I. THE ISOLATION AND CHARACTERIZATION OF A SOLUBLE WHEAT FLOUR PROTEIN II. PROTEIN CHANGES IN THE WHEAT KERNEL DURING MATURATION

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Bachelor of Science

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Stillwater, Oklahoma

1963

Submitted to the faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
July, 1967

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ACKNOWLEDGMENTS

The author gratefully acknowledges the guidance and advice by his major professor, Dr. Donald C. Abbott, during the course of this investigation and the preparation of the thesis.

Thanks are due to Dr. K. E. Ebner and Dr. G. Gorin for their stimulating discussions on protein studies. The suggestions and encouragements of Drs. R. E. Koeppe, D. R. Wilken, E. J. Eisenbraun, and G. V. Odell are sincerely appreciated.

The author would like to thank Mrs. Julia Watson for her technical help in the protein isolation studies and Miss Deretha Rainey for her assistance in the studies on developing wheat endosperm.

A very special thanks are due his wife, Sue, for her understanding and encouragement during these years of graduate studies.

Finally, he wishes to acknowledge the support of a National Defense Education Fellowship and the Oklahoma State University Biochemistry Department for facilities and financial support during these investigations.

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CHAPTER I

INTRODUCTION

The importance of wheat as a leading world staple has focused much attention on the role of flour proteins in the production of baked goods. Experience has shown that the dough properties and the quality of the loaf of bread made from a hard wheat flour are directly related to the quantity and quality of the proteins of that flour.

The earliest comprehensive investigation of wheat proteins was that done by Osborne (1) who employed various solvents to separate the proteins of wheat into five main fractions. With the impetus given by this classic study, many investigators have devoted their attention to the separation, constitution, structure, and rheological properties of the wheat proteins.

The endosperm of hard wheat flour commonly used for breadmaking contains a mixture of proteins ranging from 10 percent to 15 percent total protein. These flour proteins are complex and diverse in constitution and biological function. Gluten, the water-insoluble major fraction of flour proteins, is a mixture constituting about 80 percent of the total proteins. It is classically divided into two groups of proteins. The gliadins are prolamines which are soluble in alcohol; the glutenins are glutelins which are soluble in dilute acid and alkali and insoluble in alcohol. The elastic, cohesive properties of gluten enables it to provide a structure that holds the carbon dioxide generated

during fermentation.

The water soluble albumins and the salt soluble globulins are of interest for two principle reasons. The first is concerned with their enzymatic activity. For example, beta-amylase appears to be an albumin (2, 3), while one of the proteinases of flour may be a globulin (4). Other proteins in these groups may also prove to be enzymes which function to modify flour baking properties. The second reason for interest in the soluble proteins involves their functional properties in the baking process. It has been shown (5) that the albumins and globulins are required for maximum baking performance of all flours except durum wheat flour.

Analyses of the different classes of wheat proteins have produced some conflicting results in the literature. This may be attributed to a lack of adequate means for the separation of individual components resulting in heterogeneous protein preparations. Meaningful evaluation and comparison of physical and chemical properties of flours at the molecular level will be possible only when suitable separation and purification of the individual proteins are achieved.

A number of factors contribute to the baking quality of the mature wheat flours. Among these are weather conditions during the growth of the plant, genetic differences, soil conditions, and certain types of damage. Therefore, another approach to the understanding of basic differences among flours is the study of changes occurring in the proteins of the maturing wheat grain.

This investigation was undertaken to study wheat flour proteins by two approaches: first, by isolating and characterizing one of the water-extractable wheat proteins from a mature flour; and second, by surveying

the changes in the distribution of proteins according to their molecular weight during maturation of the wheat kernel.

CHAPTER II

LITERATURE REVIEW

The Soluble Proteins of Wheat Flour

Much of the early work on wheat proteins was carried out on the gluten proteins because of their apparent role in baking. In the last ten years, however, there has been an increase in the amount of work devoted to the study of the soluble proteins of wheat flour. This is due to two principal factors. First, it was demonstrated that the albumins and globulins were required for the maximum baking performance of a flour (5). Second, the solubility of these proteins in water or dilute salt solutions made them easier to work with than the gluten proteins.

Early work demonstrated that the albumins and globulins are highly heterogeneous. Laws and France (6) were among the first to note this heterogeneity when they demonstrated, by moving boundary electrophoresis, three protein components in the water solubles. At about the same time, Danielsson (7), in 1949, noted a molecular weight heterogeneity of globulins in wheat flour. Using the analytical ultracentrifuge, he observed two components of widely different molecular weights; one of 210,000 and one of 29,000.

Their work in the early 1950's on the effects of the soluble protein fractions on baking behavior, encouraged Pence and Elder (8) to further purify the albumins and globulins. The albumins were extracted

from flour with a dilute neutral phosphate buffer. Precipitation of the albumins between 0.4 M and 1.74 M ammonium sulfate removed gliadins and pentosans present as contaminants. Globulins were extracted from flour with one M sodium chloride and were purified by fractional precipitation with ammonium sulfate. Dialysis against distilled water precipitated the globulins, thus separating them from the albumins. Certain physical and chemical properties of these two purified fractions were determined by electrophoretic, ultracentrifugal, osmotic pressure, and chemical analyses. Moving boundry electrophoresis of the purified albumin preparation in a sodium cacodylate buffer, pH 6.0, showed it to be highly heterogeneous. Electrophoresis on filter paper in the same buffer system demonstrated the presence of at least six components. Analytical ultracentrifuge analysis indicated that these albumin components were of nearly the same molecular weight. With the globulin preparation, sedimentation patterns showed marked heterogeneity, and three components were observed.

Pence (9) further resolved the albumin fraction (prepared by the method described above) by paper electrophoresis and determined the approximate isoelectric points for eleven discernible components. He grouped these eleven components into three groups on the basis of their isoelectric points. The isoelectric points ranged from pH 4.5 to 4.8 for the four alpha group components, pH 4.9 to 5.9 for the four beta group components, and pH 6.7 to 8.7 for the three gamma group components. The relative amounts of the eleven components were estimated visually on the basis of staining intensity with a protein stain. The major components were found in the beta group, while most of the minor components occurred in the gamma group. The components of the alpha group

behaved most nearly alike in their relative migration at various pH's; whereas, the gamma group components exhibited the most diverse range of migration rates.

Perhaps the greatest stimulus to wheat protein research was the application of starch gel and polyacrylamide gel electrophoresis to wheat proteins. At present, these are the most widely used techniques for the analysis of protein extracts and the evaluation of the purity of fractions isolated by other procedures. Their sensitivity to charge and molecular size difference was first used to demonstrate the heterogeneity of flour proteins fractionated by solubility differences. Woychik et al. (10) were the first to employ the starch gel technique of Smithies (11) for this purpose. Woychik's group used an aluminum lactate buffer, pH 3.1, $\mu = 0.05$, containing three M urea. Nine electrophoretic components were observed in a water soluble fraction isolated from gluten. Elton and Ewart (12) later applied starch gel electrophoresis to purified albumins and globulins from flour. The albumin fraction, prepared by ammonium sulfate fractionation, included eight components, while the globulin fraction, prepared by ammonium sulfate fractionation and dialysis, contained three components. Kaminski (13), employing starch gel electrophoresis, showed the presence of 22 components in a distilled water extract and 23 in a salt extract of wheat flour. This is almost three times as many components as Kelley and Koenig (14) observed when they examined the composition of extracts of soft wheat flour by the use of moving boundary electrophoresis. They had reported a minimum of nine components in a 0.1 M sodium chloride extract of wheat flour. Graham (15), using vertical starch gel electrophoresis on flour extracts obtained with a variety

of solvents, demonstrated that similar protein components occurred in the various extracts. There were, however, marked differences in the proportions of the components found in the various solvents. Electrophoresis in agar gel, together with immunoelectrophoretic analysis, was used by Grabar and coworkers (16) in their study of water- and salt-soluble proteins from wheat and barley. Immunoelectrophoretic analysis revealed eight to ten protein components in wheat and 17 to 22 in barley. Of these, several were of similar mobilities but of distinct antigenic specificity. Gehrke, Oh, and Freeark (17) reported nine components in an albumin preparation obtained by repeated ammonium sulfate fractionation. They also observed seven to ten components in a globulin preparation. Four components in varying amounts were common to the albumin, globulin, and gliadin fractions. These components were termed "base" proteins.

Elton and Ewart (18) have recently reported that starch gel electrophoresis of water extracts of flour and bran from the same wheat demonstrates that bran is rich in proteins of mobilities corresponding to albumins.

The application of starch gel electrophoresis as a preparatory procedure for wheat proteins was first reported by Elton and Ewart (19). A vertical apparatus was designed with a horizontal slot across the gel, and an arrangement was included for periodic flushing of the slot with buffer in order to elute protein bands as they reached the slot during electrophoresis. Using this procedure on a water soluble protein fraction of flour they obtained two successive fractions, each of which contained the same single component. Two other fractions contained only two components. The fractions obtained in this manner were identified

with corresponding components resolved by electrophoresis of the original mixture in a separate experiment. A total of 17 components were present in the original extract. The recovery of isolated fractions from the preparatory apparatus was about 0.2 to 0.5 mg per fraction.

The advent of cellulose cation and anion exchange chromatography and gel filtration chromatography in the early 1960's provided a stimulus for renewed efforts to isolate individual "soluble" proteins, as well as the gluten components.

Coates and Simmonds (20) described the cellulose anion exchange chromatography of 0.01 M sodium pyrophosphate (pH 7) extracts of two different flours on DEAE-cellulose.[1] The separation was effected using a six mM glycine buffer, pH 9.5, at the beginning of the elution, followed by a slow linear gradient of increasing ionic strength to 0.3 M sodium chloride. Material remaining on the column was eluted in two successive fractions by the use of 0.05 M acetic acid followed by 0.1 N sodium hydroxide. Six major fractions were obtained by this procedure. Three of the fractions eluted from the column in the salt gradient were designated as peaks D, E, and F and were rechromatographed on DEAE-cellulose to yield single symmetrical peaks. When effluent fractions combined from the individual peaks were examined by moving boundry electrophoresis, a total of six components were detected. Simmonds (21) later refined this procedure to yield eight subfractions. Three of these fractions were rechromatographed on DEAE-cellulose to yield symmetrical elution patterns. These refined fractions gave symmetrical moving boundry electrophoretic patterns showing only small amounts of

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[1]Abbreviations are in accordance with the IUPAC-IUB Combined Commission on Biochemical Nomenclature, J. Biol. Chem., 241, 527 (1966).

mM phosphate buffer, pH 7.0. The proteins remaining on the CMC column were eluted with a gradient of increasing ionic strength. Those applied to the DEAE column were eluted in the same manner. Analysis of the fractions from the CMC column for acid phosphatase activity showed it to be concentrated in one peak while beta amylase activity was found in one of the peaks from the DEAE column. Carbohydrate analysis on the DEAE fractions suggested the presence of a glycoprotein in one of the peaks. Six varieties of vulgare wheat and two varieties of durum wheat were studied by these techniques. The vulgare wheat protein elution patterns were characterized by a marked intervarietal similarity. On the other hand, one buffer-soluble protein which was prominent in vulgare wheat was not found in durum wheats.

Jones et al. (25) were the first to investigate the applicability of gel filtration to the separation of wheat proteins in gluten, particularly glutenin and gliadin. Sephadex G-75, having a nominal molecular weight exclusion limit of fifty thousand, gave only partial refinement of the crude preparation. A fraction corresponding to the water soluble proteins was the last to elute from the column.

Abbott and Johnson (26) investigated the usefulness of gel filtration as a means of separating the protein components in the water extracts of flour and found the most effective combination to be Sephadex G-100 with 0.5 N lactic acid as the eluant. Starch gel electrophoresis showed that this system permitted a simplification of the protein mixture and a concentration of some components in relatively small fractions of the eluate from gel columns. Glutenins and gliadins present in the water extract were found in the fractions from the first peak. The second peak contained the components generally associated with the

water solubles.

Feillet and Bourdet (27) studied the behavior of flour albumins on Sephadex. The albumins examined were prepared by ammonium sulfate fractionation of a water extract of flour. Gel filtration of these albumins on Sephadex G-75, G-100, and G-150 gave essentially the same results for all three gel types. Four fractions were obtained, each of which represented only a partial refinement of the original preparation.

Jankiewicz and Pomeranz (28) fractionated several different types of extracts on a Sephadex G-100 column which had been calibrated by measuring the elution volumes of several proteins of known molecular weight. A five mM acetate buffer, pH 4.1, was used as eluant. One fraction obtained from a .01 M pyrophosphate buffer (pH 7) extract of flour rechromatographed as a single peak. The components in this fraction had an average molecular weight of 18,000 and migrated rapidly toward the anode at low pH during electrophoresis in polyacrylamide gel.

From the evidence reported thus far, it would appear that the isolation of individual proteins in quantities large enough to permit chemical and physical characterization must come about by the combination of two or more techniques. To date, there has been only one reported isolation of a "soluble" wheat flour protein, other than beta-amylase, in sufficient quantity and purity to permit further studies on it. This was accomplished in 1964 by Kelley (29) who isolated a salt-soluble protein from a soft wheat flour by using a combination of salting out with potassium phosphate and chromatography on DEAE-cellulose. The isolated protein was 96 percent pure as judged by moving boundry electrophoresis.

Since only a few individual proteins have been isolated, nearly

all reported physicochemical properties of soluble flour proteins pertain to the globulin or albumin classes of proteins rather than pure components. The early work in the area of physical and chemical investigation was that of Pence and Elder (8). Ultracentrifugal analysis of their isolated albumins indicated that these albumin components were of nearly the same molecular size. The single peak in 0.1 M sodium chloride had an $S_{20,w}$ value of 2.54. Osmotic pressure measurements showed the number average molecular weight of these albumins to be 28,000 in a dilute salt solution. In a dissociating medium such as 15 percent sodium salicylate or ten M urea, the molecular weight dropped to 17,000. The albumins were chemically characterized by a higher tryptophan content and a lower amide nitrogen content than the other classes of wheat proteins. The globulins were heterogeneous with respect to molecular weight. Ultracentrifugal analysis showed that a gamma-globulin ($S_{20,w}$ = 7.65) was present in the highest concentration, followed by an alpha-globulin ($S_{20,w}$ = 2.22), and a small amount of a delta-globulin ($S_{20,w}$ = 10.64). Chemically, the globulins were characterized by low tryptophan and amide-nitrogen contents and by a high arginine content. Globulin preparations containing widely different ratios of components were not significantly different in amide and tryptophan contents. However, preparations containing larger percentages of alpha globulin had significantly lower arginine contents than preparations containing less alpha globulin. It is possible that the alpha globulin fraction was actually an albumin, since its lower arginine content and its $S_{20,w}$ = 2.22 agree rather well with the chemical and physical properties observed for the albumins.

Holme (30) purified the water soluble proteins from cake flour and

demonstrated five components in the albumin fraction by moving boundry electrophoresis. Sedimentation runs in a synthetic boundary cell indicated a marked heterogeneity in the albumin fraction. Although this observation disagrees with that of Pence and Elder, the $S_{20,w}$ value calculated for the broad albumin peak was 2.1 and compares favorably with earlier values.

Coates and Simmonds (20) further studied two fractions obtained by chromatography of a dilute salt extract of flour on DEAE-cellulose. Ultracentrifugal analysis showed the presence in both fractions of two components having sedimentation coefficients of approximately 5S and 2S. The 2S component compares favorably in size to the albumin fraction of Pence and Elder (8) or to that reported by Holme (30). The heavier component appears to correspond to Pence and Elder's gamma globulin (8). Amino acid analysis of the two fractions showed that they contained a lower amide and glutamic acid content than the gluten proteins and also a higher content of arginine, aspartic acid, glycine, leucine, and tyrosine. These results also agree with those reported by Pence and Elder (8).

By chromatographing a crude extract containing essentially all of the flour proteins on a Sephadex G-200 column equilibrated with a dissociating solvent, Meredith and Wren (31) effected a separation of the proteins into three molecular weight groups. The elution volume of the smallest molecular weight group was the same as that of an albumin fraction prepared by "conventional" means. The dissociating medium employed consisted of three M urea and .01 M cetyltrimethylammonium bromide (a cationic detergent) in 0.1 M acetic acid. The column was calibrated with 15 proteins of known molecular weight so that the molecular weight

of each protein peak could be estimated. The molecular weight of the albumin fraction was 16,000 as estimated by this method. This value is in close agreement with the molecular weight observed for the albumins by Pence and Elder (8) when they employed dissociating solvents for molecular weight determination.

