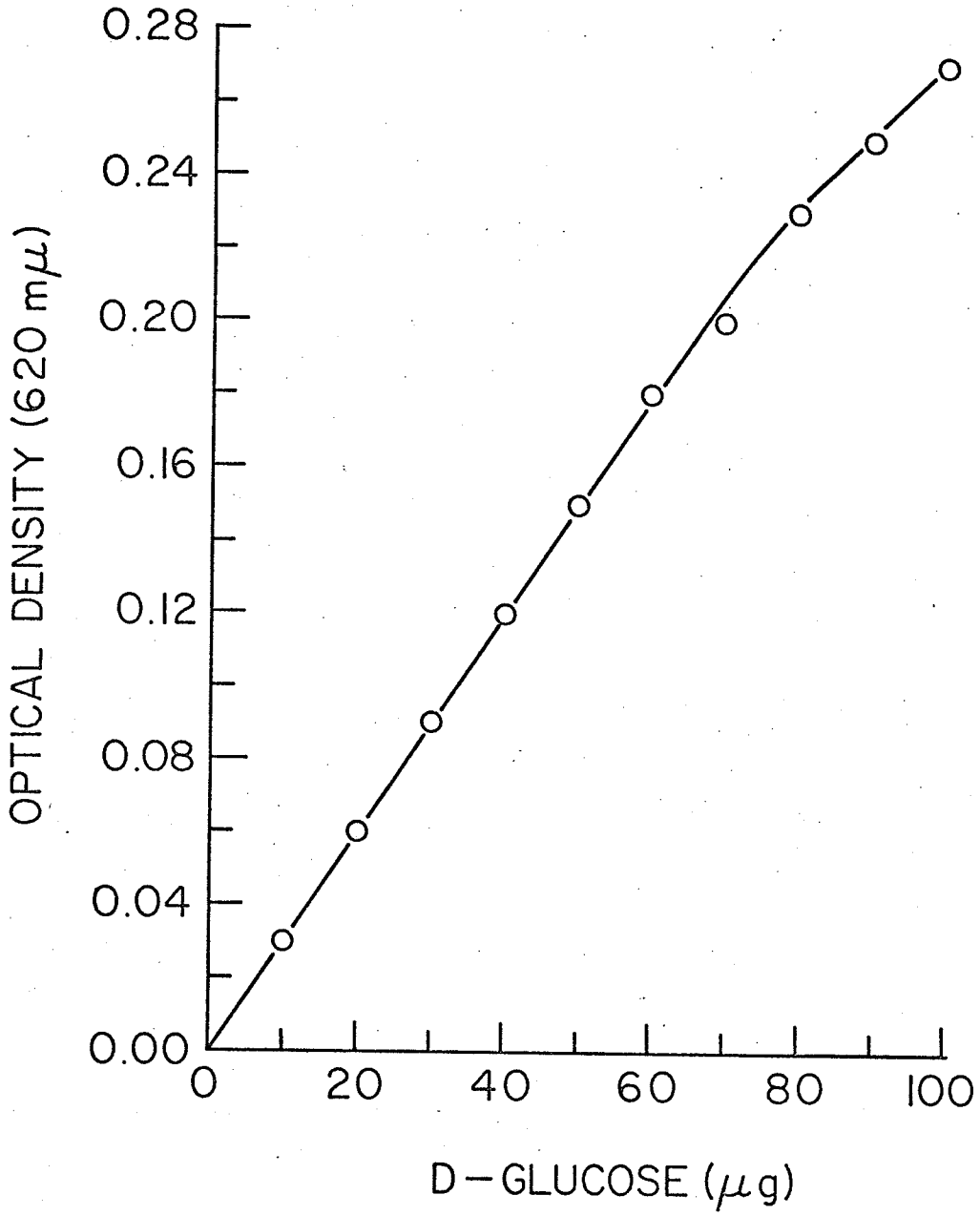
as low as 2%, gave mol wt values, 30% higher by gel-filtration when compared with the mol wts obtained by other methods (Andrews, 1965). It appears that presence of carbohydrate can induce unusual aggregations.

The carbohydrate content of each peak from gel-filtration of PEC glutenin and total PEC glutenin was determined by the anthrone method (Fig. 33). Peak I proteins showed the highest carbohydrate content (6.5%), while peaks II and III contained 1% and 0.3%, rexpectively. PEC glutenin of the cultivar Manitou contained 4.9% carbohydrate. One explanation, therefore, for the abnormal behaviour of peak I proteins on the Sephadex column might be the high carbohydrate content of these proteins. The possible implication of carbohydrates in the aggregation of peak I subunits may be relevant to functionality of glutenin in bread doughs and therefore, is worthy of detailed study.

The abnormal behaviour of peak I proteins was used in the separation of total PEC glutenin by gel-filtration chromatography into groups of fewer subunits which were easier to characterize and purify. If peak I proteins had behaved normally in gel-filtration chromatography, then peaks II and III would have contained more subunits, a condition which would have complicated the separation.

Gel-filtration chromatography of PEC glutenin was routinely performed on Sephadex G-200 in acetic acid (0.1N)-urea (3M) solvent. This will be referred to as the AU solvent. Urea was incorporated to minimize non-specific protein association through hydrogen bonding. The detergent (as in AUC solvent) was not included in the solvent as it offered no additional advantages over the AU solvent.

Figure 33. Calibration curve for carbohydrate determination by the Anthrone method. D-glucose was used as the standard sugar.


0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
OPTICAL DENSITY (620 mμ)
0
20
40
60
80
100
D-GLUCOSE (μg)

c. Rechromatography of Peak I Proteins

Peak I fraction from the gel-filtration chromatography of the PEC glutenin (cv. Manitou) was re-chromatographed on Sephadex G-200 using the AU solvent to examine if the aggregates in this fraction could be dissociated by re-chromatography.

The elution profile again showed 3 peaks. The first peak eluted with the void volume while peaks II and III eluted at the positions of peaks II and III, respectively, as obtained with chromatography (Fig. 32) of PEC glutenin. These results may be analogous to those obtained by Dalek-Zawistowska *et al.* (1975) with gel-filtration chromatography of gluten proteins.

SDS-PAGE (Fig. 34) of each peak showed that a further fractionation into smaller groups of proteins was achieved by rechromatography. Huebner and Wall (1974) also achieved a further separation of peak I (their fraction A) proteins by rechromatography on Sephadex G-200 using 6M guanidine hydrochloride as eluant. However, they did not succeed in isolating any homogeneous subunits from peak I proteins by subsequent ion-exchange chromatography of the smaller groups of subunits.

d. Molecular Weight Estimation of Subunits of Glutenin by Sephadex Gel-filtration Chromatography

PEC glutenin from the cultivar Manitou was subjected to gel-filtration chromatography in AU solvent on Sephadex G-200. Each of the 3 peaks obtained (Fig. 32) was rechromatographed on Sephadex G-200 in the same solvent to estimate the average mol wt of the proteins in each peak. Figure 35 gives the calibration curve for the mol wt estimation. Since peak I proteins eluted with the void volume, the

Figure 34. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of the rechromatography of peak I subunits:

C - Peak I subunits before rechromatography

D - Peak I subunits from rechromatography

E - Peak II " " "

F - Peak III " " "

Included, for comparison purposes, are Peak II subunits (pattern A) and Peak III subunits (pattern B) from chromatography of pyridylethlated Manitou glutenin. Electrophoresis time was 3 hr. Staining procedure 1 was used.

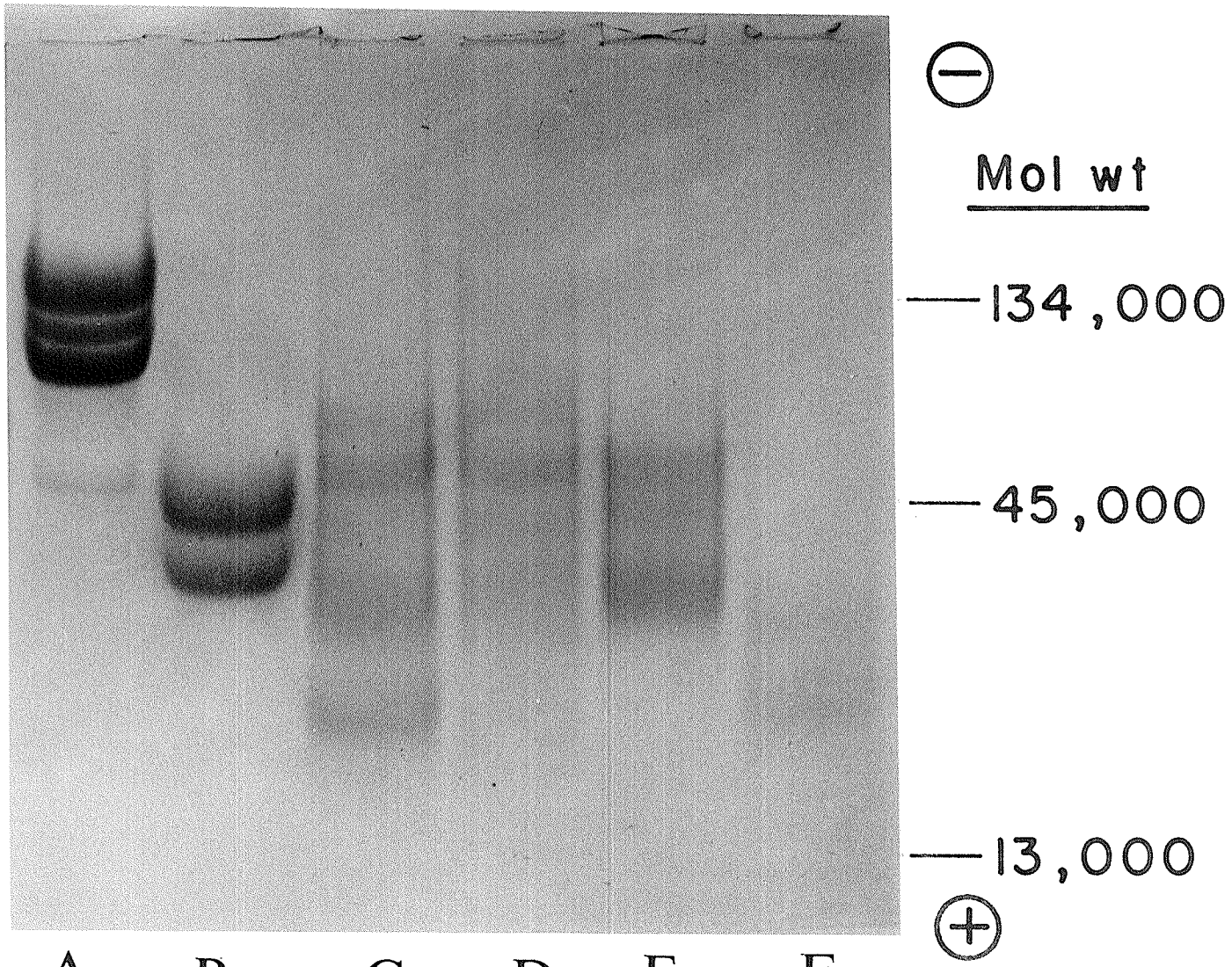
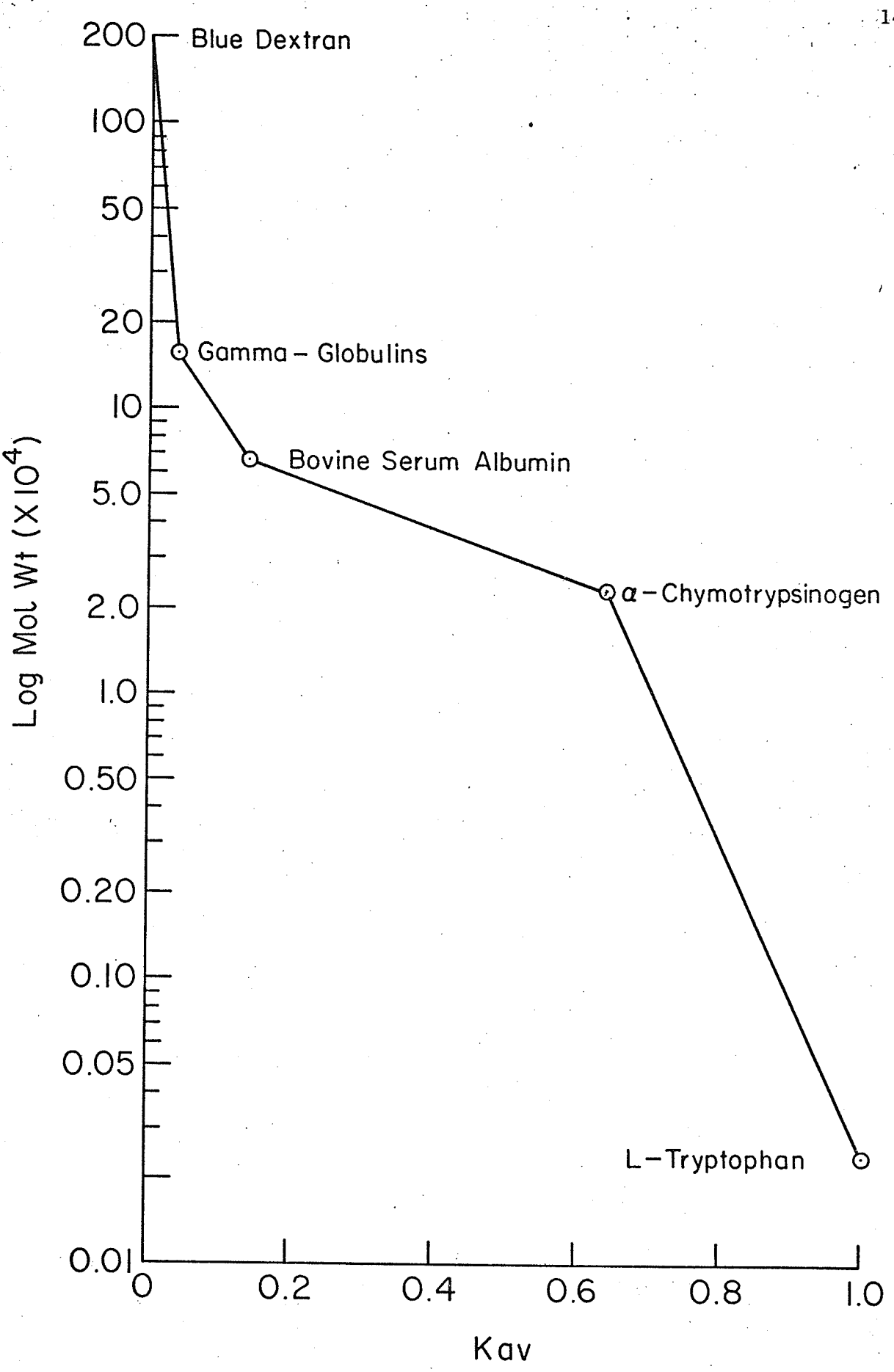

⊖
Mol wt
134,000
45,000
13,000
⊕
A
B
C
D
E
F

Figure 35. Calibration curve from Sephadex G-200 gel-filtration chromatography for molecular weight estimation of the subunits of glutenin. Column size was 2.5 x 50 cm with a bed height of 38 cm. Flow rate was 20 ml/hr.


200
100
50
20
10
5.0
2.0
1.0
0.50
0.20
0.10
0.05
0.01
Log Mol Wt (X 10^4)
Blue Dextran
Gamma – Globulins
Bovine Serum Albumin
α – Chymotrypsinogen
L – Tryptophan
0
0.2
0.4
0.6
0.8
1.0
Kav

estimated mol wt of these proteins should be between 200,000 and 800,000 depending on the shape of the protein. Peak II proteins were calculated to have an average mol wt of 110,000 while peak III proteins had an average mol wt of 47,000. If the mol wts obtained by SDS-PAGE for the subunits of peaks II and III, respectively, are averaged, the averaged values obtained are in close agreement with the average values obtained by gel-filtration chromatography.

e. Gel-filtration Chromatography of Glutenin from Cultivars of Different Mixing and Baking Properties

An attempt was made to fractionate PEC glutenin from Red River 68, Manitou, Talbot, and Stewart 63 (widely different baking properties) on Sephadex G-200 and to subject each peak obtained from gel-filtration to SDS-PAGE to examine if there are any qualitative and quantitative inter-cultivar differences that may explain differences in breadmaking properties. The first three cultivars are of the hexaploid or common wheat class while Stewart 63 belongs to the tetraploid (durum) group of wheats.

Figure 36 shows the elution profile from Sephadex G-200 chromatography of Red River 68. This profile, which is representative of the hexaploid cultivars, shows 3 peaks. Figure 37 shows the elution profile for Stewart 63, a durum wheat cultivar. Although peak I showed the largest absorbance of all the peaks at 280 mμ, it contained the least amount of material by weight, amounting to approximately 20%. Peak II contained approximately 36% while peak III contained approximately 40% of the material.

The SDS-PAGE patterns for the various peaks show a number of qualitative and quantitative differences in certain subunits. The

Figure 36. Elution profile for alkylated Red River 68 glutenin from Sephadex G-200 gel-filtration chromatography in 0.1N acetic acid-3M urea solvent. The column size was 5.0 x 100 cm with a bed height of 72 cm. Sample size was 250 mg and the flow rate was 45 ml/hr. Fraction volume was 7.0 ml/tube.

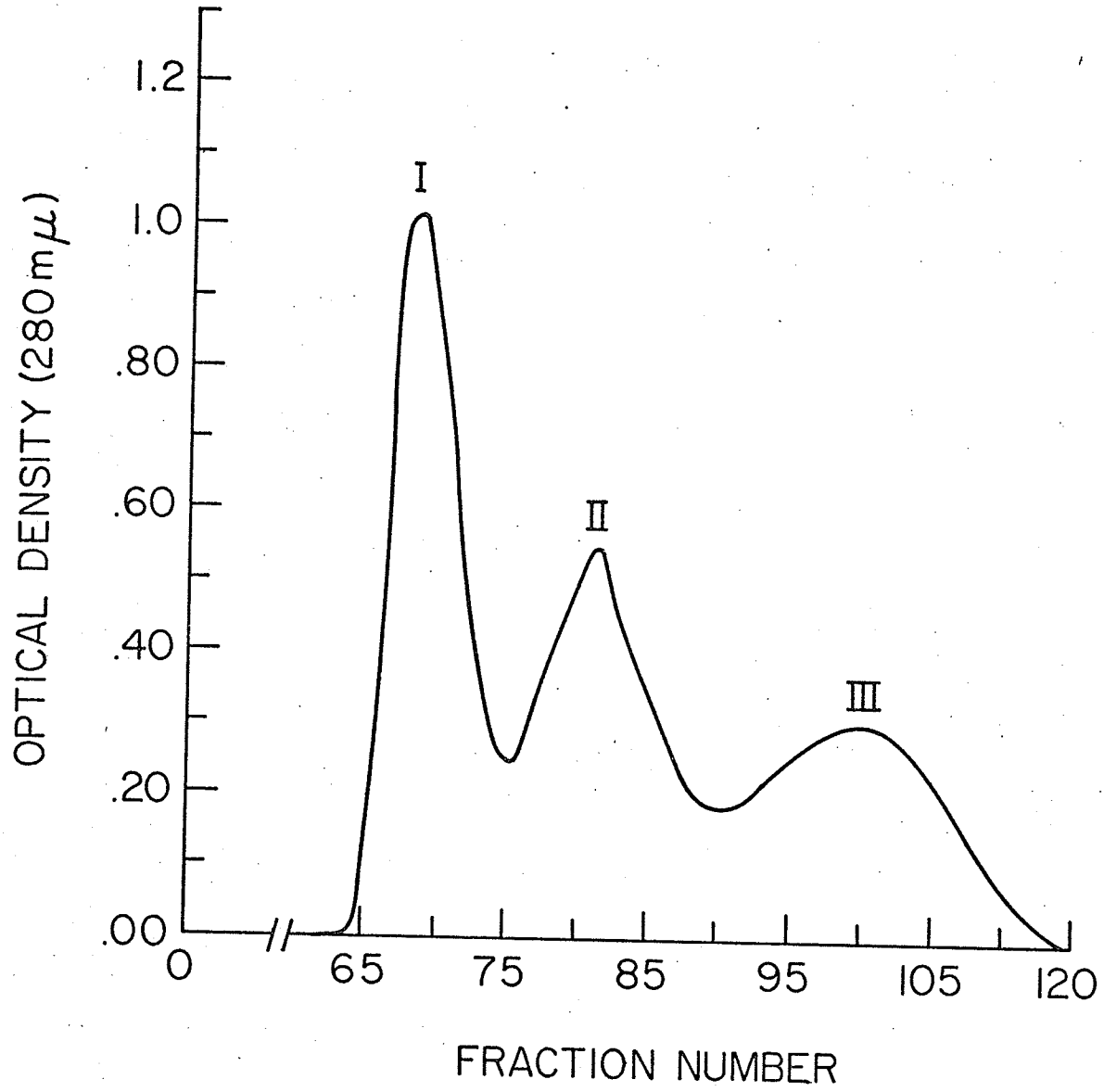
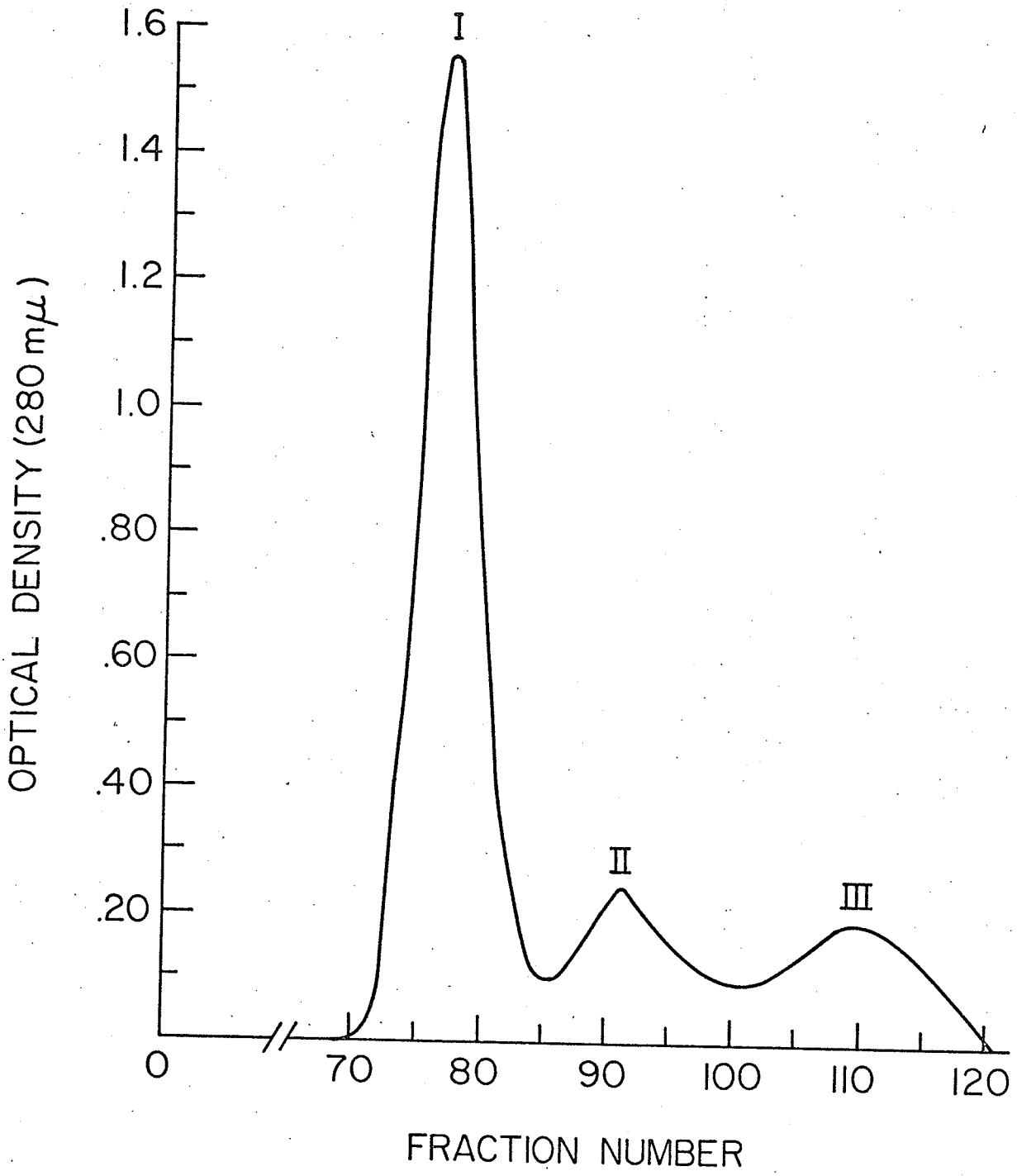

OPTICAL DENSITY (280 mμ)
1.2
1.0
.80
.60
.40
.20
.00
0
65
75
85
95
105
120
I
II
III
FRACTION NUMBER

Figure 37. Elution profile of alkylated Stewart 63 glutenin from Sephadex G-200 gel-filtration chromatography in 0.1N acetic acid-3M urea solvent. The column size was 5.0 x 100 cm with a bed height of 72 cm. The sample size was 200 mg and the flow rate was 40 ml/hr. The fraction volume was 6.7 ml/tube.