The soluble flour protein prepared by Kelley (29) was partially characterized physically and chemically. The sedimentation coefficient at infinite dilution for this protein was 5.95 S, while the weight average molecular weight was approximately 75,000. Calculated mobilities of the protein in buffers of different pH's yielded an isoelectric point of 6.2. The amino acid composition of Kelley's globulin differed from that of the water solubles and whole gluten as reported by Woychik et al. (32). The arginine content was higher and the ammonia (indicating amide-nitrogen) was considerably lower than those of the water solubles and whole gluten. This agrees with the results obtained by Pence and Elder (8) on the crude globulins. The isoelectric point of the protein at pH 6.2 is comparable to the value of 5.7 reported by Quensel (33) for gamma globulin of barley. The sedimentation coefficient of 7.65 S reported by Pence and Elder (8) for gamma globulins of wheat, however, is somewhat higher than the 5.95 S of Kelley's protein.

The chemical nature of the soluble protein fractions of flour and of a few purified preparations has been concerned principally with amino acid composition. One of the earlier amino acid analyses on wheat flour proteins was that of Woychik and co-workers (32). Their results have been used as a basis for comparison in subsequent work by many investigators. Soluble protein preparations isolated to date

exhibit only slight differences in their amino acid compositions. These preparations have been obtained by such diverse methods as gel filtration, cellulose ion-exchange chromatography, and ammonium sulfate fractionation. The over-all amino acid content of these soluble preparations is in general agreement with the values obtained for wheat albumins and globulins by Pence and Elder (8) and for the water solubles by Woychik et al. (32). On the other hand, there is a marked difference between the amino acid composition of the soluble proteins and the amino acid composition of the gliadins and glutenins.

Stevens and co-workers (34) examined the over-all amino acid composition of the proteins in the aleurone cells and found a marked similarity to the composition of the soluble fractions of flour. A high arginine content, which is a distinctive feature of the soluble components, was also noted in the aleurone proteins.

In addition to amino acid composition, a few results have been reported on the end group analysis of the protein components of flour. Using 1-fluoro-2,4-dinitrobenzene, Rohrlich and Schlussler (35) performed N-terminal amino acid analyses on all four protein solubility classes and found glutamic acid, glycine, alanine, valine, leucine, and histidine to be present. These, together with serine, threonine, and aspartic acid have been shown to be present in gluten and gliadin preparations of varying degrees of purity by numerous investigators (36). Simmonds (21) found serine as the only N-terminal amino acid in two fractions he obtained by DEAE column chromatography of a pyrophosphate extract of flour. No data have been reported for the C-terminal amino acids of the soluble proteins. The meaningful determination of end groups and amino acid sequences must await the preparation of more

homogeneous proteins.

Before the contribution made by the soluble wheat flour proteins can be clearly associated with specific biological functions in the kernel or with the baking performance of flours, each of these proteins must be isolated and characterized, both physically and chemically. Thus, this study was undertaken to isolate a water soluble wheat flour protein in sufficient amounts to permit further chemical and physical studies on the protein.

Protein Changes During Kernel Maturation

A different approach to the study of the proteins of wheat flour is the investigation of the wheat kernel during its development. A number of workers have taken this approach, and such varied methods as morphological studies, labeling studies, and chemical fractionation of the kernel constituents have been employed.

Buttrose (37) made some of the early electron microscope observations of developing wheat endosperms. Protein deposits appeared to be spherical to oval granules from 0.1 μ up to eight μ in diameter. These deposits usually appeared to be loosely enclosed within large sacs delimited by a single membrane and could be recognized as early as one week after fertilization. No morphological variations corresponding to heterogeneity of proteins either within a protein granule or between granules were observed.

Jennings, Morton, and Palk (38) also noted osmiophilic, electron-dense bodies within vacuolar structures in the developing wheat endosperm. It was concluded that these were predominantly composed of protein, and they were designated "protein bodies." The isolation of

these protein bodies was accomplished by density gradient centrifugation after homogenizing the endosperm in phosphate buffer containing one percent of a non-ionic detergent (39). Two fractions of protein bodies were obtained. The "small protein body" fraction contained protein bodies from 0.1 to 0.3 μ in diameter. The "protein body" fraction contained particles of 0.25 to 1.2 μ in diameter. The larger protein bodies (up to ten μ in diameter) could not be isolated in "pure" form. A marked enlargement of the protein bodies was observed during kernel development between the second and third weeks after flowering of the plant. The use of a novel fixation procedure showed that the protein bodies were localized within lipoprotein membranes. Some of the lipoprotein structure appeared to be an integral part of the protein body. Starch gel electrophoretic patterns of the protein bodies showed a predominance of the slow-moving components characteristic of the acetic acid-soluble proteins of whole endosperm. The high-speed supernatant from the isolation showed only proteins corresponding to the pyrophosphate soluble proteins. Incorporation of ^{35}S sulfate and ^{14}C glycine into the proteins indicated that the protein bodies were sites of accumulation of storage proteins of wheat endosperm.

Radioactive tracers have been employed to study flour protein changes associated with wheat endosperm development. In 1958, Bilinski and McConnell (40) injected acetate-1-^{14}C and acetate-2-^{14}C into wheat plant stems at selected stages of maturation and examined the distribution of ^{14}C in the proteins of the mature kernel. The earliest injections were at a time when the kernels were in "early dough" or "late milk" stage. Proteins were isolated according to the classical solubility scheme. Gliadins had the highest specific activity at the various

stages of maturity, followed by the glutenins. The specific activities of the albumins and globulins were similar to each other and were considerably lower than that of the gluten proteins. The gliadins showed a marked increase in specific activity when isotope was injected at successively later stages of maturity, whereas, the glutenins, albumins, and globulins showed comparatively little change in specific activity with later dates of injection. Similar measurements were made on glutamic acid isolated from the protein fractions. These results suggested that gliadins reached a maximum rate of biosynthesis at a later period than did glutenins, albumins, and globulins. More methyl carbon of acetate was incorporated into glutamic acid than carboxyl carbon, with the greatest difference in incorporation of the two carbons being shown by gliadin. Bran proteins showed markedly increasing radioactivity with later injection. This suggested that their formation occurs at still later stages of maturation.

Finlayson and McConnell (41, 42) later employed performic acid to oxidize a gliadin fraction soluble in dilute ammonium hydroxide. Five fractions were isolated from this performic acid-oxidized preparation by chromatography on DEAE-cellulose. The fractions differed from each other in chemical properties, amino acid composition, and carbon-14 content. The specific activities of glutamic acid isolated from acid hydrolysates of the five gliadin fractions were also different from each other. The glutamic acid specific activity of each fraction was also affected by the time at which tracer was administered. Two possible explanations for the results were offered. The oxidation of intramolecular disulfide linkages facilitated separation of different gliadin proteins, in which the relative efficiencies of carbon-14 incorporation

varied during kernel formation and maturation. Or, by rupturing interpeptide disulfide bonds, the oxidation produced polypeptide chains each with its own chemical properties, amino acid composition, and biosynthetic history. The fact that performic acid oxidation destroys tryptophan residues in a polypeptide chain thus breaking it at that point, was not mentioned as a possibility.

Lee and Reynolds (43) injected ^{35}S sulfate into the stems of maturing wheat and examined the distribution of ^{35}S in the mature plants. Injections were made 28, 23, 18, 13, and 8 days before harvest. The kernels appeared to be the most active site of ^{35}S accumulation, but only about one-half of the administered sulfate was utilized. Gluten was the most radioactive component of flour milled from the labeled kernels. The specific activity of the gliadins and glutenins was greater when the tracer was present in the plant for longer periods of time. The results indicated that at any stage during the growth period studied, the gliadin fraction increased in specific activity about 20 percent faster than the glutenin fraction.

Graham and Morton (44) reported a rapid uptake and incorporation of ^{35}S sulfate and ^{14}C glycine by wheat heads when they were removed from the plant at about 20 days after flowering and placed in a beaker containing a solution of the radioisotopes. After nine hours, approximately 80 percent of the total radioactivity was incorporated into the protein fractions. The total radioactivity of the acetic acid soluble proteins from the endosperm exceeded that of the pyrophosphate soluble proteins. However, the specific activity of the pyrophosphate soluble proteins based on µg of nitrogen was significantly greater than that of the acetic acid soluble proteins. This was true whether the ^{35}S sulfate

or the uniformly labeled ^{14}C glycine was used. Starch gel electrophoresis of the extracted proteins showed the slow-moving components in the acetic acid extract and the fast moving components in the pyrophosphate extract. Radioautographs of the starch gels showed a correspondence of label with dyed protein bands.

Fractionation of the proteins of maturing wheat endosperm with the subsequent study of their chemical and physical properties was probably the first means of studying kernel development. In the late 1930's results by McCalla (45) suggested that the potassium iodide-soluble material (gliadin) was the last protein fraction formed. It was further concluded that the earliest formed gluten fractions became the most insoluble portion (glutenins) of the protein in the mature kernels.

Recently, Jennings and Morton (46) found that, at about 19 days after flowering and thereafter, there was a rapid increase in the amount of acetic acid-soluble proteins per grain as compared with pyrophosphate-soluble proteins. The amino acid composition of the endosperm proteins remained relatively constant from 19 days after flowering to maturity. The interpretation derived from these results suggested a rapid accumulation of storage (acetic acid soluble) proteins at this early date. The amount of amide nitrogen per grain was shown to increase almost linearly during development. Since the gluten proteins of wheat flour have a relatively high proportion of glutamine residues, this was also taken as an indication of increasing amounts of gluten proteins.

Graham et al. (47) chromatographed sodium pyrophosphate soluble proteins on DEAE-cellulose and acetic acid soluble proteins on CMC. The chromatographic elution patterns from both types of columns remained

relatively constant for each variety throughout the period of kernel development. Only the quantity of protein in each fraction from the columns was shown to increase. It was suggested that these results indicated that the changes in protein composition are quantitative rather than qualitative. No attempt was made to correlate the acetic acid soluble proteins and sodium hydroxide soluble proteins with the gliadins or glutenins. Based on their observations of the increasing amounts of both the pyrophosphate extractable proteins and the acetic acid soluble proteins with maturity, these workers took exception to earlier evidence (46, 44, 41) that gliadin is formed at a later stage of development and independently of other protein components.

It has been shown (32, 48) that the amino acid compositions of the glutenins, gliadins, and soluble proteins are significantly different. On this basis, studies of the changes of the amino acid composition of developing wheat endosperm have been employed as a measure of protein changes. Jennings and Morton (49) found that there were considerable differences in the amino acid compositions of the fractions extracted by pyrophosphate, acetic acid, and dilute alkali. The composition of the fraction extracted by acetic acid remained relatively constant from 14 days after flowering through maturity. The amino acid composition changes that were noted in the pyrophosphate extract, e.g. glutamic, proline, lysine, were explained as being the result of the extraction of more of the gluten-type proteins with this solvent as the endosperm matured.

Jennings and Morton (49) also improved the technique for the isolation of the protein bodies and isolated a large proportion of the total protein bodies of the endosperm. The amino acid composition of

the protein bodies showed little change between 18 days after flowering and maturity. Their amino acid composition resembled the acetic acid soluble proteins. The smaller protein bodies were isolated by an earlier method (39), and their amino acid composition was determined. The composition of these bodies differed from that of the total protein body fraction. It more closely resembled the composition of the dilute sodium hydroxide soluble proteins than that of the acetic acid soluble proteins at 18 days after flowering. Comparisons were not made on more mature samples. The differences in the amino acid compositions of the proteins from the protein bodies suggested that these bodies were not necessarily uniform in composition.

Pomeranz et al. (50) determined the amino acid composition of flours from two hard red winter wheat varieties harvested at various stages of maturity. During maturation, the amount of lysine, aspartic acid, glycine, alanine, and valine decreased in the flour while glutamic acid and proline increased. Although no allowance was made for the free amino acids, the results agree with many of the earlier findings that the quantity of the gluten proteins (high in glutamic acid and proline) increases at the later stages of maturity.

Coulson and Sim (51), employing starch gel electrophoresis, studied changes in the wheat protein system, particularly endosperm proteins, during the life cycle of the plant. The proteins were extracted from the endosperm with dilute acetic acid. Fractions of low electrophoretic mobility were progressively degraded during germination and progressively synthesized towards the end of the ripening period. In contrast, fractions of higher mobility were less affected during germination and were utilized at a later stage. Similarly, these com-

ponents of higher electrophoretic mobility were synthesized first during ripening and remained at a relatively constant level during the build-up of apparently high molecular weight material.

Hoseney and co-workers (52) used three M urea in a pyrophosphate buffer, pH 7, to extract the proteins of two hard red winter wheat flours at different stages of kernel development. They reported a correlation between a decrease in the absorbance at 280 mμ and a gradual increase in molecular weight and complexity of the proteins in the extract as the kernels matured. The increase in formation of gluten proteins was accompanied by a decrease in water and salt soluble proteins and paralleled improvement in bread making properties of the flours.

Changes observed in compounds closely associated with protein synthesis, i.e. free amino acids and nucleic acids, have provided additional information related to the development of proteins in wheat. Jennings and Morton (47, 49) noted that the non-protein nitrogen formed a high proportion of the total nitrogen (about 25 percent) in the endosperm at 14 days after flowering but declined rapidly to about two percent at maturity. Free amino acids present in large amounts during early kernel development (14 days after flowering) were glutamic acid, alanine, glutamine and asparagine, serine, and aspartic acid. At maturity, the free amino acids present in largest amounts were glutamine and asparagine, glutamic acid, aspartic acid, alanine, and proline. The amounts of free glutamic acid and proline declined during the period in which incorporation of these amino acids into protein showed a marked increase. Aspartic acid and arginine increased in relative amounts during development, whereas most of the other amino acids showed relatively little change. These variations were interpreted to be a

reflection of the rate of supply of an amino acid to the pool as compared to the rate of its incorporation into protein.

Based on two dimensional paper chromatography of ninhydrin positive compounds, Coulson and Sim (51) made some qualitative observations about the changes in the free amino acids during the life cycle of the wheat plant. About ten weeks before maturity, the major ninhydrin positive constituents were glutamic acid, alanine, asparagine, glutamine, probably citrulline, and proline. Chromatographic separation of ninhydrin positive substances of mature endosperm revealed glutamic acid, asparagine, glutamine, proline, and probably citrulline as major constituents. These observations are in agreement with the results of Jennings and Morton (49).

Research on protein development in the wheat kernel has been carried out using a variety of techniques. Radioactive tracer studies, together with fractionation by solubility characteristics, have suggested a late and rapid synthesis of the gliadin proteins. Morphological studies have indicated a build up of protein bodies in the endosperm during maturation. Isolation of these protein bodies and subsequent electrophoresis on starch gel have shown the contents of the protein bodies to be the slow-migrating, gluten-type proteins. Ion exchange chromatography of the endosperm proteins indicates a quantitative build up of the various proteins rather than a qualitative change in the protein composition.

Wheat flour proteins range in molecular weights from about 16,000 for the albumins (31) to about one million for some of the glutenins (53). Thus, separation of the proteins according to their molecular weight offers an additional method to study the protein changes associ-

ated with maturation of the kernel. The second phase of this investigation, therefore, was the utilization of the gel-filtration technique to determine the molecular weight distributions of the flour proteins at various stages of maturity.

CHAPTER III

THE ISOLATION AND CHARACTERIZATION OF A WATER SOLUBLE WHEAT FLOUR PROTEIN

Experimental Procedure

Flour

Straight-grade experimentally milled flour from a composite of Hard Red Winter Wheats grown in 1963 at Stillwater, Oklahoma, was used. Protein (N x 5.7) and ash contents were 12.35 percent and 0.42 percent, respectively. Solutions of water soluble proteins for ion exchange chromatography were prepared by stirring flour with water at a ratio of 1:1.5 (W/V) at room temperature. The slurry was stirred at five minute intervals for 30 minutes, then centrifuged at 13,000 x g for 30 minutes at 2° C. The clear supernatant solution was dialyzed against two changes of ten volumes of 0.05 M acetate buffer, pH 4.6, for 24 hours.

Ion Exchange Column Chromatography

Carboxymethyl cellulose (1218 Mannex-CM, Lot 2042, Mann Research Lab., New York, N. Y.) was purified by washing in 0.05 N NaOH for five minutes followed by treatment in 0.5 N HCl for 15 minutes. It was then repeatedly washed with deionized water until no trace of acid remained. The CMC was then equilibrated with the starting buffer which was 0.05 M sodium acetate, pH 4.6. The pH of the CMC slurry was 4.6 and the conductivity was one millimho. The columns were poured from a slurry of CMC in this buffer.

Two sizes of columns were employed. Small glass columns with CMC bed dimensions of 2.2 cm x 15.0 cm were used for exploratory experiments while columns with 4.4 cm x 15.0 cm bed dimension of CMC were used for preparatory experiments.

In experiments using the small columns, 40 ml of dialyzed extract containing about 250 mg of protein as determined by microkjeldahl assay were applied to the top of the CMC bed. The walls of the column were washed twice with five ml of buffer, then 200 ml of starting buffer were passed through the column to remove any unadsorbed material. Stepwise elution of the adsorbed proteins was carried out using the following volumes of increasing concentrations of sodium chloride: 1.5 l of 0.15 M NaCl, 500 ml of 0.28 M NaCl, and 250 ml of 0.40 M NaCl.

The same conditions were used for the preparatory CMC columns except that four times the amounts of sample and eluting solutions were used. Effluent from the columns was collected by means of a Packard drop-counting fraction collector. For small columns, ten ml fractions were collected. Preparatory column fractions were 20 ml.

Gel Filtration Experiments

For preparatory gel filtration columns, cross-linked dextran gel, Sephadex G-75, was used. It was a commercial preparation obtained from Pharmacia, Uppsala, Sweden. The dimensions of the column gel bed were 2.0 cm x 70.0 cm. The lower ends of the columns were nearly flat and were equipped with two mm Leur joints to allow the fitting of a syringe needle. This needle was, in turn, connected to canular tubing which carried the effluent from the column to the fraction collector. Gels were retained in the column by a disc of Whatman # 54 filter paper. Protein solutions were placed on the column by layering under the

eluant. Either 0.5 M lactic acid or 0.02 M Tris, (pH 7.4, containing five mM $MgCl_2$) was used as the eluting buffer. Effluent from the columns was collected in three ml fractions.

For the estimation of the molecular weight and the diffusion coefficient of the isolated protein, a 1.3 cm x 100.0 cm column of Sephadex G-75 was used. The buffer employed was that described by Andrews (54). The gel column was calibrated with the following proteins: ovalbumin, MW = 45,000 (54), $D^{o}_{20,w} = 7.76 \times 10^{-7}$ (55); α-chymotrypsinogen, MW = 25,000 (54), $D^{o}_{20,w} = 9.5 \times 10^{-7}$ (56); cytochrome c, MW = 12,400 (54), $D^{o}_{20,w} = 13.0 \times 10^{-7}$ (57) (all from Sigma Chem. Co., St. Louis, Mo.); β-lactoglobulin, MW = 35,000 (54); α-lactalbumin, MW = 15,500 (54) (gifts from Dr. Kurt E. Ebner); papain, MW = 20,700 (58), $D^{o}_{20,w} = 10.23 \times 10^{-7}$ (58) (Difco Laboratories, Detroit, Mich.); bovine pancreatic ribonuclease, MW = 13,700 (54), $D^{o}_{20,w} = 11.9 \times 10^{-7}$ (59) (Mann Research Laboratories, New York, N. Y.).

Analysis of Flour Extracts and Fractions From the CMC and Sephadex Columns

Proteins appearing in the effluent from CMC and Sephadex columns were detected by measuring the absorbance at 280 and 260 mμ for each tube. A Beckman Model DU spectrophotometer equipped with a Gilson Medical Electronics transferator was used for absorbance measurements. The protein content in flour extracts placed on CMC columns was determined by a microkjeldahl procedure or by a Biuret method (60). In the CMC preparatory column runs, the correspondence of the 280 mμ absorbance with protein concentration was checked by analysis of the fractions by the method of Lowry et al. (61). Carbohydrate was determined by the anthrone procedure (62). The ionic strength gradient on the CMC

columns was followed by measuring the conductivity of the fractions on a Radiometer conductivity meter.

Electrophoresis Apparatus and Procedures

The apparatus and procedures for starch-gel electrophoresis were as previously reported by Abbott and Johnson (26). Thin gels on glass plates were used exclusively in this investigation.

Polyacrylamide gels contained 7.5 gm of Cyanogum 41 (American Cyanimid Co., New York, N. Y.) per 100 ml of buffer and were prepared in the same manner as the thin starch gels with the heat step being excluded.

Electrophoresis on Sepraphore III strips (Gelman Instrument Co., Ann Arbor, Mich.) was conducted on a Buchler Universal Electrophoresis Cell using a Buchler D.C. power supply (Buchler Instruments, Fort Lee, N. J.). Strips were soaked in buffer for four hours; the excess buffer was then blotted off, and the protein sample applied with a Gelman sample applicator.

Three buffer systems were employed for electrophoresis. An eight mM aluminum lactate - three M urea buffer, pH 3.3 (11) was used for the analysis of the column fractions by starch gel electrophoresis. Two additional buffers were used for the electrophoretic examination of the isolated protein. One was a 20 mM sodium cacodylate-HCl buffer, pH 6.1. The third buffer contained 15 mM Tris, three mM citric acid, eight mM boric acid, and 0.5 M urea. Sufficient sodium hydroxide was added to give a final pH of 8.6.

Protein bands in the gels were detected by staining with one of three stains: a saturated solution of Buffalo Black NBR (Amido Black 10B) (Allied Chemical Corp., New York, N. Y.) in five percent acetic

acid for 30 minutes, 0.007 percent Nigrosine (Allied Chemical Corp., New York, N. Y.) in two percent trichloroacetic acid for 20 hours, or 0.2 percent Ponceau S (Allied Chemical Corp., New York, N. Y.) in five percent trichloroacetic acid for two hours. Excess stain was removed by allowing the gels to stand in a five percent acetic acid wash solution for about 48 hours with occasional agitation. The solvent was changed occasionally, and washing was continued until the gels were suitable for photography. The Sepraphore III strips were stained in the Ponceau S stain for 15 minutes. Washing of the strips was accomplished by passing the stained strips through a bath of five percent acetic acid.

Photography of Starch Gels

The stained starch gels were mounted on glass plates and photographed by transmitted light. The plates were placed on the opal glass cover of a light box equipped with twelve 50-watt bulbs. An enlarger equipped with film holders was used as a camera. Panatomic X film was employed, and a Wratten Series A (red) filter used on the camera to increase the contrast. Film was developed in DK-50 developer while prints were developed in Dektol.

Amino Acid Analysis

Amino acid analyses were carried out on a Beckman model 120 C automatic amino acid analyzer according to the method of Moore and Stein (63). Samples were hydrolyzed at 110° C for 12, 24, 48, and 72 hour periods in sealed evacuated tubes by placing the sealed tubes in refluxing toluene. Hydrolysis was incomplete in 12 hours and resulted in low values for all amino acids. The values for threonine, serine, and half-cystine were determined by extrapolation of the data to zero time of hydrolysis. No significant change with time in the values for

the other amino acids was observed. Half-cystine was also determined by oxidizing the cystine residues to cysteic acid by the performic acid oxidation procedure of Schram, Moore, and Bigwood (64). Nitrogen recovery after chromatography of the hydrolysates was about 98 percent. Tryptophan content and the ratio of tyrosine to tryptophan were determined by the spectrophotometric method of Goodwin and Morton (65) as outlined by Beaven and Holiday (66) using a Cary 14 recording spectrophotometer. The value for ammonia was obtained by subtracting losses in serine, threonine, and half-cystine after 24 hours hydrolysis, together with twice the tryptophan content, from the observed ammonia value at 24 hours.

Free sulfhydryl groups were determined by the spectrophotometric method of Boyer (67) according to the procedure of Fraenkel-Conrat (68). Ovalbumin was run at the same time as a check. Samples were assayed in buffer with and without 0.6 percent sodium lauryl sulfate.

Reduction and Alkylation of Disulfide Bonds

Reduction of the disulfide bonds was performed at pH 8.0 in 0.05 M Tris buffer containing five M guanidine hydrochloride. Five mg (about 0.25 μmoles) of protein and 40 μmoles of dithiothreitol (Calbiochem, Los Angeles, Calif.) were added to one ml of solvent and incubated at 25° C for two hours.

A ten percent molar excess (over reagent -SH) of iodoacetamide (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M Tris, pH 7.4, was added in equal volume to the solution of reduced protein. The pH was adjusted to 8.0 with dilute base, and the reaction mixture allowed to incubate one hour.

Carboxy- and Amino-terminal Amino Acid Determinations

The fluorodinitrobenzene method for amino end group analysis was carried out according to the method of Fraenkel-Conrat et al. (69). The dinitrophenylamino acid derivatives were identified by two dimensional paper chromatography by comparison with known derivatives.

The Edman phenylisothiocyanate method for step-wise degradation from the amino end was done by the paper strip technique (70). Egg white lysozyme (Nutritional Biochemicals Corporation, Cleveland, Ohio) was degraded at the same time as a means of checking techniques. The phenylthiohydantoins of the amino acids were identified by one dimensional paper chromatography according to the method of Sjöquist (71). Standards were synthesized from the free amino acids and twice recrystallized (70).

For carboxyl-terminal studies, Carboxypeptidase A treated with diisopropylfluorophosphate was used at a one to fifteen or a one to fifty molar ratio of enzyme to substrate. The protein substrate was suspended in cold five percent trichloroacetic acid (one mg protein/100 µl) and centrifuged down to remove free amino acids or polypeptide impurities. The protein substrate was then treated with carboxypeptidase at pH 8.0 and 25° C. Aliquots were removed at intervals during an eight hour incubation period. The addition of trichloroacetic acid to a final concentration of five percent served to terminate the reaction as well as to precipitate residual protein, which was then removed by centrifugation. The supernatant solutions were then spotted on Whatman #1 chromatography paper. The descending chromatogram was developed with the upper phase of an n-butanol-acetic acid-water (4:1:5) mixture.

Results and Discussion

Preliminary CMC Chromatography Studies

Abbott and Johnson (26) employed Sephadex G-100 to resolve the complex mixture of water soluble flour proteins into considerably less heterogeneous fractions. One component, appearing as the most heavily stained band in a water extract of flour subjected to starch gel electrophoresis, was concentrated in one of the fractions. Using a preparative starch gel electrophoresis technique, they isolated small amounts of this protein from the fraction. It appeared that a different method of separation based on charge differences might be successfully applied to the isolation of this protein from the G-100 fraction. Therefore, a study of the use of ion-exchange chromatography on CMC was undertaken for this purpose.

In preliminary experiments, the fraction prepared according to the procedure of Abbott and Johnson (26), was chromatographed on a CMC column. Figure 1 shows a typical elution pattern together with a description of the experimental conditions. The starch gel electrophoresis patterns of the fractions from this column are shown in Figure 2. The first peak contained material which was not adsorbed on the column and no bands were observed upon starch gel electrophoresis. The fractions eluted by the 0.1 M NaCl contained a portion of nearly all the proteins. The linear gradient from 0.1 to 0.18 M NaCl yielded two peaks. The first of these peaks appeared to contain about equal amounts of three proteins, one of them being the protein of interest. The second peak contained predominantly this major soluble protein. There was insufficient material in the remaining fractions to be visible on starch gel.

Although ion exchange chromatography had further purified the

Figure 1. CMC Column Chromatography of the Water Solubles Fraction From a Sephadex G-100 Column

Two hundred fifteen mg of the protein fraction from the Sephadex G-100 column were dissolved in five ml of 0.05 M acetate buffer, pH 4.6, and applied to a CMC column (2.2 x 8 cm) previously equilibrated with the buffer. After washing the column with 75 ml of the same buffer, the proteins remaining on the column were eluted with a series of continuous and discontinuous gradients, (-----), from 0.00 to 0.28 M NaCl. (———), protein distribution in the eluate fractions.

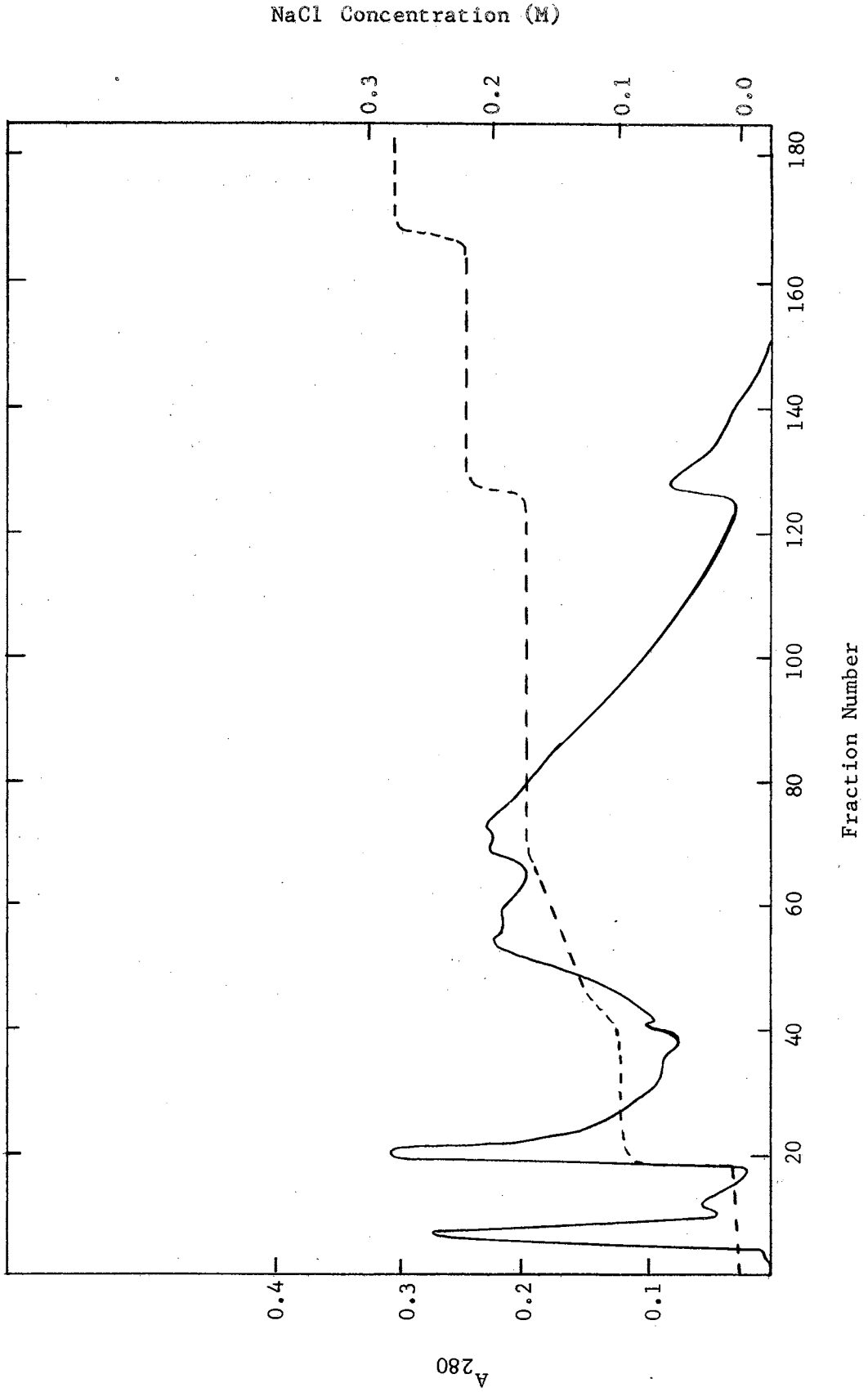
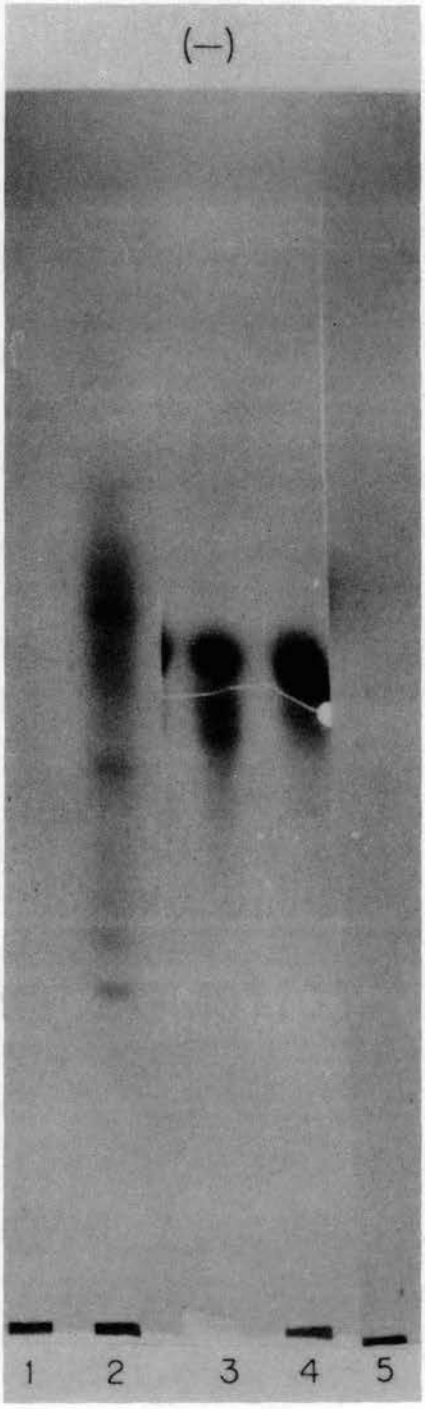

NaCl Concentration (M)
0.3
0.2
0.1
0.0
180
160
140
120
100
80
60
40
20
Fraction Number
0.4
0.3
0.2
0.1
A_{280}

Figure 2. Starch Gel Electrophoresis of Fractions From CMC Chromatography of a Sephadex G-100 Fraction

Electrophoresis was for 15 hours at six V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B in five percent acetic acid.