1.6
1.4
1.2
1.0
.80
.60
.40
.20
0
70
80
90
100
110
120
I
II
III
OPTICAL DENSITY (280 mμ)
FRACTION NUMBER

patterns of peak I (Fig. 38) show that RR68 has both the 72,000 and 68,000 mol wt subunits (subunits 7 and 8, respectively) whereas the other cultivars seem to have only one subunit (8, 68,000 mol wt). Also, the subunits with mol wts of 60,000 and lower are much fainter in the pattern for Talbot (G), a soft white winter wheat. It should also be noted that the bands in the region of the 72,000 and 68,000 subunits are very faint in the patterns of RR68, Manitou and Talbot (A, C, and E), whereas these two subunits in the pattern of Stewart 63 (H) seem to be of the same intensity before and after gel-filtration.

Peak II (Fig. 39) showed predominantly the 134,000, 132,000, 110,000, 98,000, and 90,000 mol wt subunits with three faint bands at 72,000, 68,000 and 60,000 mol wts for the hexaploids. The pattern for Stewart 63, is different from that of the hexaploid cultivars in that it does not have the 134,000 and 132,000 mol wt subunits. However, it has the 110,000 and the 98,000 mol wt subunits. In addition, the pattern of Stewart 63 showed three other clearly, well resolved, subunits of 72,000, 68,000 and 63,000 mol wts, which appeared as extremely faint bands in the patterns of the hexaploid wheats.

Peak III (Fig. 40) comprised predominantly subunits of 45,000 and 35,000 mol wts with fainter bands at 60,000, 40,000 and 30,000 for the hexaploid cultivars. Stewart 63, a tetraploid wheat, showed six subunits of 80,000, 60,000, 45,000, 40,000, 35,000 and 30,000 mol wts.

f. Amino Acid Composition of Protein Fractions from Gel-filtration Chromatography

The amino acid composition (Table 4) of the various protein fractions (peaks) from gel-filtration chromatography of PEC glutenin showed

Figure 38. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of alkylated glutenin and peak I subunits from gel-filtration chromatography of alkylated glutenin:

A - Alkylated Red River 68 glutenin

B - Peak I subunits from Red River 68

C - Alkylated Manitou glutenin

D - Peak I subunits from Manitou glutenin

E - Alkylated Talbot glutenin

F - Peak I subunits from Talbot glutenin

G - Alkylated Stewart 63 glutenin

H - Peak I subunits from Stewart 63 glutenin

Electrophoresis time was 3 hr. Staining procedure 1 was used.

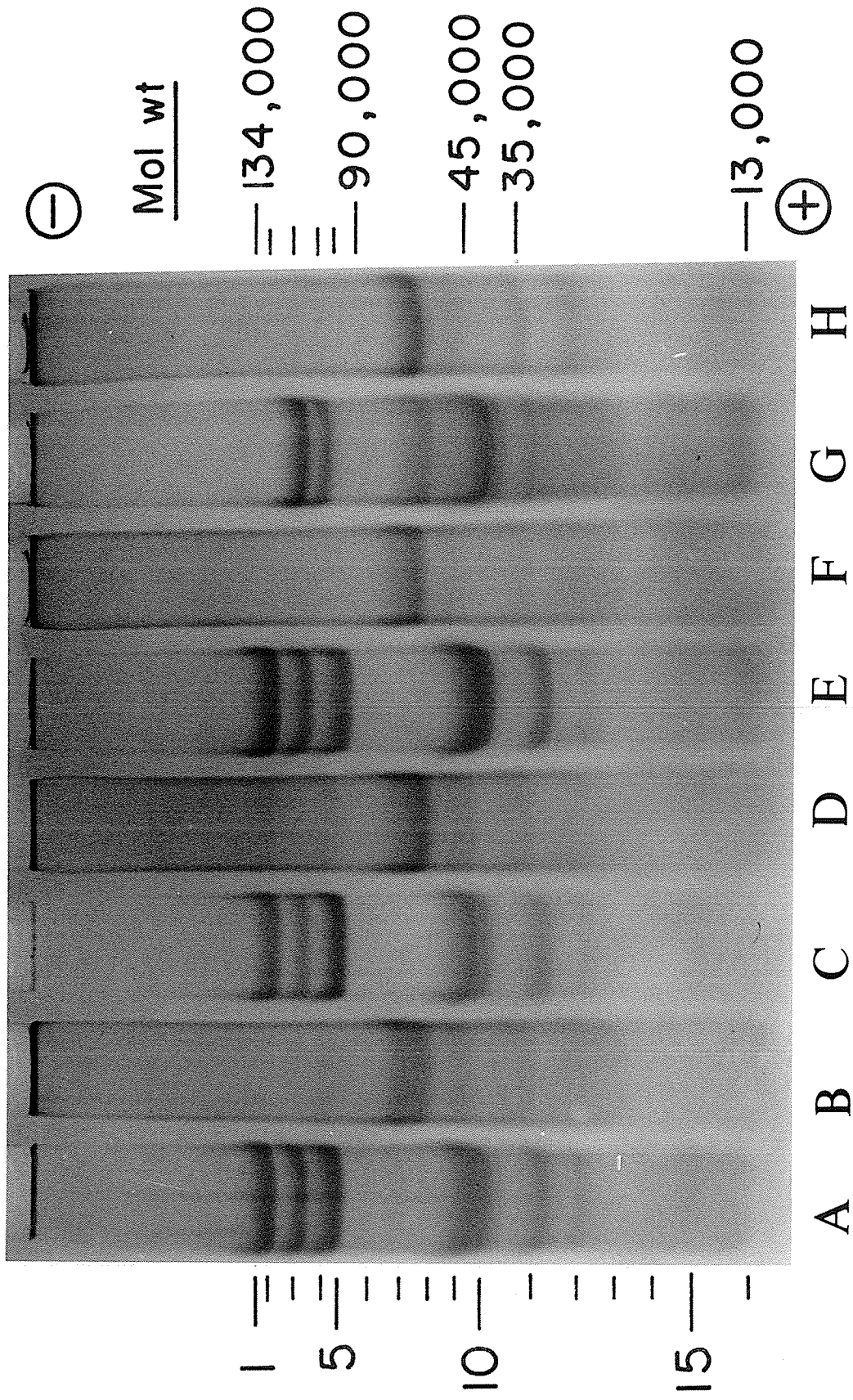

⊖
Mol wt
134,000
90,000
45,000
35,000
13,000
⊕
A
B
C
D
E
F
G
H
1
5
10
15

Figure 39. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of alkylated glutenin and peak II subunits from gel-filtration chromatography of alkylated glutenin:

A - Alkylated Red River 68 glutenin

B - Peak II subunits from Red River 68 glutenin

C - Alkylated Manitou glutenin

D - Peak II subunits from Manitou glutenin

E - Alkylated Talbot glutenin

F - Peak II subunits from Talbot glutenin

G - Alkylated Stewart 63 glutenin

H - Peak II subunits from Stewart 63 glutenin

Electrophoresis time was 3 hr. Staining procedure 1 was used.

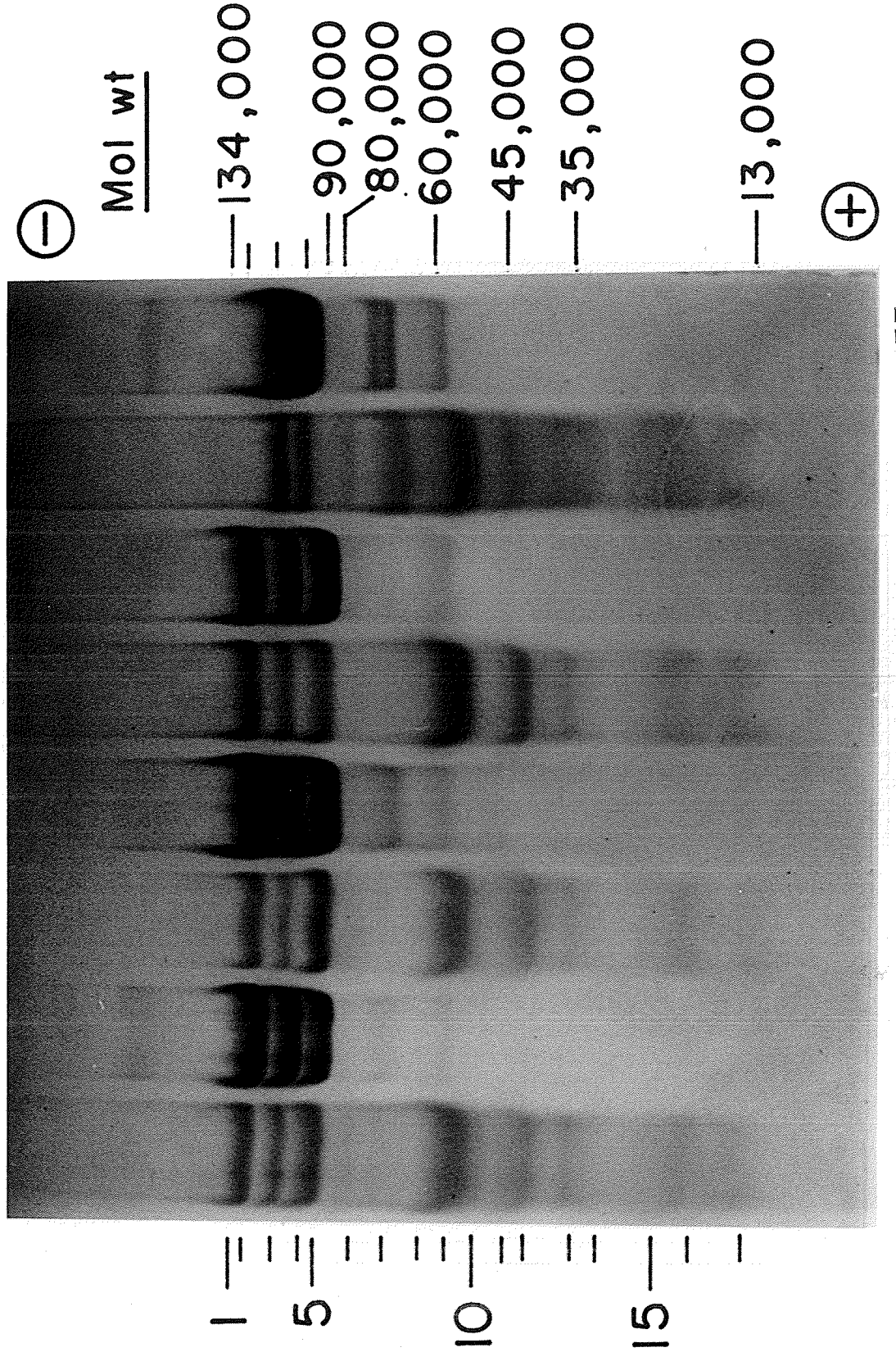

Mol wt
134,000
90,000
80,000
60,000
45,000
35,000
13,000
A B C D E F G H
1
5
10
15

Figure 40. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of alkylated glutenin and peak III subunits from gel-filtration chromatography of alkylated glutenin:

A - Alkylated Red River 68 glutenin

B - Peak III subunits from Red River 68 glutenin

C - Alkylated Manitou glutenin

D - Peak III subunits from Manitou glutenin

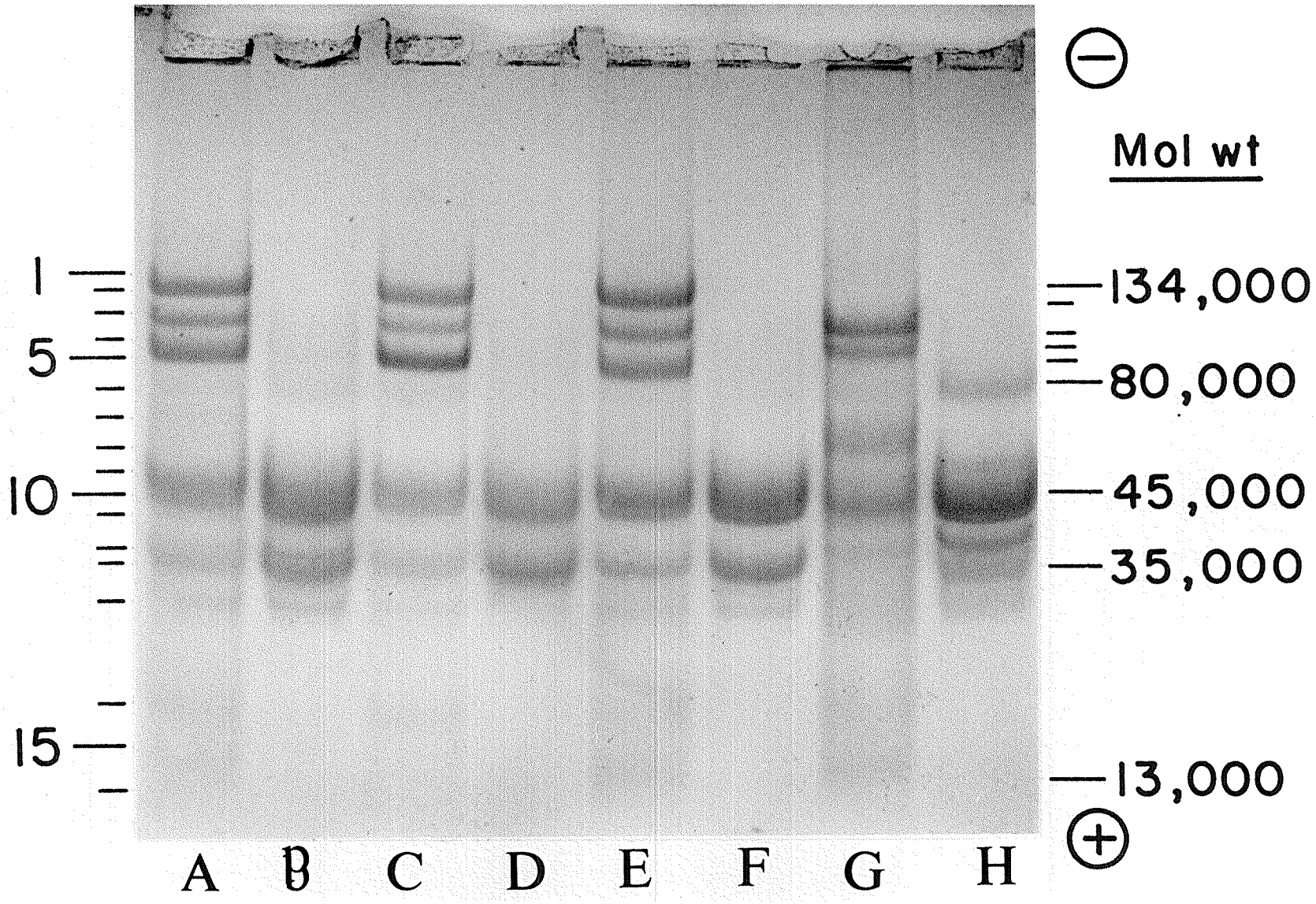
E - Alkylated Talbot glutenin

F - Peak III subunits from Talbot glutenin

G - Alkylated Stewart 63 glutenin

H - Peak III subunits from Stewart 63 glutenin

Electrophoresis time was 3 hr. Staining procedure 1 was used.


Mol wt
1
5
10
15
134,000
80,000
45,000
35,000
13,000
A
B
C
D
E
F
G
H

differences between peaks for the same cultivar. Differences among cultivars were also evident.

Peak I proteins for each cultivar have a higher content of the basic amino acids, lysine, histidine, and arginine. Peak I also contained more aspartic acid but less glutamic acid. Methionine was also higher in peak I proteins. In general, peak I proteins contained a higher proportion of the hydrophobic amino acids, alanine, valine, leucine, isoleucine and methionine. Peak I proteins seem to resemble albumins (water-soluble) and globulins (salt-soluble) proteins in amino acid composition (Huebner *et al.*, 1974).

The most outstanding characteristic of the amino acid composition of peak II proteins is the very high content of glycine. Glycine is generally high in structural proteins such as collagen. This high content of glycine in peak II proteins may reflect a structure-function relationship (as yet unknown). Peak II proteins also contain a high content of glutamic acid, proline, and tyrosine, but a very low content of phenylalanine.

The most outstanding characteristics of peak III proteins are the high glutamic acid and proline contents, and a very low glycine content. Peak III proteins resemble gliadin proteins in amino acid composition in having higher contents of glutamic acid and proline (Huebner *et al.*, 1974).

Intercultivar differences in amino acid composition occurred mainly in peak II proteins. Variations occurred in the basic amino acids lysine, histidine and arginine, and in aspartic acid. The rest of the amino acid contents among the cultivars were generally consistent

TABLE 4. Comparison of the Amino Acid Compositions[1] of the Various Peaks from Gel-filtration Chromatography of PEC[2] Glutenin (PEC Glu)

Amino acids[3]	Cultivars															
	Red River 68				Manitou				Talbot				Stewart 63			
	PEC Glu	PK I	PK II	PK III	PEC Glu	PK I	PK II	PK III	PEC Glu	PK I	PK II	PK III	PEC Glu	PK I	PK II	PK III
Lysine	1.69	4.78	0.99	0.93	1.91	3.81	0.73	0.83	1.98	4.20	0.88	0.78	2.67	5.05	1.47	1.22
Histidine	1.58	2.13	0.85	1.67	1.66	2.02	0.73	1.59	1.77	2.32	0.95	1.66	1.81	2.34	1.19	1.60
Arginine	4.09	5.04	1.99	3.49	4.01	4.76	2.14	3.66	2.81	5.02	1.75	3.97	4.91	5.54	3.83	4.03
Aspartic acid	3.01	9.20	1.38	2.35	3.48	7.63	0.69	2.32	4.19	9.25	1.54	2.12	5.63	9.56	2.75	2.68
Threonine	3.15	4.42	3.17	2.83	3.20	4.06	3.03	2.66	3.32	4.20	3.02	2.56	3.52	4.43	3.35	2.82
Serine	6.52	6.35	6.44	6.97	6.26	6.45	6.34	6.49	6.59	6.33	6.48	6.75	6.62	6.49	7.27	6.87
Glutamic acid	34.12	15.02	38.42	38.93	32.24	19.99	40.42	39.32	32.63	16.90	39.27	38.83	28.97	13.11	34.10	37.43
Proline	12.31	6.18	12.27	14.90	12.15	7.93	12.30	14.99	12.09	7.22	13.03	14.82	10.25	5.66	10.51	14.22
Glycine	8.98	9.43	18.08	4.02	9.04	9.75	17.72	3.59	8.88	9.34	15.77	3.76	8.66	9.14	16.13	4.62
Alanine	3.83	8.17	3.14	2.74	4.06	6.96	2.79	2.72	4.27	7.60	2.90	2.50	5.15	8.53	4.18	2.99
Valine	3.92	6.55	1.77	4.46	4.10	5.84	1.58	4.25	4.33	6.44	2.09	4.25	4.46	6.77	2.61	3.98
Methionine	1.12	2.10	0.10	0.14	1.57	1.82	0.29	0.71	0.71	1.00	Trace	0.93	1.48	2.28	0.59	0.90
Isoleucine	2.94	4.39	0.96	3.67	3.01	3.92	0.87	3.67	3.11	4.17	1.34	3.64	3.40	4.55	1.87	3.62
Leucine	6.50	9.03	4.43	7.04	6.79	8.25	4.30	7.34	6.89	8.73	5.07	7.22	6.92	9.27	4.85	6.93
Tyrosine	3.06	3.34	5.44	1.49	3.04	3.41	5.41	1.42	3.02	3.34	4.62	1.70	3.15	3.28	5.00	1.76
Phenylalanine	3.15	3.85	0.59	4.38	3.43	3.39	0.63	4.40	3.34	3.93	1.23	4.50	3.54	3.99	1.57	4.38

[1]Expressed as mole percent.

[2]Reduced glutenin alkylated with 4-vinylpyridine.

[3]Cysteine and Tryptophan were not determined.

between peaks except Stewart 63, a tetraploid or durum wheat, which showed a lower glutamic acid and proline contents in peak II proteins. The lower content of these two amino acids in peak II proteins of Stewart 63 could be attributed to the absence of the first two high mol wt subunits of glutenin (Fig. 39, pattern G). These two subunits are present only in hexaploid wheats.

2. Isolation of the First Two High Mol Wt Subunits of Glutenin by SDS-PAGE

A simple and rapid method was sought to isolate the 134,000 and 132,000 mol wt subunits of glutenin from different cultivars to examine their amino acid compositions for intercultivar differences. Peak II fraction (from gel-filtration chromatography of PEC glutenin) was used for the isolation of these two subunits. Since the 134,000 and 132,000 mol wt subunits were eluted together, they will be referred to here as the 133,000 mol wt subunit.

The method of Weber and Kuter (1971) with slight modifications (see Materials and Methods section), was used for the elution of the 133,000 mol wt subunit from SDS-gels. After elution and recovery, the subunits were re-run by SDS-PAGE as a check for their presence in the eluate and purity. Figure 41 shows the SDS-PAGE patterns of the eluted subunits of the cultivars Chinese Spring, Red River 68, Manitou, Ponca and Talbot. These results show that there is an additional protein band of approximately 160,000 mol wt on the SDS-gel, a band which is not present in the SDS-PAGE patterns of unfractionated PEC glutenin. The presence of this additional band cannot be explained and was not investigated further.

Figure 41. SDS-PAGE electrophoretograms at pH 7.3 (0.02M phosphate buffer) of the 133,000 (134,000 and 132,000) mol wt subunit eluted from SDS gels:

A - Chinese Spring

B - Red River 68

C - Ponca

D - Talbot

E - Manitou

Electrophoresis time was 3 hr. Staining procedure 1 was used.

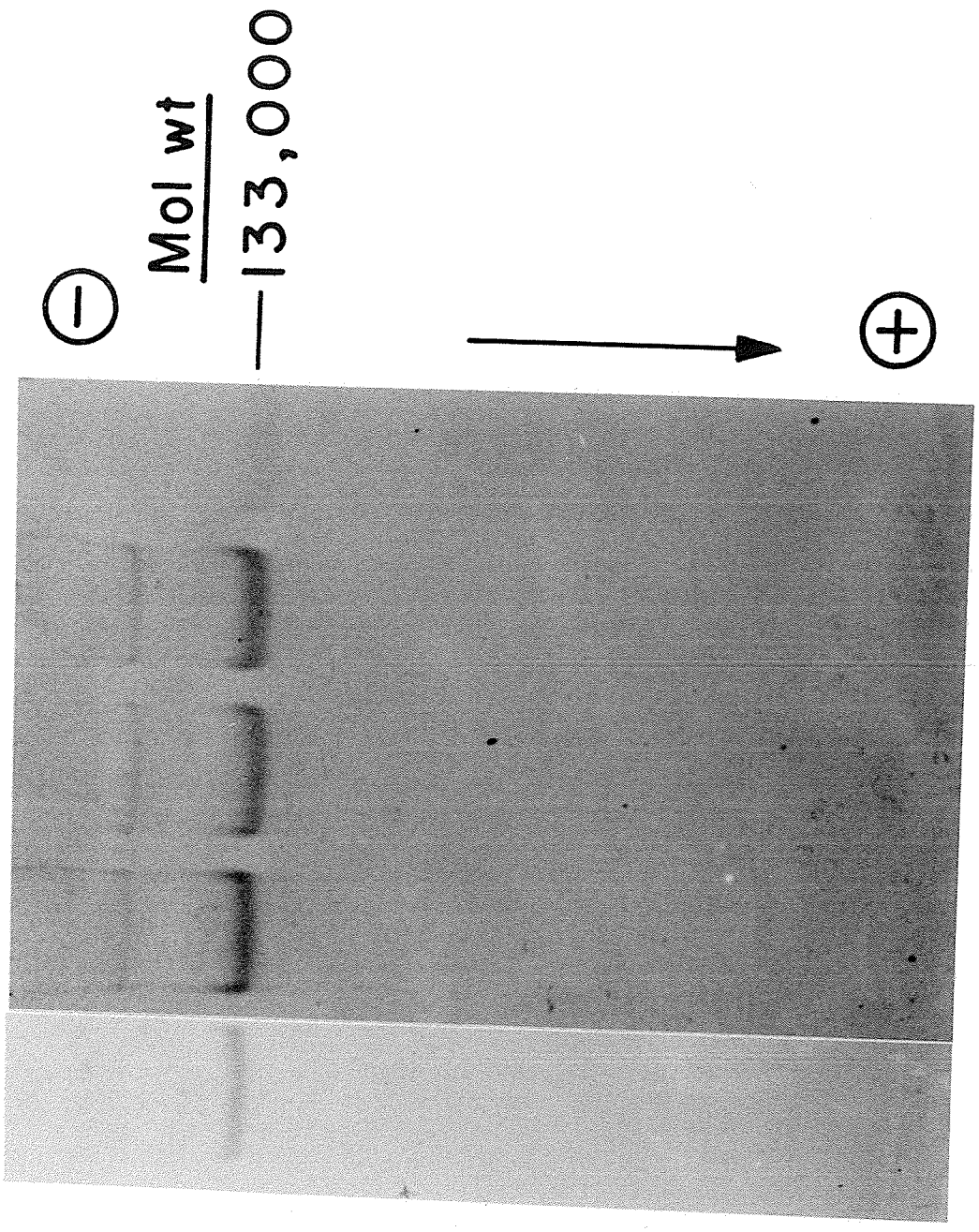

⊖
Mol wt
—133,000
⊕
A B C D E

Table 5 shows the amino acid compositions of the 133,000 subunit of the five cultivars used in this experiment. Chinese Spring showed a higher lysine, histidine, serine and alanine content but a lower glutamic acid, proline, and glycine content than the other cultivars. Marked variations occurred in the basic amino acids lysine, histidine and arginine, and also in serine, proline, alanine, valine, methionine, isoleucine and phenylalanine among the various cultivars.

The amino acid composition (Table 5) showed sufficient differences between cultivars to merit the formulation of a preparative procedure to isolate the high mol wt subunits of glutenin for further investigation. Isoelectric focusing, because of its high resolution, was adopted for this purpose.

The SDS-PAGE method of Orth and Bushuk (1973b) at pH 7.3 for reduced glutenin is especially suitable for the elution procedure described above since the high mol wt subunits are visible as bright white bands upon immersion of the SDS-gel in 10% TCA for 1/2 hr to 45 min. The white protein bands can then be cut out accurately. However, due to the close proximity of the high mol wt subunits there could be contamination of the 133,000 mol wt subunit by the 110,000 mol wt subunit if the bands are not cut out very carefully. In contrast, the high mol wt subunits are only faintly visible when pH 8.9 SDS-gels are immersed in 10% TCA, so that chances of contamination are higher if this system is used.

TABLE 5. Comparison of the Amino Acid Composition[1] of the 133,000 Molecular Weight Subunit of Glutenin Isolated by SDS-PAGE from Different Cultivars

Amino acid[2]	Cultivars				
	Chinese Spring	Red River 68	Manitou	Ponca	Talbot
Lysine	5.09	2.12	2.53	3.53	2.04
Histidine	0.96	0.46	0.32	Trace	0.48
Arginine	1.44	1.52	1.39	1.30	1.90
Aspartic acid	3.59	2.94	2.89	3.19	2.64
Threonine	3.47	3.24	3.35	3.53	3.19
Serine	13.01	8.44	9.46	11.46	8.44
Glutamic acid	27.23	34.34	35.29	32.84	34.50
Proline	8.24	11.26	10.20	9.63	11.42
Glycine	18.72	20.41	20.31	19.83	20.60
Alanine	5.81	3.29	2.93	4.88	3.40
Valine	2.44	1.60	1.58	2.41	1.45
Methionine	0.47	Trace	0.46	0.12	Trace
Isoleucine	1.56	1.31	1.27	1.48	1.29
Leucine	3.84	4.83	4.26	4.44	4.82
Tyrosine	3.16	3.32	2.60	0.23	3.28
Phenylalanine	0.96	0.94	1.18	1.11	0.82

[1]Expressed as mole percent.

[2]Cysteine and Tryptophan were not determined.

3. Isoelectric Focusing Experiments

a. Isoelectric Focusing of Alkylated Glutenin of cv. Manitou

PEC glutenin was subjected to isoelectric focusing in 6M urea on a 3-10 pH gradient of carrier ampholytes. The elution profile (Fig. 42) showed 10 peaks ranging in pI from 5.0 to 9.0. The SDS-PAGE patterns (not shown) of each peak showed considerable overlap in the subunit compositions. It was concluded that total PEC glutenin cannot be used to isolate single subunits of glutenin. However, since the pI of the high mol wt subunits fell in the 5-9 pH range, it was decided to use this narrower pH range with peak II (high mol wt) subunits from gel-filtration in an attempt to isolate homogeneous subunits.

b. Isoelectric Focusing of Peak II Subunits on a 5-9 pH Gradient

The high mol wt subunits (peak II) of PEC Manitou glutenin from gel-filtration on Sephadex G-200 were subjected to isoelectric focusing in 6M urea on a 5-9 pH gradient of carrier ampholytes. The elution profile (not shown) showed nine peaks in the pH range 6.0 to 8.5. The SDS-PAGE pattern (not shown) of each peak showed better separation of these subunits than on the 3-10 pH gradient. However, it was obvious that a narrower pH gradient was needed if individual subunits were to be isolated.

c. Isoelectric Focusing of Peak II Subunits on a 6-8 pH Gradient

The high mol wt subunits of PEC glutenin from Manitou were subjected to isoelectric focusing on a 6-8 pH gradient of carrier ampholytes in 6M urea. The elution profile (Fig. 43) showed 12 peaks in the pH range 4 to 9. The SDS-PAGE patterns of each peak is shown in Fig. 44. Peak 3 (pattern D) contains only the 134,000 subunit while peak 9

Figure 42. Elution profile of alkylated Manitou glutenin from isoelectric focusing on a 3 to 10 pH gradient of ampholine carrier ampholytes in 6M urea. Sample size was 40 mg. Initial voltage was 400 (approximately 7 mA) while final voltage was 420 (approximately 1.5 mA). Focusing time was 72 hr.

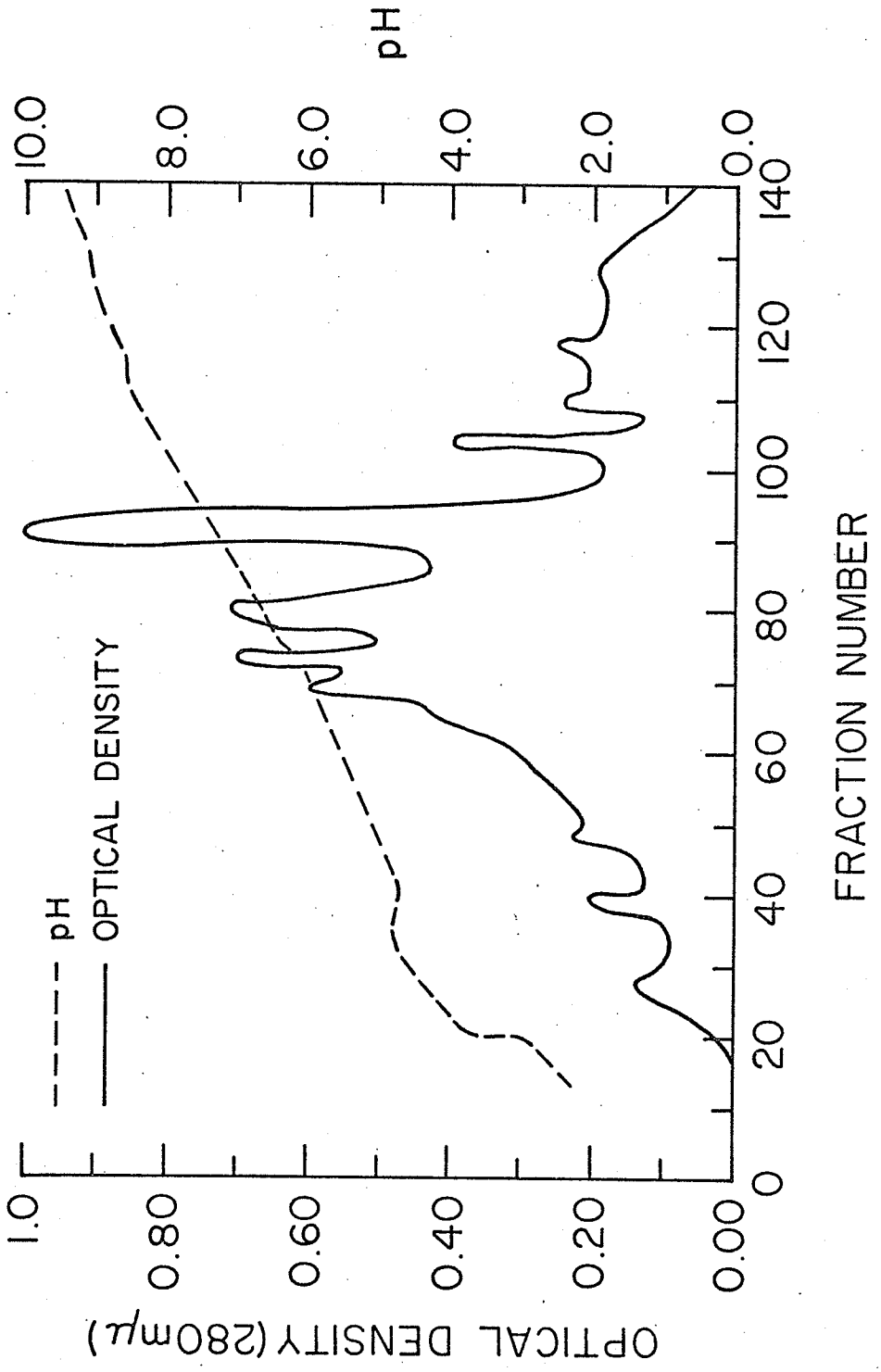
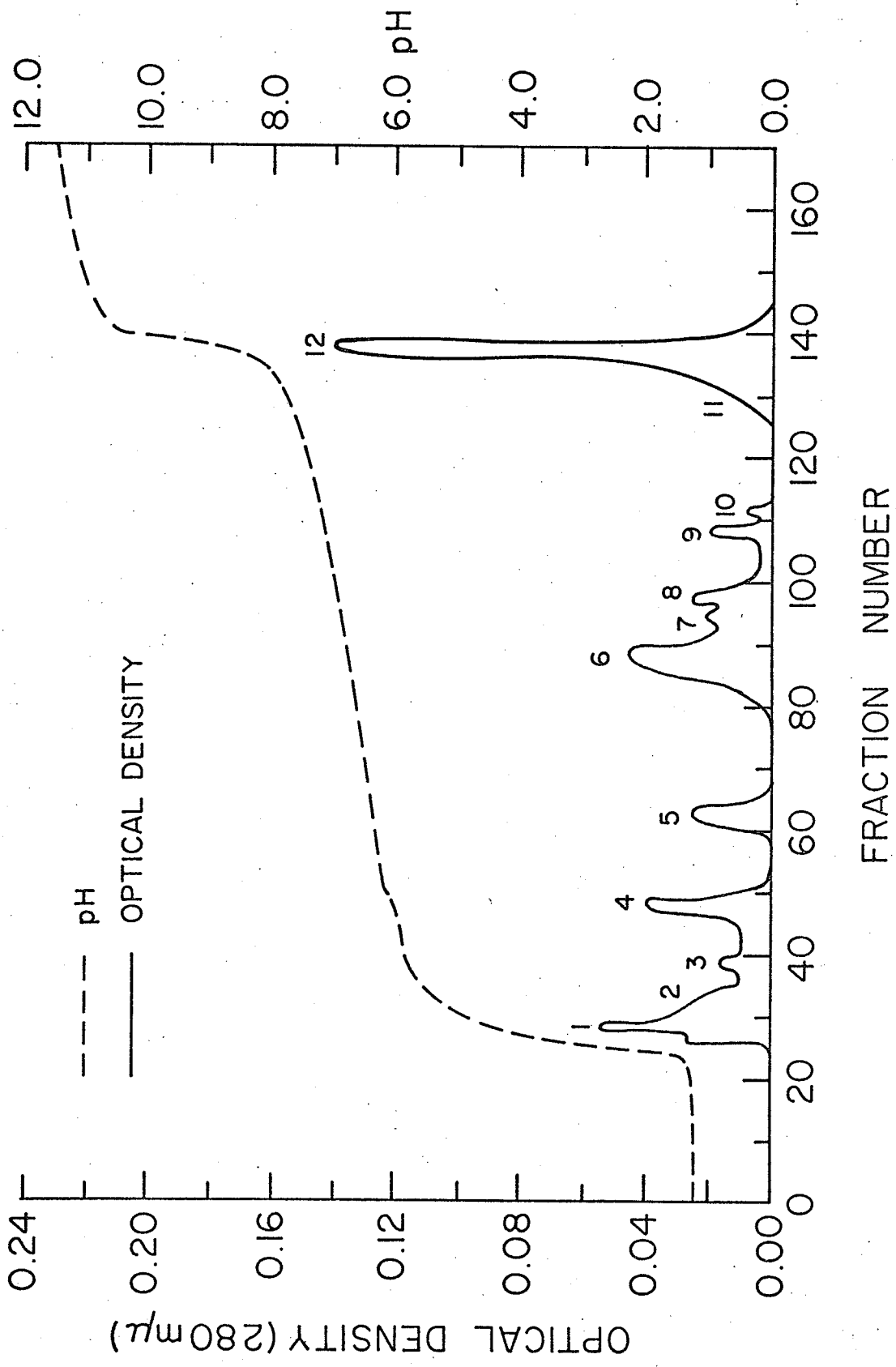

pH
10.0
8.0
6.0
4.0
2.0
0.0
140
120
100
80
60
40
20
0
FRACTION NUMBER
OPTICAL DENSITY (280mμ)
1.0
0.80
0.60
0.40
0.20
0.00
pH
OPTICAL DENSITY

Figure 43. Elution profile of peak II subunits (from gel-filtration chromatography of alkylated Manitou glutenin) from isoelectric focusing on a 6-8 pH gradient of ampholine carrier ampholytes in 6M urea. Sample size was 40 mg. A voltage of 600 volts was applied for 120 hr.