1. Fractions # 6 - # 16
2. Fractions # 20 - # 36
3. Fractions # 40 - # 64
4. Fractions # 65 - # 117
5. Fractions # 127 - # 150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5

fraction from the Sephadex column, the size of a Sephadex G-100 column that would be required to prepare this starting protein fraction in large quantity and the time required to make such a run were prohibitive. It did not appear that this problem could be solved by utilizing the faster flow rate of Sephadex G-75 since Abbott and Johnson (26) had shown that this gel was inferior to G-100 in resolving the proteins of a crude water extract of flour. However, other workers have reported the use of cellulose ion exchange columns to partially resolve crude preparations of wheat flour proteins (20, 23). Thus, an attempt was made to prepare a fraction rich in the protein of interest by CMC chromatography of a water extract of flour without a preliminary G-100 treatment. The crude extract was applied to the column and all unadsorbed material eluted from the column with buffer. This was followed by a series of continuous and discontinuous gradients of increasing ionic strength. A typical elution pattern from a column of this type is shown in Figure 3, and its corresponding starch gel is shown in Figure 4. When material eluted by the buffer was subjected to starch gel electrophoresis, no proteins were detected. The fractions eluted between 0.00 and 0.18 M NaCl contained mainly the slow moving components. Although it appeared that some of the protein of interest was eluted from the column at lower ionic strengths, most of it appeared in the first peak of the gradient from 0.18 to 0.4 M NaCl. The peak tube in this fraction eluted at a sodium chloride concentration of about 0.28 M.

Since it was apparent that some concentration and purification of the desired protein had been effected by use of CMC chromatography, further experiments were conducted to improve the purification, if

Figure 3. CMC Column Chromatography of a Water Extract of Flour

The extract (40 ml) which contained 230 mg of protein (N x 5.7) was dialyzed against 0.05 M acetate buffer, pH 4.6, overnight and applied to a CMC column (2.2 x 15 cm) previously equilibrated with the buffer. After washing the column with 150 ml of buffer, the proteins were eluted by a series of stepwise and linear gradients (-----) of NaCl from 0.1 M to 0.6 M. (———), protein distribution in eluate fractions as measured by the absorbance at 280 mμ.

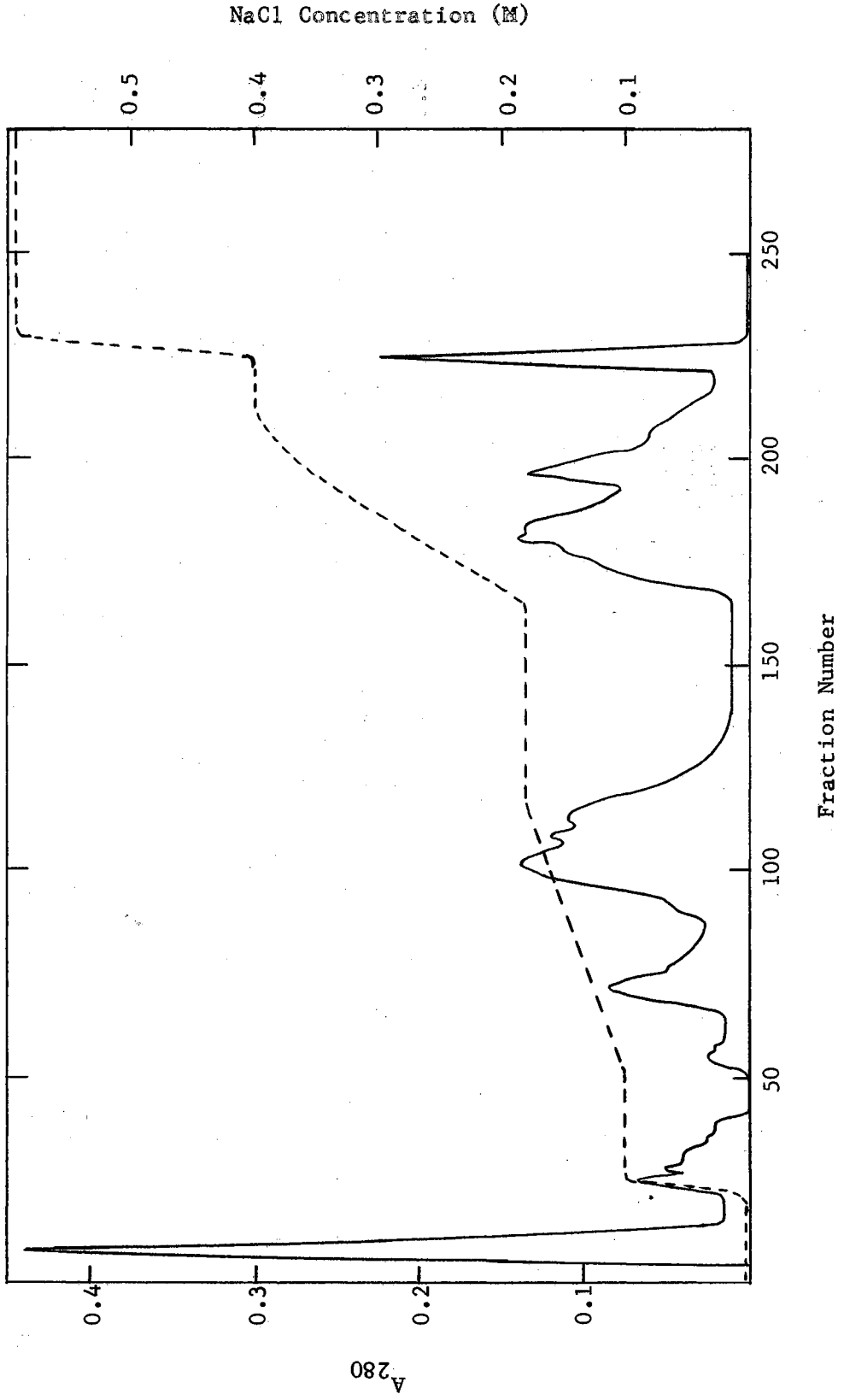
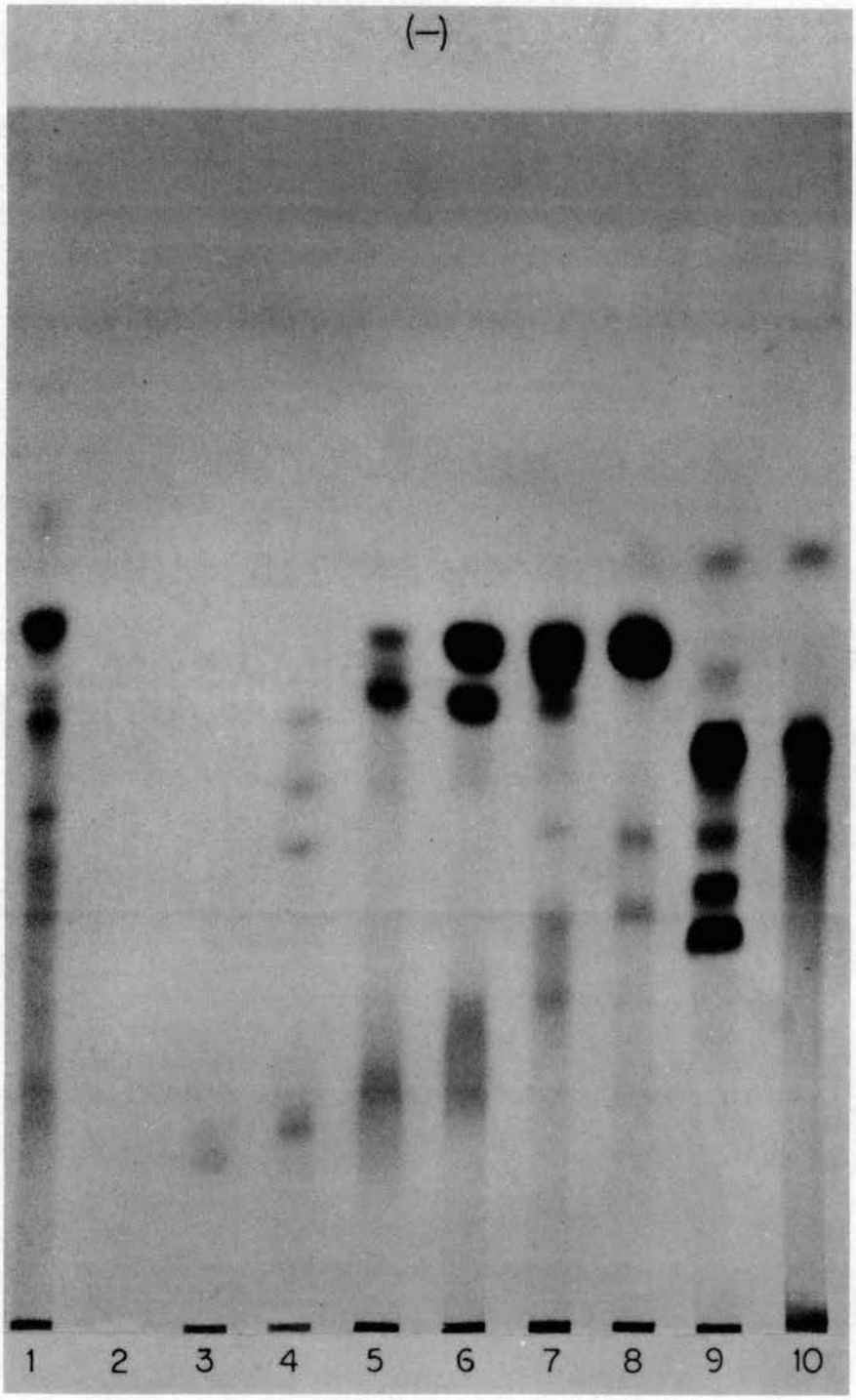

NaCl Concentration (M)
0.5
0.4
0.3
0.2
0.1
250
200
150
100
50
Fraction Number
0.4
0.3
0.2
0.1
A_{280}

Figure 4. Starch Gel Electrophoresis of Fractions From CMC Chromatography of a Water Extract of Flour

Electrophoresis was for 15 hours at six V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B in five percent acetic acid.

1. Water extract
2. Fractions # 6 - # 12
3. Fractions # 20 - # 40
4. Fractions # 50 - # 65
5. Fractions # 65 - # 85
6. Fractions # 90 - # 105
7. Fractions # 106 - # 125
8. Fractions # 170 - # 193
9. Fractions # 194 - # 215
10. Fractions # 220 - # 230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10

possible. A discontinuous, or stepwise, elution schedule, based on previous results, was investigated with typical results shown in Figures 5 and 6. After washing unadsorbed material through the column with buffer, 0.15 M NaCl was passed through the column until no further protein could be detected as measured by the absorbance at 280 mμ. Proteins remaining on the column were eluted by successive 0.28 and 0.40 M NaCl steps. As in the previous experiment, the protein of concern was found predominantly in the fraction eluted with 0.28 M NaCl. A small amount of this protein was also eluted in the preceding peak together with another protein of slightly slower migration rate. This second protein was always found associated with the protein of interest, and eluting with 0.15 M NaCl until the broad, plateau-shaped region (Figure 5, elution volume from 900 ml to 1400 ml) appeared was necessary to separate the contaminating protein from the major portion of the protein being isolated. No further work was done to determine if these two proteins might associate or whether the charges on the two proteins just happened to be very similar. Thirty milligrams of material (essentially 100 percent protein by the Lowry protein assay on lyophilized material) were obtained from the fraction eluted with 0.28 M NaCl when 200 milligrams of protein (N x 5.7) were applied to a column. Rechromatography of the material in this fraction with the same elution procedure yielded a single peak which eluted with the 0.28 M NaCl step.

Efforts to further improve the separation of components by the use of various types of continuous gradients between 0.15 M and 0.28 M NaCl were unsuccessful. Therefore, the CMC fractionation procedure employing the discontinuous elution system was scaled up for use in preparatory work.

Figure 5. CMC Column Chromatography of a Water Extract of Flour Eluted by a Discontinuous Gradient

The extract (40 ml) which contained 210 mg of protein (N x 5.7) was dialyzed against 0.05 M acetate buffer, pH 4.6, for 24 hours and applied to a CMC column (2.2 x 15 cm) previously equilibrated with the buffer. Proteins were eluted from the column by a stepwise gradient (— - —) from 0.0 to 0.4 M NaCl. (———), absorbance of each fraction at 280 mμ. (- - - - -), absorbance of each fraction at 260 mμ.

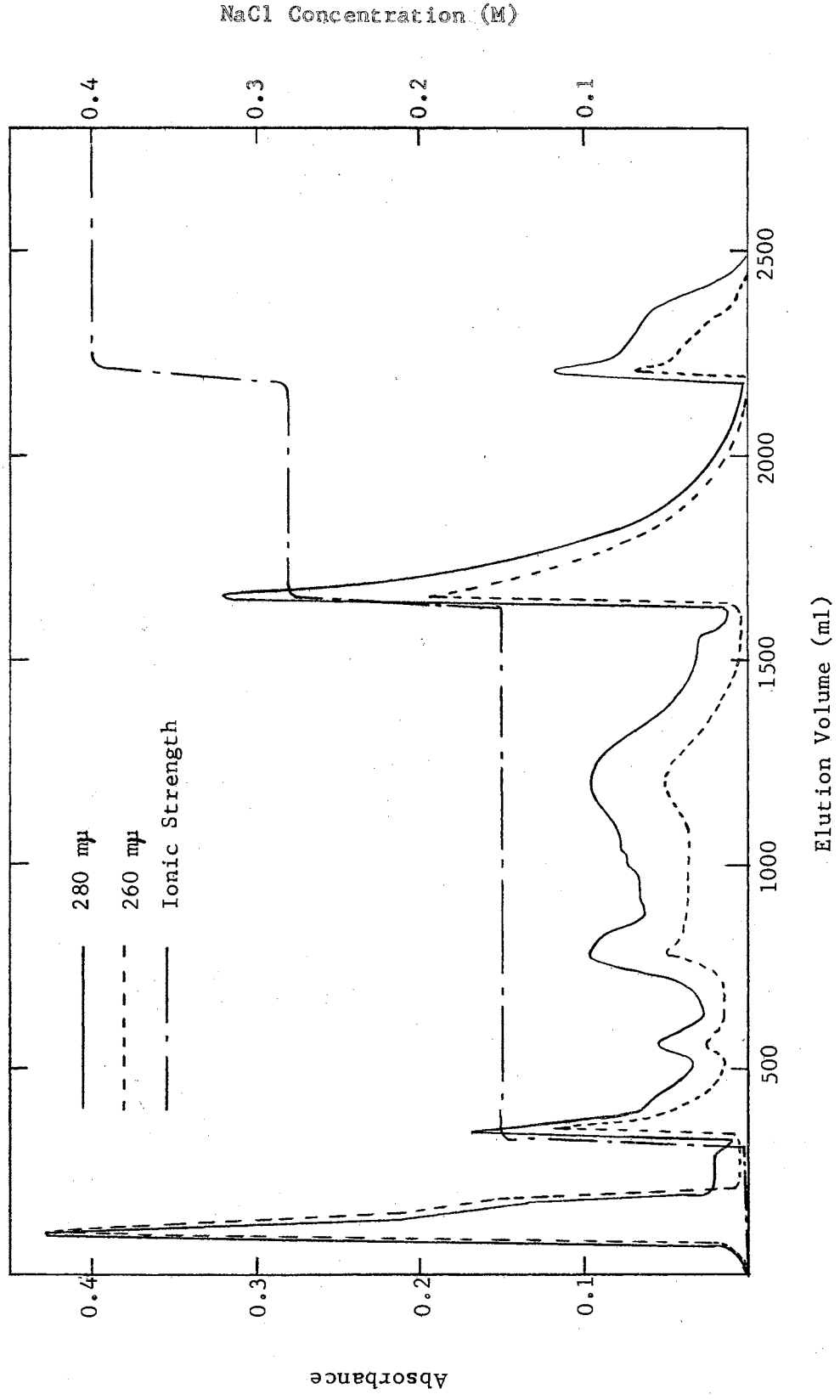
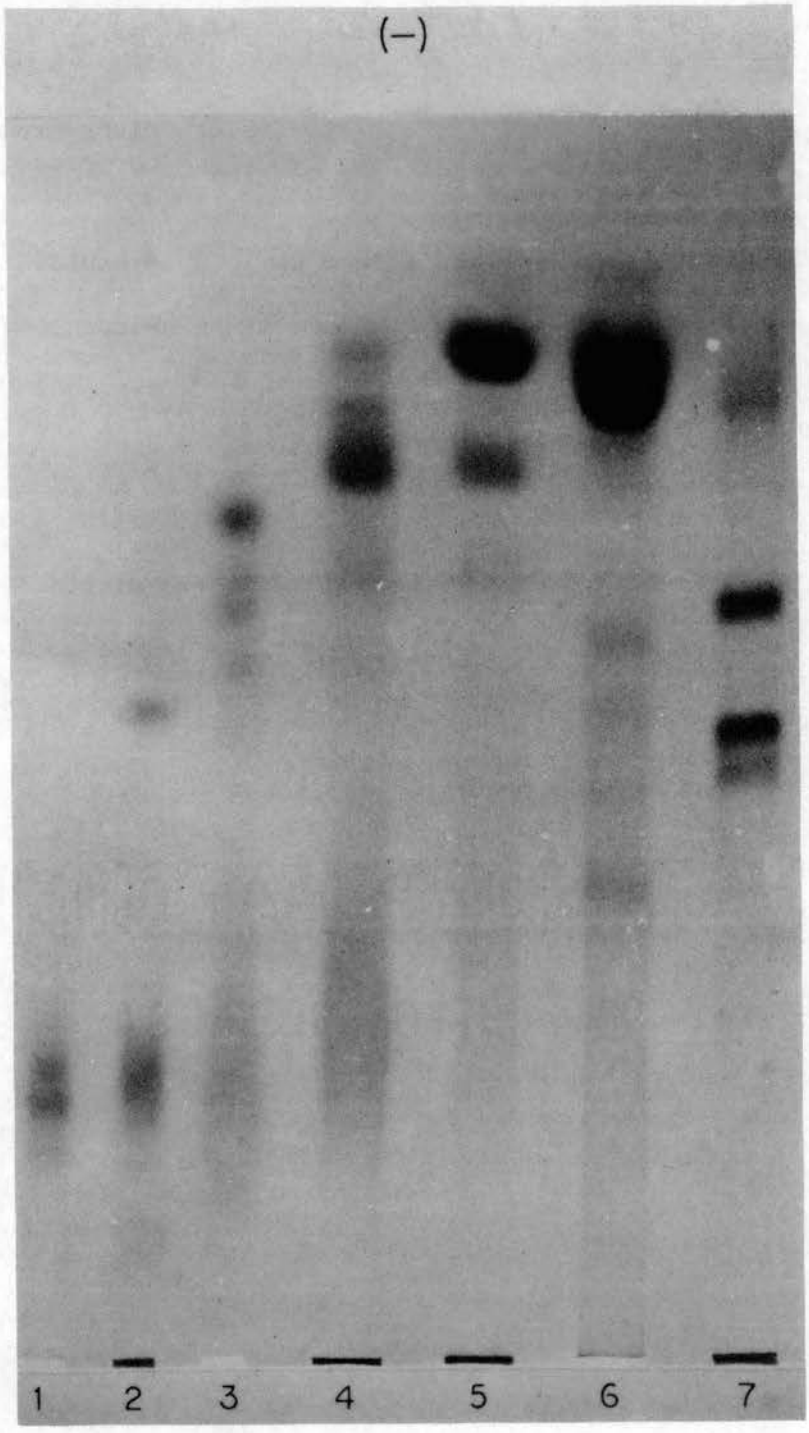

NaCl Concentration (M)
0.4
0.3
0.2
0.1
2500
2000
1500
1000
500
280 mμ
260 mμ
Ionic Strength
Absorbance
Elution Volume (ml)

Figure 6. Starch Gel Electrophoresis of Fractions From a Stepwise Elution of Water Soluble Flour Proteins From CMC

About 700 to 900 µg sample was applied to each wick. Electrophoresis was for 17 hours at five V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B in five percent acetic acid.

1. Fractions from elution volume of 320 ml to 470 ml
2. Fractions from elution volume of 520 ml to 640 ml
3. Fractions from elution volume of 710 ml to 860 ml
4. Fractions from elution volume of 890 ml to 1100 ml
5. Fractions from elution volume of 1120 ml to 1400 ml
6. Fractions from elution volume of 1630 ml to 1900 ml
7. Fractions from elution volume of 2190 ml to 2350 ml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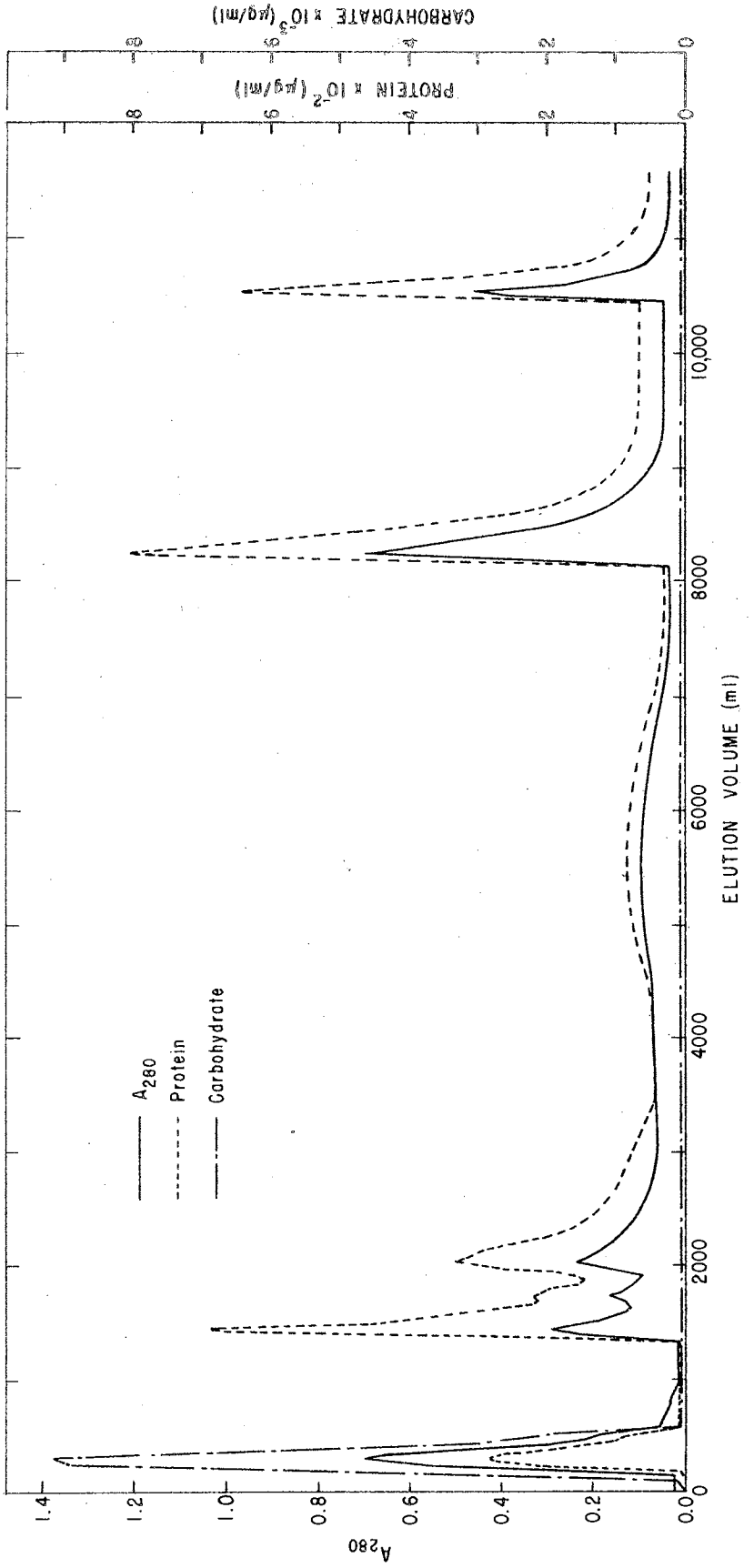
Preparative CMC Column Chromatography

A column of twice the diameter as the column used in the preliminary experiments was employed; this permitted a four-fold increase in the amount of protein applied to a column. The elution pattern from the large column was very similar to that of the smaller column as measured by the absorbance at 280 mμ.

Since the elution patterns of the columns had been monitored only by measuring the absorbance of each fraction at 280 and 260 mμ, it was of interest to determine the protein and carbohydrate content of each fraction. Protein assays by the Lowry procedure (61) and carbohydrate assays by the anthrone method (62) were performed on each tube from a large CMC column. The results, together with the absorbance readings at 280 mμ, are shown in Figure 7. These data show that, indeed, each absorbance peak contained protein. All 280 mμ absorbance peaks, except the first one, contained large amounts of protein and essentially no carbohydrate. The first peak, however, contained about 40 mg of carbohydrate for each mg of protein. This would not permit enough protein to be placed on a starch gel to yield visible dyed protein bands after electrophoresis. The material in the first peak also gave a positive carbohydrate test by the Molish test and a positive test for pentose sugars by the Bial's procedure. Coates and Simmonds (20) isolated a fraction which passed unretarded through both a DEAE-cellulose column and a CMC column. The fraction contained both carbohydrate and protein, and yielded arabinose and xylose upon acid hydrolysis. They suggested that this material corresponded to the pentosan fraction of wheat flour. These preliminary results indicate that the unadsorbed material from the CMC column corresponds to the pentosan fraction of Coates and Simmonds.

Figure 7. Preparative CMC Column Chromatography of a Water Extract of Flour

One gram of protein (N x 5.7) in 190 ml of extract was dialyzed against 0.05 M acetate buffer, pH 4.6, for 36 hours and applied to a CMC column (4.4 x 15 cm) previously equilibrated with acetate buffer. Proteins were eluted from the column by a discontinuous gradient of NaCl from 0.0 M to 0.4 M. (———) absorbance of each fraction at 280 mμ. (- - - - -) protein in ug/ml in each fraction as measured by the Lowry method. (— - —) carbohydrate in μg/ml in each fraction as measured by the anthrone procedure.


A280
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2000
4000
6000
8000
10,000
ELUTION VOLUME (ml)
A280
Protein
Carbohydrate
PROTEIN x 10-2 (μg/ml)
CARBOHYDRATE x 10-3 (μg/ml)

After analysis for carbohydrate and protein, the tubes from the column were pooled into appropriate fractions, dialyzed to remove the NaCl, and lyophilized. The fractions were then examined by starch gel electrophoresis, with the results shown in Figure 8. Both Amido Black and Ponceau S stains revealed the same protein bands in a fraction with corresponding relative intensities. The protein of interest again was found in the fraction eluted with 0.28 M NaCl (elution volume 8160 ml to 8900 ml).

Fractionation by Gel Filtration of Protein Eluted From CMC by 0.28 M NaCl

Earlier work by other groups indicated that gel filtration on Sephadex G-75 did not effect a clean separation of albumin and gluten proteins in a crude water extract (25, 26). However, it appeared that such a separation might be possible with a more refined starting material since the molecular weights reported for wheat flour albumins have been from 15,000 (31) to 28,000 (8), and Sephadex G-75 has a nominal exclusion limit of fifty thousand. Therefore, a solution (ten mg/ml) of the protein which eluted from a CMC column in 0.28 M NaCl was placed on a Sephadex G-75 column and eluted with 0.5 N lactic acid. The elution pattern for a typical experiment is shown in Figure 9, and the starch gel electrophoresis pattern of the appropriately pooled fractions is shown in Figure 10. Proteins in the first peak were totally excluded from the gel matrix and were eluted from the column in the void volume. Their migration rates in starch gel were similar to those of the gliadin proteins. The second peak contained the protein of interest which appeared to be nearly homogeneous electrophoretically. Occasionally, one or two faint protein bands were observed when large amounts of some

Figure 8. Starch Gel Electrophoresis of Fractions From a Preparative CMC Column

From 300 to 800 µg of each sample were applied to each wick. Electrophoresis was for 20 hours at five V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B in five percent acetic acid.

1. Fractions from elution volume of 1400 ml to 1460 ml
2. Fractions from elution volume of 1700 ml to 1760 ml
3. Fractions from elution volume of 2000 ml to 2080 ml
4. Fractions from elution volume of 5340 ml to 5520 ml
5. Fractions from elution volume of 8160 ml to 8900 ml
6. Fractions from elution volume of 10,460 ml to 10,800 ml

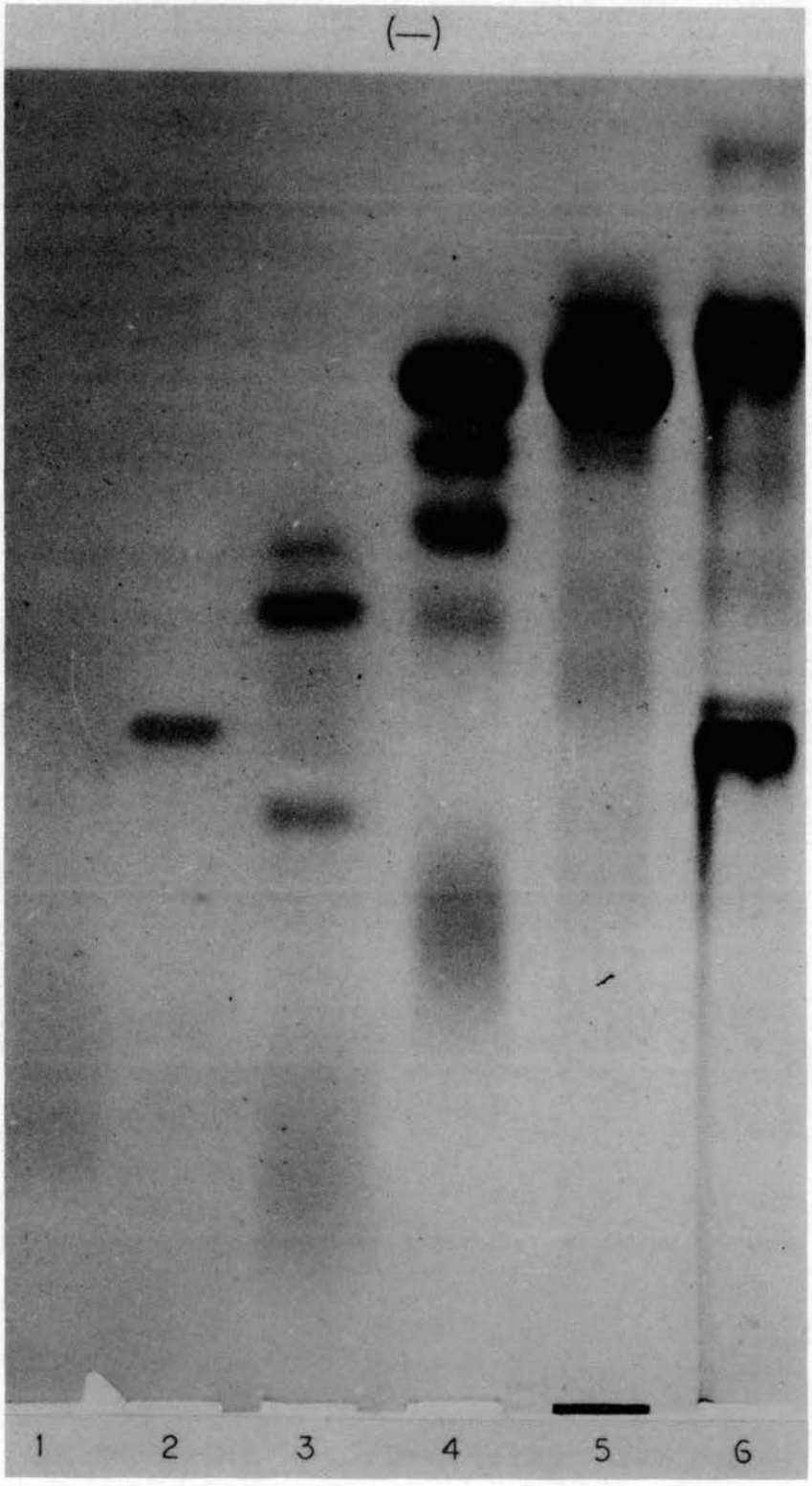

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Figure 9. Gel Filtration on Sephadex G-75 of the Protein Eluted From CMC by 0.28 M NaCl

Thirty mg of protein from the CMC preparation were dissolved in three ml of 0.5 N lactic acid and applied to a 2 x 70 cm G-75 column. Protein was eluted with 0.5 N lactic acid. O, protein distribution in the eluate fractions.