12.0
10.0
8.0
6.0 pH
4.0
2.0
0.0
0.24
0.20
0.16
0.12
0.08
0.04
0.00
0
20
40
60
80
100
120
140
160
pH
OPTICAL DENSITY
OPTICAL DENSITY (280mμ)
FRACTION NUMBER
2
3
4
5
6
7
8
9
10
11
12

(pattern J) contains only the 90,000 subunit. The homogeneity of these two subunits was checked by N-terminal amino acid analysis. The N-terminal residue of the 134,000 mol wt subunit was found to be aspartic acid while that of the 90,000 mol wt subunit was glutamic acid.

Isoelectric focusing of the high mol wt subunits of glutenin revealed some further complexities of this protein not realized before by other techniques. Peak 1, non-protein absorbing material, most probably ampholytes, showed no protein bands but streaking on SDS-gels. Peak 2 showed 2 subunits of 134,000 and 110,000 mol wt with a pI of 5.5. Peak 3 showed one subunit of 134,000 mol wt with a pI of 5.9. Peak 4 showed a 132,000 mol wt subunit and a very faint 110,000 mol wt subunit with a pI of 6.2 while peak 5 (pattern F) showed a 132,000 mol wt subunit with a pI of 6.4. Peak 6 (pattern G) and peak 7 (pattern H) showed a 132,000, a faint 110,000 and 98,000, a 90,000, and a 60,000 mol wt subunit with pI's of 6.8 and 7.0, respectively. Peak 8 (pattern I) showed very faint 132,000, 110,000, and 60,000 mol wt subunits but a brightly-stained 90,000 mol wt subunit with a pI of 7.1. Peak 9 (pattern J) showed one subunit of 90,000 mol wt with a pI of 7.2. Peak 10 (pattern K) showed extremely faint 110,000, and 60,000 mol wt subunits but a brightly-stained 90,000 mol wt subunit with a pI of 7.3. Peak 11 (pattern L) showed a 110,000, 98,000, and a faint 90,000 mol wt subunit with a pI of 8.0 while peak 12 (pattern M) showed brightly stained 110,000 and 98,000, and a faintly-stained 80,000 mol wt subunit with a pI of 8.5

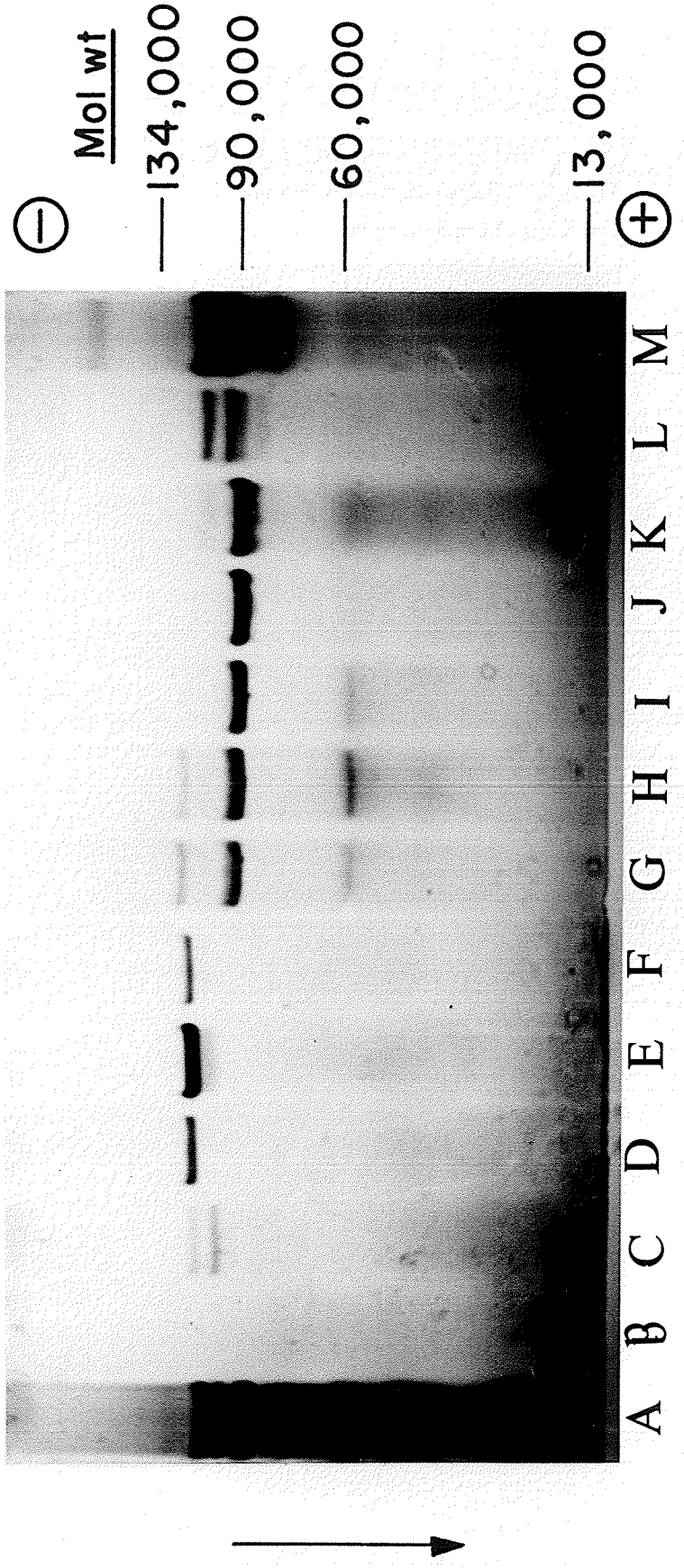
If the proteins of each peak represent different species, then

Figure 44. SDS-PAGE electrophoretograms at pH 8.9 (0.125M Tris-borate buffer) of the protein fractions from isoelectric focusing of peak II proteins (from gel-filtration chromatography):

A - Aklylated Manitou glutenin

B - M - Represent subunits from peaks 1 to 12 of Fig. 43.

Electrophoresis time was 3 hr. Staining procedure 2 was used.


⊖
Mol wt
134,000
90,000
60,000
13,000
⊕
A B C D E F G H I J K L M

there are at least 20 different subunits in peak II (from gel-filtration on Sephadex G-200). Huebner and Wall (1974) used ion-exchange chromotography to isolate the high mol wt subunits of PEC Ponca glutenin. They obtained 9 protein peaks which, when examined by starch-gel and SDS-gel electrophoresis, showed 9 different protein species. It seems that preparative isoelectric focusing can separate the highly heterogeneous high mol wt subunits of glutenin into its individual components better than the ion-exchange chromatography technique. Mita and Yonezawa (1970) subjected cyanoethyl-glutenin to isoelectric focusing on a 3-10 pH gradient of carrier ampholytes in 6M urea but they succeeded in isolating only one partially homogeneous subunit.

The possibility exists, however, that the high mol wt subunits may have been bound to the ampholytes at different pI's. This binding could result in multiple protein subunits of the same mol wt as observed for peak II subunits. However, one argument to discredit this possibility is the fact that Wrigley (1970) found approximately 40 protein species for the gliadin proteins by a combined technique of isoelectric focusing in polyacrylamide gels in one dimension and starch-gel electrophoresis in a second dimension. Nevertheless, the possibility of ampholytes binding to the subunits of glutenin should be investigated.

Figure 45 summarizes, in schematic form, the overall purification procedure for isolating two homogeneous subunits from the high mol wt subunits of glutenin based on experience in the present study.

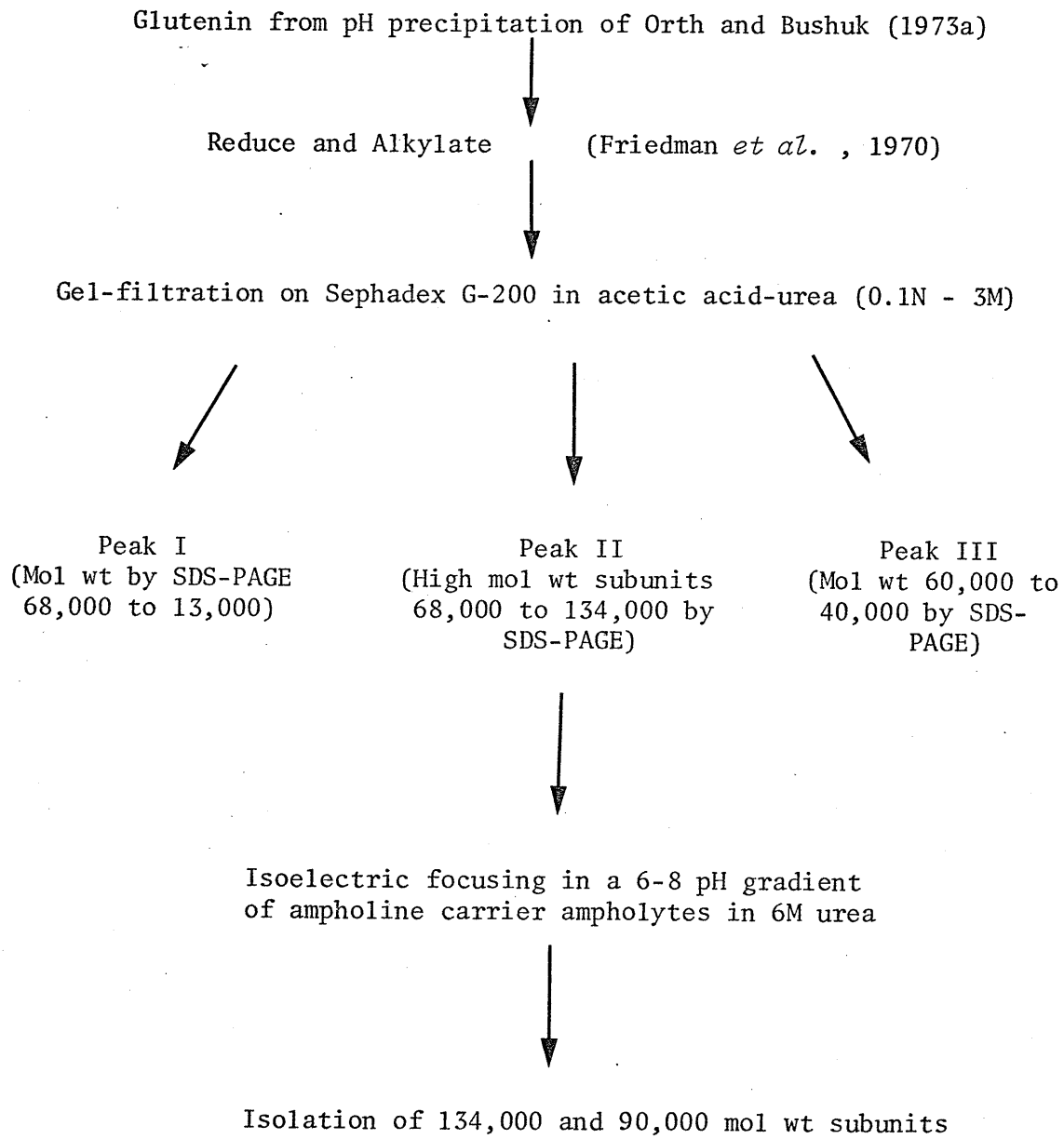

Glutenin from pH precipitation of Orth and Bushuk (1973a)
Reduce and Alkylate (Friedman *et al.*, 1970)
Gel-filtration on Sephadex G-200 in acetic acid-urea (0.1N - 3M)
Peak I
(Mol wt by SDS-PAGE
68,000 to 13,000)
Peak II
(High mol wt subunits
68,000 to 134,000 by
SDS-PAGE)
Peak III
(Mol wt 60,000 to
40,000 by SDS-
PAGE)
Isoelectric focusing in a 6-8 pH gradient
of ampholine carrier ampholytes in 6M urea
Isolation of 134,000 and 90,000 mol wt subunits

Figure 45. Schematic diagram of the overall purification procedure for isolating two high mol wt subunits of Manitou glutenin.

d. Amino Acid Composition of Fractions from Isoelectric Focusing

Table 6 shows the amino acid compositions of the subunits from isoelectric focusing of peak II fraction (from gel-filtration chromatography). Differences were obtained in the basic amino acids, lysine, histidine and arginine, and in aspartic acid. Other variations occurred in the glutamic acid, proline, and glycine contents which fluctuated from 32 to 38 mole %, 8 to 19.8 mole %, and 11 to 19.9 mole %, respectively. The phenylalanine content also showed wide fluctuations, from trace amounts to as high as 2 mole %. Variations occurred in the other amino acids but they were not as pronounced as the variations already mentioned.

Values for ammonia were not reported in Tables 4, 5, and 6 because of contamination from nitrogen containing substances such as urea and carrier ampholytes used in experiments.