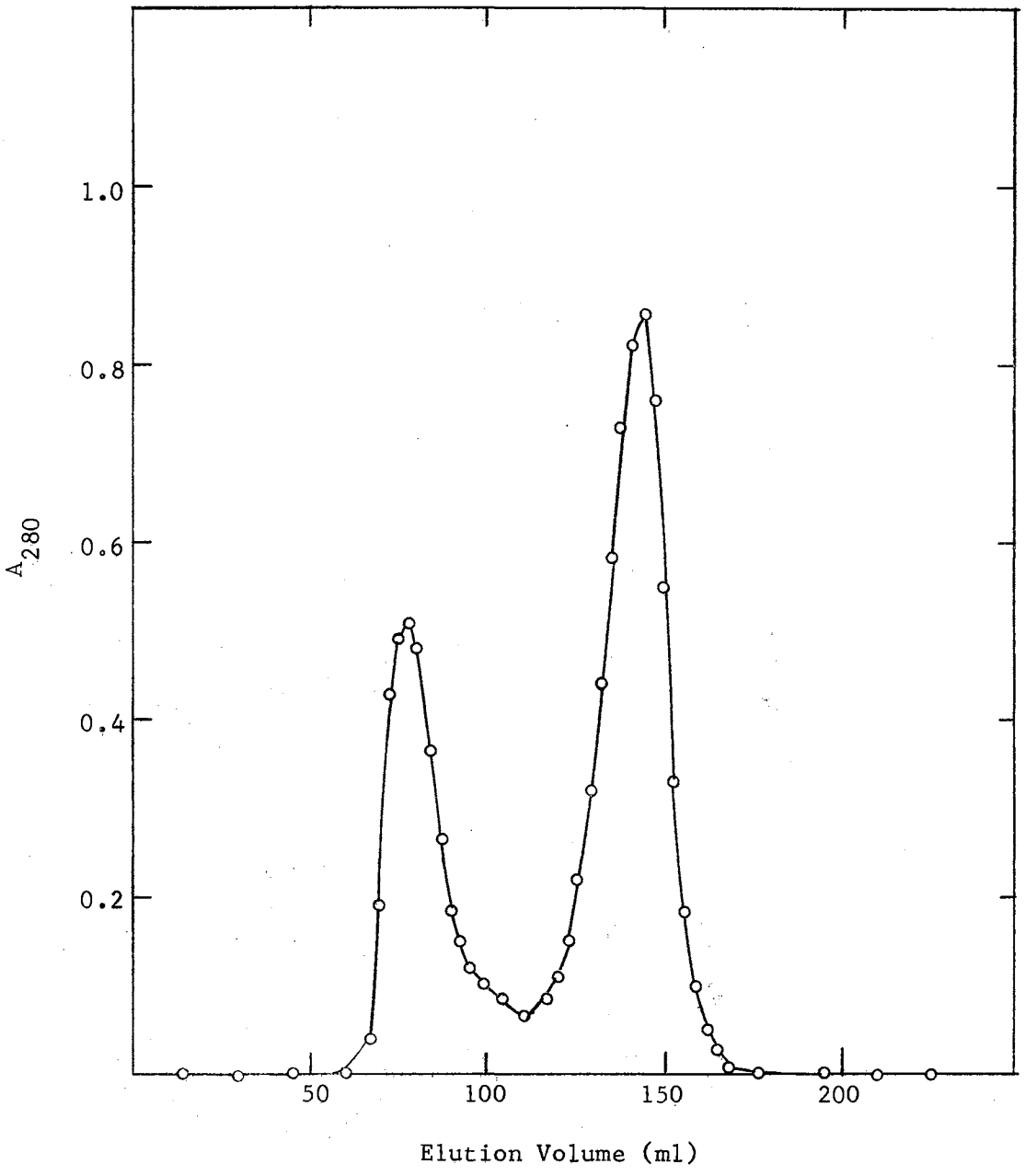
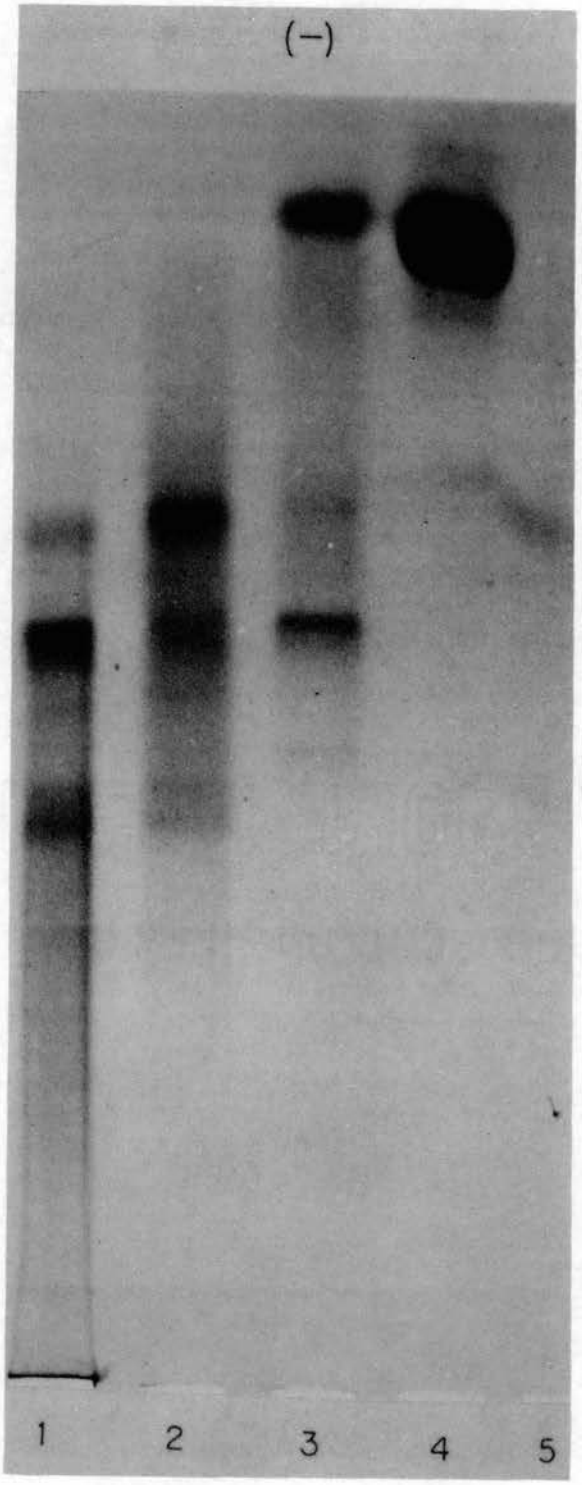

1.0
0.8
0.6
0.4
0.2
A_{280}
50
100
150
200
Elution Volume (ml)

Figure 10. Starch Gel Electrophoresis of the Fractions From a Sephadex G-75 Column

About 800 µg of sample were applied to each wick. Electrophoresis was for 20 hours at five V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B in five percent acetic acid.

1. Fractions from elution volume of 66 ml to 90 ml
2. Fractions from elution volume of 90 ml to 108 ml
3. Fractions from elution volume of 108 ml to 123 ml
4. Fractions from elution volume of 123 ml to 156 ml
5. Fractions from elution volume of 156 ml to 180 ml


(−)
1
2
3
4
5

preparations were subjected to starch gel electrophoresis (see Figure 10, protein sample 4). Lowry protein assays on the fractions from a Sephadex G-75 column showed that two-thirds of the protein placed on the column was in the second peak. The same results were obtained when a five mM Tris buffer, pH 7.4, was used as the eluant instead of 0.5 N lactic acid.

Reversible aggregation of wheat protein fractions has been reported by several investigators (72, 73, 74). Therefore, the possibility of some of the proteins in the first peak from the G-75 column being an aggregate of the protein in the second peak was investigated. When the material in the second peak from a G-75 column was re-chromatographed on the same column, no protein, as measured by the absorbance at 280 mu, was observed in the elution volume corresponding to the first peak (Figure 11). Thus, it appears that the isolated protein is not a monomer unit of some of the larger proteins in the first peak. This is not conclusive, however, since this experiment was not repeated at any higher protein concentrations.

Test of Purity of the Isolated Protein by Electrophoresis

The isolated protein was subjected to electrophoresis in various buffer systems of different pH and in both starch and polyacrylamide gels as the supporting media. Figure 12 shows the results of these experiments. The protein as revealed with any one of several protein stains, generally migrated as a single band at pH 3.3 in both types of support. As shown in gel 6, electrophoresis of greater amounts of the preparation revealed a trailing shadow area behind the protein band when the gel was stained by the sensitive dye, nigrosine. At pH 6.0, the protein migrated very slowly toward the cathode, and a diffuse area

Figure 11. Chromatography and Rechromatography of the Isolated Protein on Sephadex G-75

Fifteen mg of protein from the 0.28 M NaCl peak of a CMC column were dissolved in 1.5 ml of 0.5 N lactic acid and eluted from a Sephadex G-75 column (2 x 71 cm). The second peak from this fraction was freed of lactic acid by dialysis and lyophilized. The dried material was dissolved in 1.5 ml of 0.5 N lactic acid and chromatographed on the same column. O, protein distribution in the eluate fractions after chromatography of the CMC preparation. ●, protein distribution in the eluate fractions upon rechromatography of the second peak.

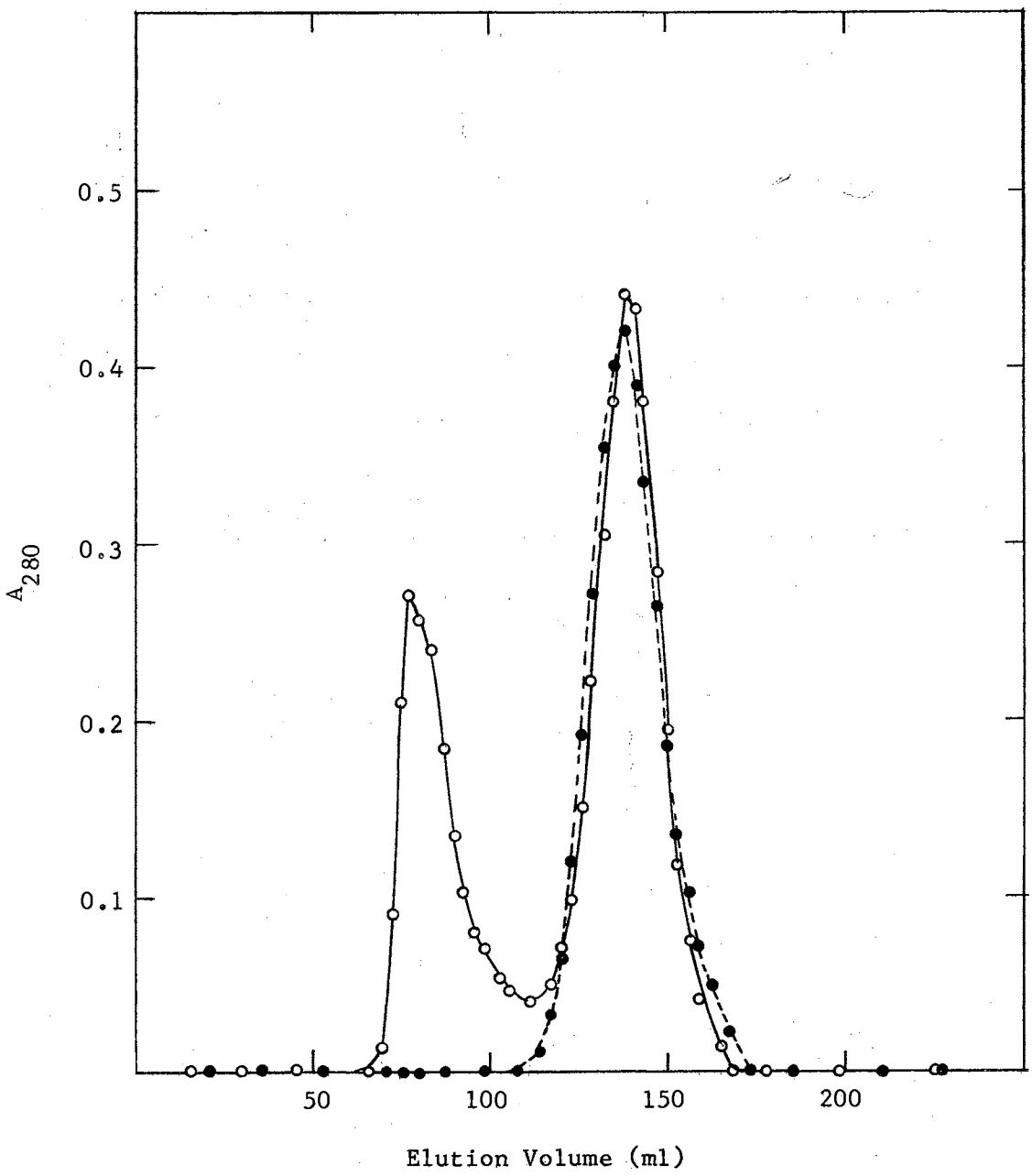

0.5
0.4
0.3
0.2
0.1
A_{280}
50
100
150
200
Elution Volume (ml)

Figure 12. Electrophoresis of the Isolated Protein in Starch Gel and Polyacrylamide Gel at Various pH's

Gel 1. Electrophoresis in starch gel, pH 8.6, at five V/cm for 18 hours. Five hundred μg of protein were applied to the wick. Protein bands were stained with Amido Black 10 B.

Gel 2. Electrophoresis in polyacrylamide gel, pH 8.6, at five V/cm for 18 hours. Five hundred μg of protein were applied to the wick. Protein bands were stained with Amido Black 10 B.

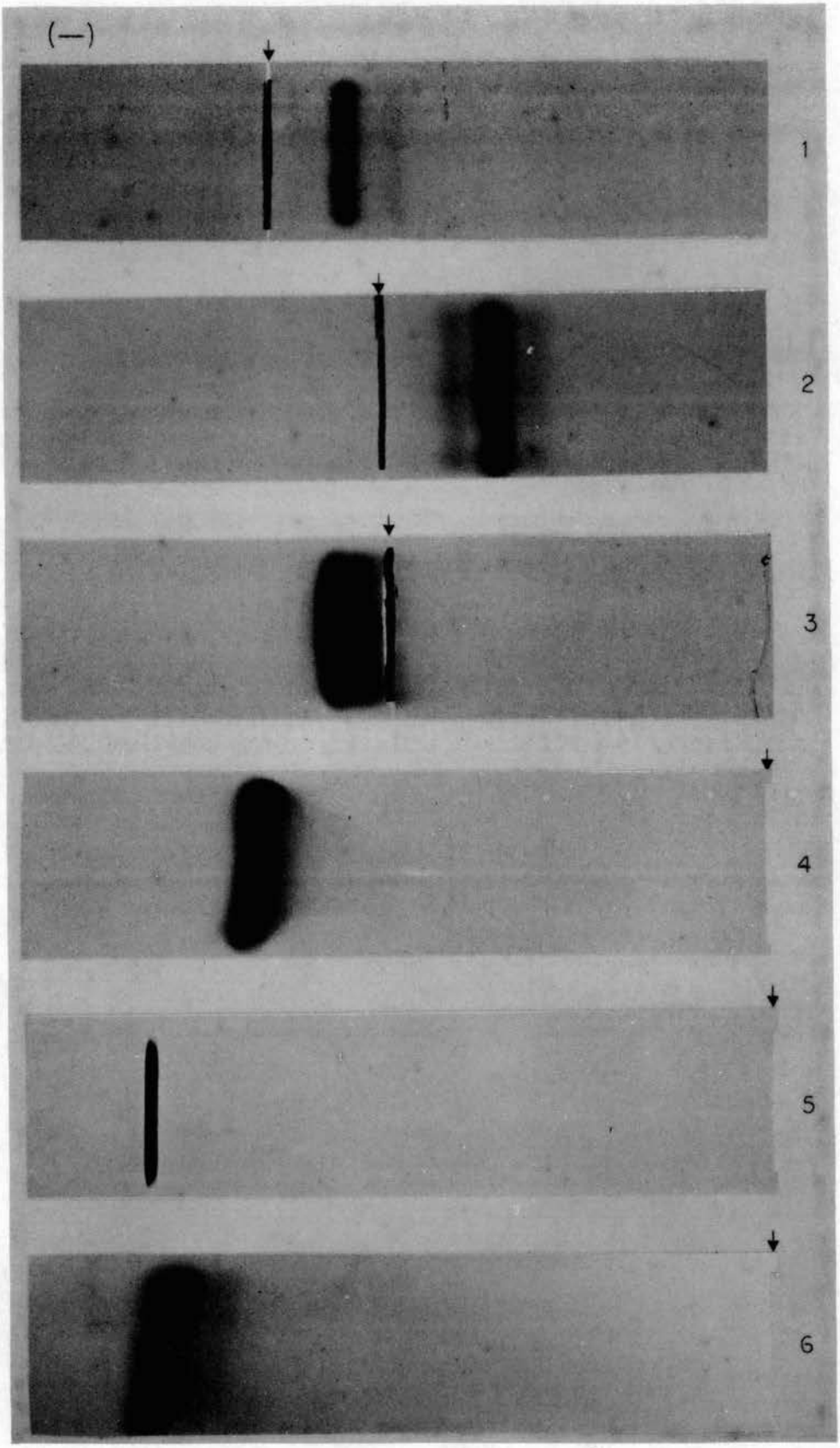
Gel 3. Electrophoresis in polyacrylamide gel, pH 5.95, at four V/cm for 14 hours. Seven hundred μg of protein were applied to the wick. Protein bands were stained with Amido Black 10 B.

Gel 4. Electrophoresis in starch gel, pH 3.3, at five V/cm for 17 hours. Seven hundred μg of protein were applied to the wick. Protein bands were stained with Amido Black 10 B.

Gel 5. Electrophoresis in polyacrylamide gel, pH 3.3, at five V/cm for 21 hours. Three hundred μg of protein were applied to the wick. Protein bands were stained with Amido Black 10 B.

Gel 6. Electrophoresis in starch gel, pH 3.3, at five V/cm for 14 hours. Seven hundred μg of protein were applied to the wick. Protein bands were stained with Nigrosine.

The cathode end of the gels is to the left of the Figure. The origins are marked with arrows.


(—)
1
2
3
4
5
6

appeared behind the main component. At pH 8.6, three protein bands, two faint and one heavy, were clearly seen in both starch gel and polyacrylamide gel. The number of protein bands appearing at this pH were the same regardless of the protein stain employed. A picture of a Ponceau S-stained gel was not included due to the difficulty of photographing the red stain.

Scherr (75) demonstrated that the binding of Ponceau S dye to each of the blood serum proteins produced a chromoprotein which obeyed Beer's law over a wide range of protein concentrations. In this report, the protein bands in blood serum subjected to electrophoresis on cellulose acetate strips were stained with Ponceau S dye and the relative amount of each of the bands was determined. This technique was therefore applied to the isolated protein as a means of estimating its purity. Electrophoresis of the isolated protein fraction on cellulose acetate strips at pH 8.6 revealed three protein bands when stained with Ponceau S as was found for starch and acrylamide gel at this pH. After each band was sectioned from the strip and dissolved in a chloroform-ethanol solvent, the absorbance of each solution was measured against an appropriate blank. The percentage of each protein band was determined by dividing its absorbance by the sum of the absorbances of the three protein bands. The absorbance of the dye-protein complex of each band was found to be a linear function of the amount of sample applied to the gel. The heavy band constituted 90 percent of the fraction, while the slower migrating protein made up about two percent and the faster migrating component about eight percent of the fraction.

Absorption Spectra

The ultraviolet absorption spectra of the isolated protein

(Figure 13) was similar to that of most proteins and exhibited an absorption maximum at 278 mμ. The extinction coefficient of a one percent solution of the protein, $E_{278}^{1\%}$, was 13.1. Based on nitrogen, the extinction coefficient, E_{278}^{mgN}, was 8.5. These results agree favorably with the results of Jankiewicz and Pomeranz (28) who observed an absorption maximum at about 278 mμ for a pyrophosphate extract of flour. The extinction coefficient for their extract, based on mg of nitrogen, was approximately 8.8. The absorption spectra of the isolated protein in 0.1 N NaOH indicated that there were tryptophan residues present in the protein. The tyrosine to tryptophan ratio was 2.1 when calculated from the absorption spectra in base by the method of Goodwin and Morton (65) as described by Beaven and Holiday (66).

Physical Studies Employing Gel Filtration

Andrews (54) showed that the use of gel-filtration columns calibrated with globular proteins of known molecular weight provides a reasonably accurate estimation of the molecular weight of other globular proteins. This technique was therefore employed to obtain a molecular weight estimate for the isolated protein. Figure 14 shows a semi-log plot of the elution volume from Sephadex G-75 versus the molecular weight for eight protein standards and the isolated wheat albumin protein. The isolated protein eluted in the same volume over a concentration range of one mg/ml to ten mg/ml in this system. A least squares regression line was fitted to the experimental points. The molecular weight of the isolated protein calculated from the regression equation was 19,300 $\pm$ 2000 at the 95 percent confidence level. This value falls in the general range of reported molecular weights for the wheat flour albumins [16,000 (31) to 28,000 (8)]. It might be noted that the large

Figure 13. Ultraviolet Absorption Spectra of the Isolated Soluble Wheat Flour Protein

The protein (0.622 mg per ml) was dissolved in five mM lactic acid, pH 4.0.

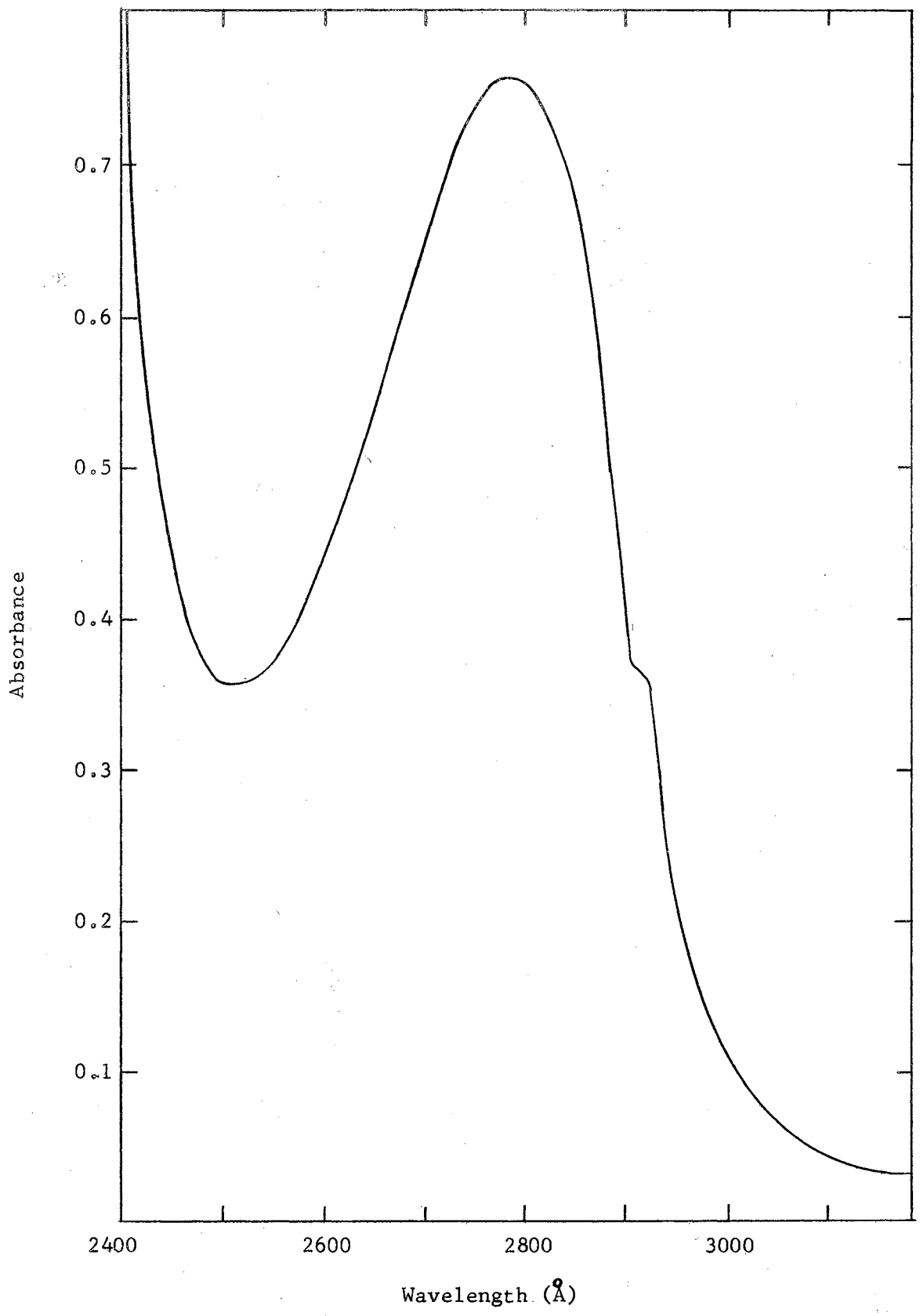
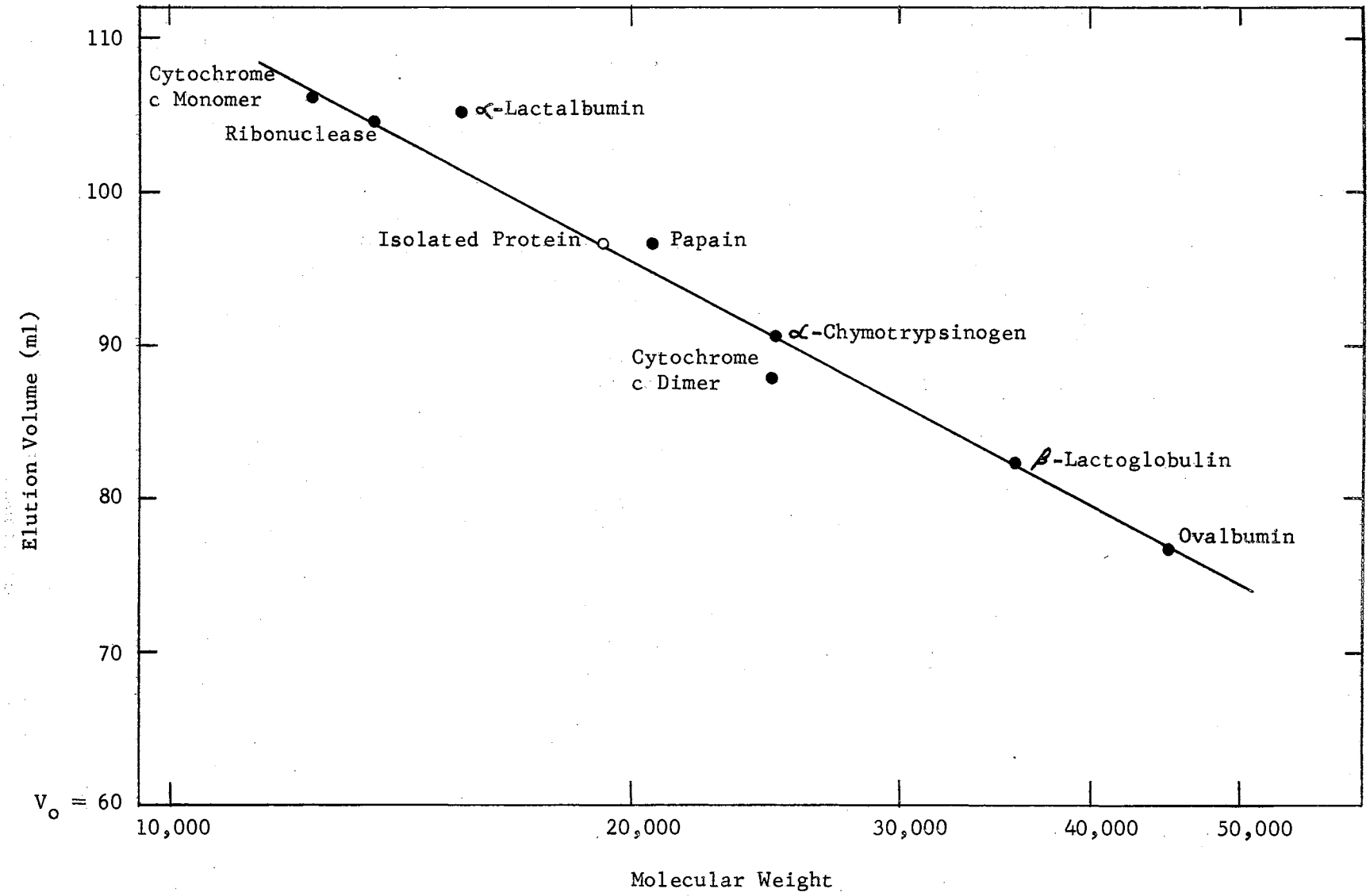

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
2400
2600
2800
3000
Wavelength (Å)

Figure 14. Molecular Weight Estimation of the Isolated Protein by the Gel Filtration Technique

Semi-log plot of elution volume versus molecular weight for seven proteins of known molecular weight. The molecular weight of the isolated protein extrapolated from its elution volume (96.5 ml) was 19,300.


110
100
90
80
70
V_o = 60
Elution Volume (ml)
Cytochrome c Monomer
Ribonuclease
α-Lactalbumin
Isolated Protein
Papain
α-Chymotrypsinogen
Cytochrome c Dimer
β-Lactoglobulin
Ovalbumin
10,000
20,000
30,000
40,000
50,000
Molecular Weight

deviation of α-lactalbumin from the standard curve may be due to an error in its assumed molecular weight. Recent evidence[2] indicates a molecular weight of 12,500 to 13,000 for bovine milk α-lactalbumin rather than 15,500 which was the value used in this experiment.

A relationship between the gel filtration behavior of proteins and their equivalent hydrodynamic radii, r, has been demonstrated by Laurent and Killander (76) and Ackers (77) in their theoretical treatments of the gel filtration process. Since the Stokes-Einstein law for the free diffusion of spherical particles of radius, r, and the diffusion coefficient, D, are inversely related, a useful correlation between the elution volume (or K_d) and the diffusion coefficient, D, was shown to exist. Although there is considerable uncertainty in many of the diffusion coefficients, and free diffusion coefficients are not strictly applicable to restricted diffusion through porous gels, a reasonable estimation of the diffusion coefficient may be obtained from gel filtration data (78). As shown in Figure 15, a linear relation was obtained when the elution volumes of the standard proteins employed in this experiment were plotted versus the reciprocal of the diffusion coefficients. A straight line was fitted to the experimental points by the method of least squares. The diffusion coefficient of the isolated protein calculated from the regression equation at the 95 percent confidence level was $10.47 \pm .09 \times 10^{-7}$ cm^2/sec.