TABLE 6. Amino Acid Compositions[1] of the Various Protein Fractions[2] Isolated by Isoelectric Focusing of Peak II Proteins from Gel-filtration Chromatography of PEC Manitou Glutenin

Amino acids[3]	Protein fractions									
	II	III	IV	V	VI	VII	VIII	IX	X	XII
Lysine	2.65	1.38	1.02	1.99	1.91	1.56	1.30	1.43	1.84	0.35
Histidine	0.71	Trace	0.37	0.81	0.89	1.24	1.09	1.34	1.27	0.79
Arginine	0.53	Trace	0.88	0.81	0.67	1.92	1.52	2.02	0.99	1.59
Aspartic acid	2.30	1.53	0.97	1.25	2.69	2.60	3.13	1.89	1.41	1.02
Threonine	3.36	2.76	2.83	2.52	3.26	3.04	3.13	3.23	3.39	3.18
Serine	9.82	7.36	6.12	6.22	8.44	6.85	6.78	7.52	7.21	7.28
Glutamic acid	37.43	38.80	38.49	32.42	36.45	34.55	38.02	37.35	36.63	38.39
Proline	11.50	19.90	13.31	8.51	13.84	14.59	14.27	13.78	15.63	12.95
Glycine	19.56	18.09	18.32	17.09	15.18	11.38	11.14	12.81	16.55	19.00
Alanine	3.00	2.15	2.36	2.44	3.15	3.13	2.78	2.89	2.89	2.93
Valine	1.15	Trace	1.02	0.88	2.47	3.17	3.26	3.02	2.19	1.59
Methionine	0.44	Trace	Trace	Trace	Trace	Trace	Trace	0.25	Trace	Trace
Isoleucine	1.15	0.77	0.69	0.59	2.14	3.61	2.65	2.10	1.34	0.95
Leucine	3.63	3.83	3.89	3.18	5.17	7.37	5.35	5.79	4.38	3.56
Tyrosine	3.89	3.52	4.35	3.11	2.69	2.96	2.69	3.15	3.67	5.65
Phenylalanine	0.26	Trace	0.93	Trace	1.01	2.00	1.87	1.30	0.56	0.22

[1]Expressed as mole percent.

[2]See also Fig. 43 for fraction numbers and Fig. 44 for SDS-PAGE of fractions.

[3]Cysteine and Tryptophan were not determined.

V. GENERAL DISCUSSION

The results from the present (this) study on glutenin have revealed many new physicochemical characteristics of this protein. It is therefore, necessary to re-evaluate and re-interpret its structure to function (breadmaking) relationships.

The results from this study showed that previous researchers were working with two types of glutenin, depending on their preparative procedures and research objectives. The first type was used by researchers who were investigating its technological aspects such as the role in breadmaking quality using a variety of reconstitution experiments. The glutenin(s) that these workers used were the soluble (acetic acid-soluble) and the insoluble (residue) glutenin obtained by the Osborne or similar solubility fractionation procedures. Results from these studies, which are all in general agreement, showed that the amount of soluble glutenin is negatively correlated with breadmaking quality (as expressed by loaf volume) while the insoluble glutenin (residue proteins) is positively correlated with the same parameter (Orth and Bushuk, 1972; Orth *et al.*, 1972; Shogren *et al.*, 1969). It has also been shown that flours of good baking quality wheat cultivars contain more insoluble glutenin than the flours of poor quality cultivars (Bietz and Wall, 1975; Pomeranz, 1968). Both the soluble (Orth and Bushuk, 1973b; Bietz and Wall, 1975) and the insoluble (Bietz and Wall, 1975) glutenins have been characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE), but only in the reduced state. The SDS-PAGE patterns for the reduced glutenins (soluble and insoluble) are qualitatively identical. It seems, however, that more meaningful results might be obtained by examining the soluble and insoluble glutenins by SDS-PAGE in the unreduced state. These experiments were made in the present study.

The soluble glutenin, when examined by SDS-PAGE without reducing agent, contains many prominent protein components that enter the SDS-gel. The major portion of protein remains at the point of application (origin) in the electrophoresis gel. The protein that remains at the origin is generally considered to be glutenin since it has been postulated (Ewart, 1977) that glutenin is composed of polypeptide subunits joined by inter-polypeptide disulfide bonds to form large molecules which cannot enter the 5% polyacrylamide gel. However, as has been shown in this study, the acetic acid-soluble glutenin in the unreduced state, not only contains high mol wt protein that remains at the origin, but also many protein components that enter the SDS-gel. The role of these protein components in breadmaking quality has not been investigated.

The AUC (aqueous acetic acid-urea-cetyltrimethyl ammonium bromide solvent) and guanidine hydrochloride extracts of the insoluble glutenin (residue protein), when examined in the unreduced state by SDS-PAGE, also contain many protein components that entered the SDS-gel. These components have the same mobilities as those of the analogous components of the acetic acid-soluble glutenin. The implications, in breadmaking quality, of the fraction of insoluble glutenin that enters the gel must also be addressed to.

Are the proteins in unreduced glutenin that enter SDS-gels albumin,

globulin, and gliadin contaminants that were not extracted by the salt and alcohol solutions in the preparation of the glutenin? It is well known that many proteins can become insoluble by denaturation. Denaturation can be caused by factors such as nature of solvent, pH, ionic strength, metal ions, temperature, mechanical action (e.g., stirring), to name a few. It is not possible to rule out, on the basis of electrophoretic mobility, the possibility that the protein components that enter the SDS-gels from unreduced glutenin are indeed albumin, globulin, and gliadin contaminants. This point remains to be clarified by other biochemical techniques that are available for determining the identity of proteins (e.g., immunoelectrophoresis, peptide mapping, amino acid sequencing, etc.).

Interaction, rather than denaturation, may be another explanation for the presence of contaminants in glutenin. The relative ease with which the glutenin preparations can be reproduced suggest a high degree of specificity in the interaction of various wheat flour proteins. This may be extremely important to our understanding of the functionality of the flour proteins in the breadmaking process and remains to be investigated.

The second type of glutenin used by researchers is that obtained for physicochemical characterization with the aim of obtaining information on the structure-function relationship. This glutenin has been usually obtained by purifying the acetic acid-soluble glutenin or by purifying the glutenin isolated from gluten by various procedures (Jones *et al.*, 1959; Bietz and Wall, 1972; Orth and Bushuk, 1973a). The main aim of those purification procedures was to remove low mol wt

proteins (that enter SDS-gels from the unreduced glutenin). In those studies the low mol wt proteins were considered to be contaminants (albumins, globulins, and gliadins) that tenaciously associate with the glutenin. As has already been mentioned, glutenin has been defined as the flour protein that does not enter the resolving portion of a 5% polyacrylamide gel. Bietz and Wall (1972) used the purification procedure of Jones *et al.* (1959) which involves precipitation of the glutenin from an alcohol-acetic acid solution to remove the low mol wt contaminants. In this purification procedure the low mol wt components are presumably removed in the supernatant. Orth and Bushuk (1973a), in addition to precipitation, have used an ion-exchanger, sulfoethyl-Sephadex, to remove the low mol wt contaminants. Starch-gel (Bietz and Wall, 1972) and analytical disc-gel (Orth and Bushuk, 1973a) electrophoresis showed that the low mol wt proteins were indeed removed from the glutenin by those procedures. It should be noted that the electrophoresis procedures used in those studies did not use SDS.

The results from this study show that when these so-called pure preparations of glutenin (unreduced) were subjected to SDS-PAGE, a number of low mol wt proteins entered the resolving portion of the SDS-gel. Even after a very extensive purification, there were still many protein components that entered the gel. Many of these protein bands (from unreduced glutenin) have identical mobilities to those of some subunits of reduced glutenin. It appears, therefore, that the low mol wt "contaminants" may indeed be part of the integral structure of glutenin.

One of the objectives of the research on physicochemical properties of glutenin was to obtain information on the behaviour of this protein

in vitro, in the hope that the information might be useful in explaining the functional role of this protein in dough and thereby, its influence on breadmaking quality. Another objective was to obtain information that might explain intercultivar differences in breadmaking quality. The ultimate objective of most such studies, however, is to develop a molecular model from physicochemical data that would explain the behaviour (and role) of glutenin in the breadmaking process for any wheat cultivar. It is unfortunate that one group of researchers (functionality studies) used the acetic acid-soluble and insoluble glutenin (residue), from the Osborne or related solubility procedures, without purification, while the other group (fundamental physicochemical studies) used extensively purified glutenin. This study has examined both types of glutenin in an attempt to inter-relate the findings of the two groups.

Glutenin protein is not as simple as has been previously thought. Results from this study emphasize the tremendous complexity of this protein (fraction).

For example, the present SDS-PAGE study of three hexaploid wheat cultivars and their extracted AABB tetraploids showed that the glutenin of the hexaploids contains at least 5 high mol wt subunits whereas only 4 of these subunits were reported previously (Orth and Bushuk, 1973c; Bietz *et al.*, 1975). On the other hand, this study showed that the glutenin of the hexaploid cultivar Chinese Spring (used extensively in cytogenetic studies) has only 4 high mol wt subunits. In this respect, this historic cultivar appears to be different from other hexaploid wheats.

Other complexities of the subunit structure of glutenin were found

by SDS-PAGE using buffers of different ionic strength. Certain subunits of glutenin responded differently during electrophoresis on SDS-gels with buffers of different ionic strength. This response of ionic strength may be relevant to functional behaviour of glutenin in the breadmaking process. It is well known that doughs of different cultivars respond differently to addition of a constant amount of salt. The answer to this behaviour may be in the effect of salt on the physical properties of glutenin.

Analysis by SDS-PAGE of unreduced purified glutenin from different extraction procedures revealed additional complexities of this protein. The subunits that enter SDS-gels appear to form part of the glutenin complex by strong association. Apparently this association was not disrupted by solvent action during extraction and purification of the glutenin fraction. Perhaps, the subunits from unreduced glutenin that enter SDS-gels may be analogous to the peak II proteins obtained by Dalek-Zawistowska *et al.* (1975) from the rechromatography of the glutenin (peak I) obtained by gel-filtration chromatography. If the glutenin aggregate or micelle is the "functional" unit in dough making, then the subunits that enter SDS-gels from unreduced glutenin may be extremely important in determining this functionality.

Gel-filtration chromatography, isoelectric focusing and amino acid composition analyses revealed additional complexities of the glutenin fraction. The three peaks obtained by gel-filtration chromatography of total (reduced) glutenin contained groups of different subunits with different physical (mobility in SDS gels) and chemical (amino acid composition) properties. Peak I protein comprised mainly subunits of

68,000 (by SDS-PAGE) and lower mol wts. These subunits exhibited a strong tendency to associate during gel-filtration since they always eluted with the void volume (mol wt > 200,000). Peak II proteins, on the other hand, contained subunits of the highest mol wt present in glutenin (68,000 to 134,000 by SDS-PAGE), while peak III contained those subunits that have the same mobilities as the major gliadin proteins (mol wt 45,000 and 35,000 by SDS-PAGE).

Isoelectric focusing of peak II proteins (obtained by gel-filtration chromatography) revealed further complexities of glutenin. There were at least 20 different protein components when the fractions from isoelectric focusing were examined by SDS-PAGE. The 20 components had mol wts (by SDS-PAGE) that fell in the range of 68,000 to 134,000. Many components that were different by isoelectric focusing had the same mol wt by SDS-PAGE. Obviously, many peak II subunits (components) have different amino acid compositions (hence, differences in pI), but similar mol wt.

The amino acid compositions of the 3 fractions from gel-filtration chromatography (peaks I, II, and III) confirmed differences indicated by the physical techniques (isoelectric focusing and SDS-PAGE). Peak I proteins contained more basic, acidic, and hydrophobic amino acids than peak II and III proteins. On the other hand, peak II contained very high proportions of glutamic acid (glutamine), proline, and glycine. Amino acid compositions of fractions from isoelectric focusing of peak II proteins confirmed their unique nature. The amino acid composition of peak III proteins is similar to that of gliadin.

The differences in mol wts and contents of certain amino acids from

the 3 groups of subunits (the three peaks from gel filtration) suggest that the functional roles of these proteins in breadmaking may be different. For example, the subunits of peak II proteins may form the main covalent backbone of glutenin with the proteins of peaks I and III forming the non-covalent links of the structure to maintain the integrity of the functional molecule (aggregate) in dough formation, viscosity and elasticity.

Peak II proteins merit further investigation because of their unique amino acid compositions. Complete elucidation of the involvement of these amino acids in the functionality of those proteins in breadmaking will require determination of their primary structure (amino acid sequence).

The glutamic acid (glutamine) (30 - 38%), proline (11 - 19%) and glycine (11 - 19%) contents are unusually high in peak II fraction. Glutamine is necessary for hydrogen bonding during dough formation (Wall and Beckwith, 1969). Differences in the degree of cooperative hydrogen bonding may be an important factor in the intercultivar differences in breadmaking quality. The frequency of occurrence of the glutamine residues in the polypeptide subunits of peak II proteins (and subunits from peaks I and III) would give an indication of the strength of the co-operative hydrogen bonding.

The high glycine content of peak II protein deserves comment. Glycine is present in high amounts in structural proteins such as collagen. Collagens are generally insoluble in common protein solvents because their molecules tend to aggregate. The main reason for the tendency to aggregate is that the polypeptide chain takes on a

β-conformation instead of the α-helical conformation present in soluble proteins. The polypeptide chain that is in a β-conformation is more extended than the α-helical chain. Cluskey and Wu (1966) concluded from optical rotatory dispersion studies that glutenin molecules are mainly in random coil configuration in acetic acid and aluminum lactate solvents. The high glycine content, because of its implication in the β-conformation of proteins, may be important in the functional properties of glutenin.

The proline content is also very high in peak II subunits. Proline forms a kink or bend wherever it is located in a polypeptide chain and thereby disrupts the regular secondary structure. Because of the unusually high proportion of proline, it is assumed that this amino acid may be involved in functionality of peak II proteins of glutenin.

Elucidation of the role of the three main amino acids of peak II proteins will require knowledge of the primary structure of these proteins. This remains a task for future research.

The high contents of glutamine, glycine and proline in glutenin may also be the cause of abnormal behaviour of the subunits of peak II proteins in SDS-PAGE. The section on SDS-PAGE in the Literature Review has cited examples of proteins that exhibit abnormal behaviour due to abnormal SDS-binding. Abnormal SDS-binding can result from peculiar compositions or sequences of amino acids in a polypeptide chain. Since certain glutenin subunits contain high amounts of glutamine, glycine, and proline, it is possible that these subunits may bind more or less SDS and, thereby exhibit abnormal electrophoretic mobility relative to their mol wt. The possibility of abnormal SDS-binding by glutenin sub-

units was not investigated in this study.

The solubility and electrophoretic results obtained in this study suggest a modified definition and a somewhat different interpretation of the structure, properties, and functionality of glutenin. These are presented in the discussion that follows.

Previously glutenin was defined as the protein that remained as residue after thorough extraction of the sample with salt and alcohol solutions; or the protein that was precipitated from an acetic acid solution of gluten by raising the pH to between 6.4 and 6.6 (Orth and Bushuk, 1973a; Bietz and Wall, 1972; Jones *et al.*, 1959).

SDS-PAGE results of this study indicate that the glutenin complex (aggregate or micelle) comprises two types of protein. The first type (designated as glutenin I) are proteins that are bound in the complex by strong non-covalent forces. Glutenin I proteins enter SDS-gels when a glutenin preparation in the unreduced state is subjected to SDS-PAGE. Glutenin I consists mainly of those subunits with mol wts of 68,000 and lower. The second type of glutenin proteins (designated as glutenin II) comprise subunits that are either joined by interpolypeptide disulfide bonds to form large molecules or are aggregates held by very strong specific non-covalent forces that resist disruption by the strongest of dissociating agents. Recent results of Kobrehel and Bushuk (1977) which showed that glutenin can be completely solubilized by sodium stearate or similar soaps suggest that the aggregating secondary forces may be hydrophobic. Glutenin II, if not reduced, remains at the origin of SDS-gels since it cannot enter a 5% polyacrylamide gel because of its large size. Upon reduction of its disulfide bonds, however,

glutenin II dissociates into subunits. Glutenin II consists mainly of the high mol wt subunits of 68,000 and higher.

It should be noted that not all of glutenin I enters SDS-gels from an unreduced total glutenin preparation since some of it appears to be tightly bound or trapped within compactly folded glutenin II. When the structure of glutenin II is destroyed by reduction, glutenin I components are released. Contrary to these suggestions, Bietz and Wall (1972) have provided some evidence that these subunits with mol wts below 68,000 may be covalently bound in the glutenin II complex.

Based on the physicochemical data of this study, the following descriptive molecular model of glutenin is presented to explain some of the known rheological properties of gluten and dough.

The model postulated here is based on the two types of glutenin, glutenin I (Glu I) and glutenin II (Glu II), indicated above. It is postulated that the strength of the association, or conversely, the ease of extraction, of Glu I from different cultivars is related to the functional properties of a flour[1]. Further, it is postulated that Glu I forms the mobile linkages in dough structure between the high mol wt (or particle wt) Glu II in which the subunits are joined by interpolypeptide disulfide bonds or by specific non-covalent forces.

It is further postulated that Glu II is heterogeneous in molecular (particle) size. The Glu II may be more or less compactly folded depending on its size and amount. The greater the amount and the more compact Glu II is, the lesser the amount of acetic acid-soluble proteins that can be solubilized from total glutenin and, therefore, the

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[1] The work of Arakawa *et al.*, 1977 (Agric. Biol. Chem. *41*: 995-1001) on gluten and glutenin from different cultivars supports this conclusion. The author was not aware of this publication at the time of compilation of this thesis.

greater the amount of proteins that remain in the residue. Solubility fractionations (Orth and Bushuk, 1972; Bietz and Wall, 1975) have shown that certain cultivars, classified as good breadmaking quality cultivars, have less acetic acid-soluble glutenin and more insoluble (residue) glutenin. SDS-PAGE results of this study suggest that acetic acid soluble glutenin is mainly Glu I whereas the residue protein is mainly Glu II.

The influence of the quantity and size of Glu II becomes evident during a very critical stage of the breadmaking process, dough mixing. Cultivars that show a greater proportion of Glu II will show a greater degree of elasticity. Dough of such cultivars would have longer development times and withstand greater mixing stress (have longer mixing tolerance). Glu I forms non-covalent cross links among Glu II molecules (particles). Because of the non-covalent nature of these cross linkages, they would provide for the viscous component of the rheological behaviour of total glutenin. The magnitudes of the viscous and elastic components would depend on the relative amounts of Glu I and Glu II, respectively. Rheological properties of doughs would depend on those of glutenin as modified by other flour constituents. Doughs from flours of cultivars that contain a greater proportion of Glu II will, therefore, be more elastic and, therefore, have longer development times (during mixing) and greater mixing tolerance. This hypothesis is in general agreement with the correlations obtained by Orth and Bushuk (1972) between the amounts of soluble and insoluble glutenins and the various indices of rheological behaviour.

The degree and strength of interaction of Glu I with Glu II can

also explain most other rheological properties of dough and gluten such as stress relaxation, work hardening, resistance, overmixing, "unmixing" (Kilborn and Tipples, 1975), rupture, and improver action of oxidizing agents. Since this model is purely descriptive an attempt will not be made here to justify the model in terms of the rheological properties mentioned.

The model presented above assumes minimal disulfide bond breakage (Ewart, 1977; Tanaka and Bushuk, 1973c) during dough mixing. Also sulfhydryl-disulfide interchange is not essential to the maintenance of integrity of a dough. If such interchange does occur it is minimal and probably contributes little to functional properties of glutenin.

Two sets of results from this study support the hypothesis that functional glutenin may be an aggregate (complex or micelle) of subunits held by non-covalent forces. The strength of these non-covalent forces may not be the same throughout the aggregate but may vary from relatively weak to extremely strong, the latter of which cannot be broken even with the strongest dissociating agents. The ability of the subunits to bind specifically apparently depends on the presence of disulfide bonds. These bonds would be intrapolypeptide type and would constrain the subunit to the conformation that is required for strong interaction and aggregation (Kasarda *et al.*, 1975).

The first set of results that seem to support the aggregate hypothesis are those obtained for glutenin treated with mercuric chloride ($HgCl_2$). It was shown that the aggregates can be dissociated by $HgCl_2$ but this dissociation can be reversed by EDTA, a metal chelating agent. It seems improbable that the subunits that entered the SDS-gel from

$HgCl_2$-treated glutenin would revert to an oxidized state by reformation of interpolypeptide disulfide bonds on the addition of EDTA to form long, concatenated structures as proposed by Ewart (1977). It seems more probable that when EDTA chelates the mercuric ion from its binding site on glutenin subunits, these polypeptides perhaps take up the original conformation after reformation of intrapolypeptide disulfide bonds. These constrained subunits then associate to form large aggregates which cannot enter a 5% polyacrylamide SDS-gel. It is surprising that the aggregation can occur in the presence of dissociating agents such as SDS and urea. Perhaps, the co-operative non-covalent forces are so strong that they can overcome the dissociating effects of SDS and urea. Contrary to the above interpretation of results, the work of Beckwith and Wall (1966) would suggest a more random aggregation of subunits since they found that reoxidation of glutenin could proceed to several products differing in degree of intermolecular crosslinking, depending on conditions. Obviously, further research is needed on the specific nature of the forces that cause glutenin subunits to aggregate.

The second set of results that supports the aggregate hypothesis is that from the isoelectric focusing experiments on peak II proteins from gel-filtration of PEC glutenin. Isoelectric focusing results showed that peak II proteins comprise approximately 20 different protein species. It seems unlikely that this large number of different protein species would join together by interpolypeptide disulfide bonds to form long, concatenated structures. The disulfide content (Ewart, 1972a), estimated from titration on reduction of glutenin, is too low to accommodate all of the subunits according to the modified model of Ewart (1977) which

requires four disulfides for each interior subunit. However, the cysteine content found for these high mol wt subunits by Huebner *et al.* (1974) may just be sufficient to link these subunits through interpolypeptide disulfide bonds.

In summary, the discussion of the results from this study has emphasized the complex nature of glutenin. The discussion has also emphasized the heterogeneity of the glutenin protein obtained by various preparative procedures. It was shown that the glutenin complex comprises two types of protein. From solubility and electrophoretic results, a hypothesis was developed on the formation of functional glutenin aggregate by the interaction of these two types of glutenin. This hypothesis emphasized protein interaction as an explanation for rheological properties of glutenin, gluten, and dough. Further research is needed to verify (or refute) some of the details of the postulated hypothesis. It appears that complete determination of the primary structure of the key subunits will be required before the structure to functionality relationship of glutenin can be elucidated. This could well be the next major thrust in glutenin research.

VI. CONTRIBUTIONS TO KNOWLEDGE

This study was carried out to broaden the knowledge on the physicochemical properties and the subunit composition of glutenin and to develop a procedure for the isolation of some of the subunits for further characterization. The major contributions to knowledge arising out of this investigation are:

1. The use of SDS-PAGE for determination of mol wts of the subunits of reduced glutenin requires the use of a calibration curve obtained with standard proteins in the presence of the same reducing agent used for glutenin. If the reducing agent is left out, the mol wts are overestimated.

2. SDS-PAGE at pH 7.3 and pH 8.9 of the reduced glutenin from the hexaploid wheat cultivars Prelude, Rescue, and Thatcher, and their extracted AABB tetraploids revealed that the hexaploids contain 5 high mol wt subunits (mol wts 134,000, 132,000, 110,000, 98,000, 90,000) whereas the extracted tetraploids contain only 3 of the 5 high mol wt subunits: Tetraprelude and Tetrathatcher lack the 134,000 and 90,000 subunits whereas Tetrarescue lacks the 132,000 and 90,000 subunits.

3. SDS-PAGE at pH 7.3 and pH 8.9 revealed that the historic hexaploid cultivar Chinese Spring, used extensively in cytogenetic studies, contains only 4 of the 5 high mol wt subunits present in other hexaploids. Chinese Spring lacks the 132,000 subunit.

4. SDS-PAGE with different buffers and with buffers of different ionic strength revealed that the subunits of glutenin, especially the first 5 high mol wt subunits, show differences in resolution; these differences in resolution vary among wheat cultivars.

5. Extensively purified glutenin, when electrophoresed in its unreduced state by SDS-PAGE, contained many protein components that entered the resolving portion of a 5% polyacrylamide gel.

6. The unreduced and reduced acetic acid-soluble, the mercuric chloride-soluble, and the β-mercaptoethanol-soluble glutenins from cultivars of widely different mixing and baking properties, when examined by SDS-PAGE, revealed both qualitative and quantitative differences in subunit composition that may be related to breadmaking quality.

7. SDS-PAGE revealed that glutenin treated with 0.2 mM $HgCl_2$ is partially reduced.

8. SDS-PAGE showed that reduction of glutenin with 1.0×10^{-3}M mercuric chloride can be reversed with EDTA, a metal chelating agent.

9. SDS-PAGE showed that glutenin of one wheat cultivar reduced with different chemical agents were similar in subunit patterns.

10. Gel-filtration chromatography was used to fractionate PEC glutenin from cultivars of widely different mixing and baking qualities.

11. SDS-PAGE of the various fractions from gel filtration chromatography of PEC glutenin showed qualitative and quantitative differences among the cultivars examined.

12. Amino acid compositions of the various fractions from gel-filtration chromatography of PEC glutenin showed differences among the cultivars examined.

13. Isoelectric focusing on a 6-8 pH gradient of ampholine carrier ampholytes was used to isolate two (134,000 and 90,000 mol wt) subunits in homogeneous form from the first 5 high mol wt group of subunits. The two subunits differed in amino acid composition and N-terminal amino acids.

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