Analytical Ultracentrifuge Analysis

The sedimentation properties of the isolated protein were analyzed in a Beckman Model E ultracentrifuge. The isolated protein sedimented

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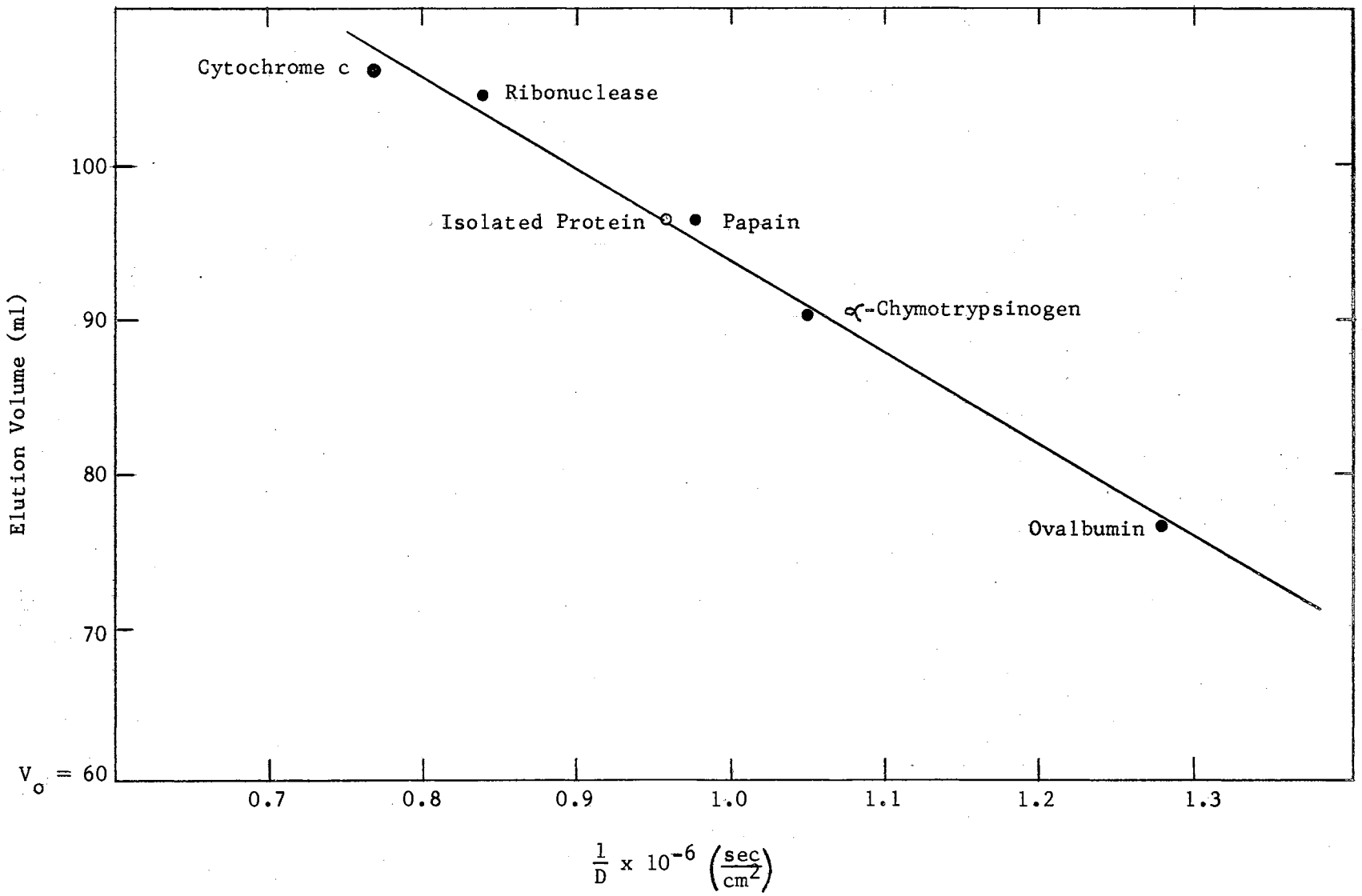
[2]Dr. K. E. Ebner, personal communication.

Figure 15. Estimation of the Diffusion Coefficient of the Isolated Protein by the Gel Filtration Technique

The elution volume versus the reciprocal of the diffusion coefficient for five standard proteins. The diffusion coefficient of the isolated protein extrapolated from its elution volume (96.5 ml) was 10.47×10^{-7} cm^2/sec.


Cytochrome c
Ribonuclease
Isolated Protein
Papain
α-Chymotrypsinogen
Ovalbumin
Elution Volume (ml)
100
90
80
70
V_o = 60
0.7
0.8
0.9
1.0
1.1
1.2
1.3
$\frac{1}{D} \times 10^{-6} \left(\frac{sec}{cm^2}\right)$

as a single symmetrical peak in 0.1 M NaCl as shown in Figure 16. The dependence of the sedimentation coefficient ($S_{20,w}$) upon protein concentration is shown in Figure 17. A sedimentation coefficient of 2.45 was obtained by extrapolation of the data to infinite dilution. The increase in the sedimentation coefficient with increasing protein concentration is not the typical behavior of most proteins. Schachman (79), however, has reported that associating-dissociating single component systems involving rapidly attained equilibria exhibit this phenomenon. A single, almost symmetrical boundry is usually observed, and S increases with concentration in dilute solutions. The increase in S with concentration in dilute solutions is presumed to be the result of a shift in the equilibrium toward the higher aggregates as the concentration increases. Several other wheat protein preparations have been shown to exhibit the same behavior (29, 72, 73).

A molecular weight of 20,400 for the isolated protein was calculated from the sedimentation coefficient, the diffusion coefficient, and the partial specific volume (calculated from the amino acid composition). This value is in reasonable agreement with the molecular weight estimated by gel filtration.

Amino Acid Analysis

The raw data for the amino acid analyses of the isolated protein after different periods of hydrolysis are shown in Table I. Literature values for amino acid compositions of wheat proteins have been expressed in several different units. Therefore, the amino acid values found in this investigation were expressed in four different ways to facilitate comparison with the literature. These calculated results are presented in Table II. Also included in this table are values for half-cystine

Figure 16. Schlieren Pattern of the Isolated Protein

The sedimentation pattern of the isolated protein at a concentration of 6.5 mg/ml in 0.1 M NaCl, pH 6.95, in a synthetic boundary cell. Photographs were taken at the time indicated after the full rotor speed of 59,780 r.p.m. was attained. The temperature was 20° C, and the phase plate angle was 70°. Sedimentation is from right to left.

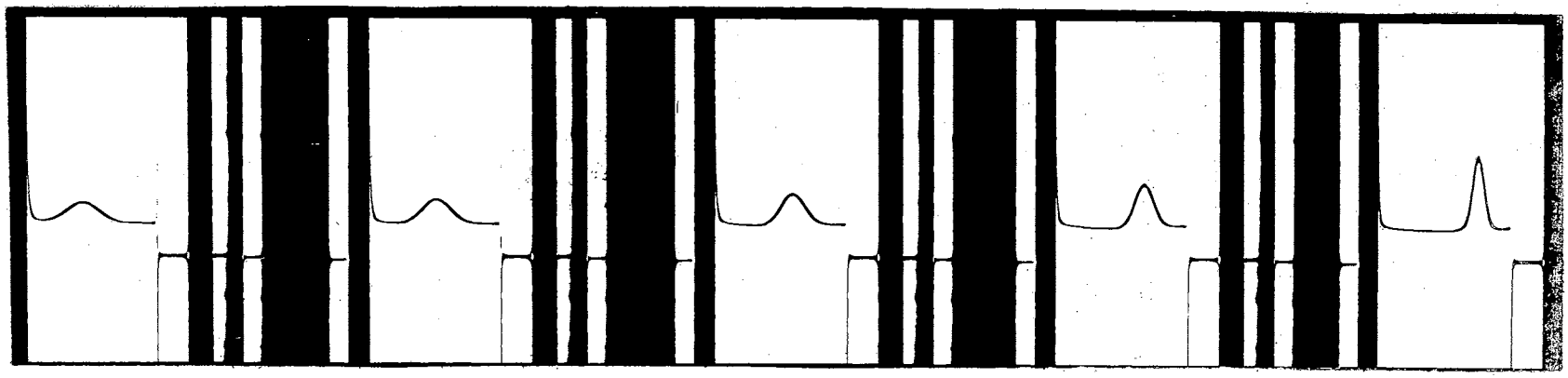

60
45
30
15
0
Time in Minutes

Figure 17. Effect of Concentration on the Sedimentation Coefficient ($S_{20,w}$) of the Isolated Protein

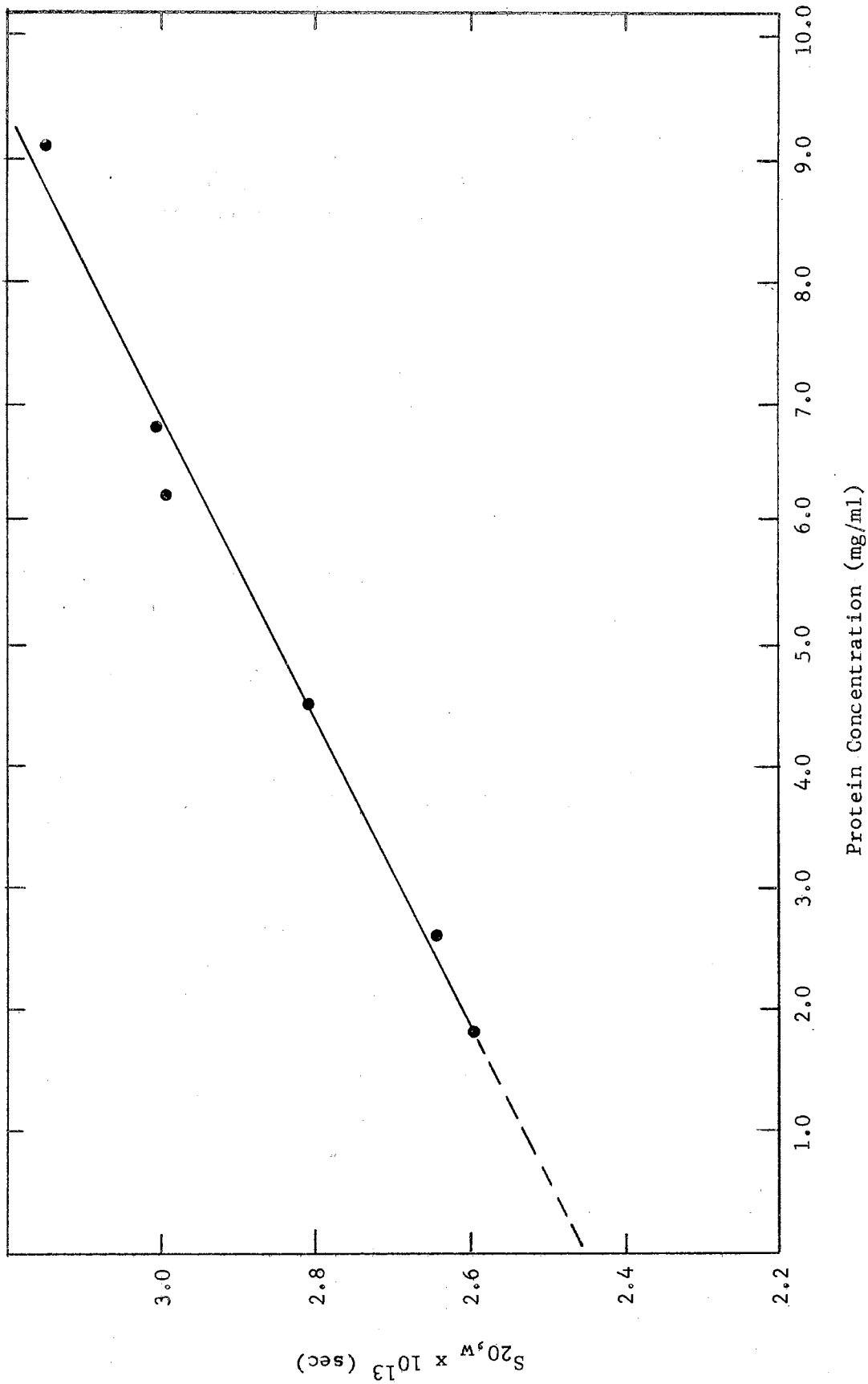

$S_{20,w} \times 10^{13}$ (sec)
3.0
2.8
2.6
2.4
2.2
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Protein Concentration (mg/ml)

TABLE I

AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS[1]

Amino Acid	12 Hour Hydrolysis (1)	24 Hour Hydrolysis (1)	24 Hour Hydrolysis (2)	24 Hour Hydrolysis (3)	48 Hour Hydrolysis (1)	72 Hour Hydrolysis (1)	72 Hour Hydrolysis (2)
Lys	16.61	18.78	22.40	21.47	20.81	20.74	21.18
His	8.31	9.39	11.60	10.69	10.30	10.37	11.30
NH_3	72.67[2]	71.99	94.08	99.28	95.07	91.85	123.23
Arg	35.30	42.25	46.30	42.75	44.12	42.96	42.36
Asp	37.38	42.25	44.80	42.75	44.12	42.96	40.44
Thr	16.61	21.91	19.41	16.79	18.01	16.30	15.40
Ser	37.38	39.13	43.31	38.17	36.74	34.07	32.74
Glu	68.52	75.11	86.62	76.33	80.24	81.48	77.02
Pro	49.83	51.65	56.75	53.43	55.01	57.78	53.92
Gly	53.99	59.47	68.69	61.07	62.86	63.72	61.62
Ala	83.06	89.20	104.54	99.24	97.72	93.33	96.28
(Cys-)	41.53	51.65	53.76	48.85	48.62	45.93	46.21
Val	43.60	53.21	58.24	50.38	57.63	60.74	51.99
Met	14.53	18.78	19.41	16.79	18.58	17.79	15.40
Ile	12.46	17.21	17.92	16.79	17.39	17.79	17.33
Leu	49.83	54.77	62.72	54.96	58.56	57.78	55.92
Tyr	24.92	28.17	29.86	27.48	28.76	28.15	26.96
Phe	10.38	10.95	13.44	12.21	12.89	11.85	11.65

[1]Values are given as millimoles amino acid per 100 g protein.

[2]Shoulder on ammonia peak.

TABLE II

AMINO ACID COMPOSITION OF THE ISOLATED WHEAT FLOUR PROTEIN

Amino Acid	mmoles / 100 g Pro.	g / 100 g Pro.	a.a. N as % Total N	Residues[1] Molecule
Lys	20.89	3.06	4.29	4
His[2]	10.60	1.64	3.26	2
NH_3[2]	48.29	0.82	4.96	9
Arg	43.45	7.56	17.80	8
Asp[3]	43.45	5.78	4.45	8
Thr[3]	21.50	2.74	2.36	4
Ser[3]	42.00	4.41	4.31	8
Glu	79.46	11.76	8.20	15
Pro	54.90	6.32	5.63	10
Gly	63.24	4.74	6.49	12
Ala	96.71	8.61	9.91	18
(Cys-)[3]	53.50	6.54	5.49	10
as $-SO_3H$	51.70	6.21		
Val	55.36	6.97	6.11	11
Met	17.79	2.72	1.87	3 (or 4)
Ile	17.40	2.31	1.81	3
Leu	57.56	7.56	5.91	11
Tyr[4]	28.23	5.20	2.95	6
Tyr[4]	32.40	5.87		
Trp[5]	15.30	3.12	2.96	3
Trp[5]	14.40	2.94		
Phe	12.10	2.00	1.24	2 (or 3)

[1]Assumed molecular weight of 19,300.

[2]Adjusted for loss of Trp, Cys, Thr, and Ser at 24 hour, see Materials and Methods.

[3]Extrapolated value.

[4]From UV analysis.

[5]From Tyr/Trp = 2.1.

determined as cysteic acid and for tyrosine determined by ultraviolet spectral analysis in conjunction with the tryptophan determination.

Microkjeldahl analysis of the isolated protein showed it to contain 15.5 percent nitrogen.

The apparent specific volume of the isolated protein was estimated from its amino acid composition by the method outlined by McMeekin and Marshall (80). The apparent specific volumes of the amino acids were those reported by Cohn and Edsall (81). To take into account the ammonia present as amide groups, it was assumed that these were equally distributed between the glutamic and aspartic residues. The partial specific volume calculated in this manner was 0.727 cm^3/g.

The minimal molecular weight of the isolated protein was calculated from its amino acid composition assuming one mole of histidine per mole of protein. This gave a value of 9,550. If two histidines were present per molecule of protein, the molecular weight would then be about 19,100. This is in reasonable agreement with the values estimated from the sedimentation coefficient and by gel filtration.

In order to illustrate the similarity of the isolated protein to other soluble flour protein preparations, the amino acid composition of several soluble flour protein preparations, together with a gliadin preparation are shown in Table III. The fraction described by Nimmo et al. (22) was from a water extract of flour fractionated on DEAE-cellulose while that of Coates and Simmonds (20) was from a dilute pyrophosphate buffer extract chromatographed on DEAE. The water solubles preparation used by Woychik et al. (32) was obtained by fractionation on CMC. Waggle and co-workers (48) obtained their preparation by chromatography of a phosphate buffer extract of flour on Sephadex G-100.

TABLE III

AMINO ACID COMPOSITION OF "SOLUBLE" FRACTIONS AND GAMMA GLIADIN FROM WHEAT FLOUR[1]

Amino Acid	Nimmo et al. (22) Fract. 5	Woychik et al. (32) H_2O Solubles	Coates & Simmonds (20) Peaks D+E	Waggle et al. (48) Fract. 6	Kelley (29) Globulin	Woychik et al. (32) γ-Gliadin
Lys	5.2	4.6	6.4	4.4	3.2	0.8
His	4.7	5.6	6.2	2.8	3.6	2.7
NH_3	7.3	17.0	10.7	16.8	11.8	26.4
Arg	16.0	11.4	18.6	9.2	18.1	3.4
Asp	4.5	3.8	6.4	4.3	5.1	1.2
Thr	2.0	2.7	3.0	2.3	4.1	1.5
Ser	3.7	3.9	4.5	4.1	4.4	3.1
Glu	8.7	11.7	12.5	18.2	8.9	25.5
Pro	5.3	5.7		9.8	8.5	12.7
Gly	5.8	4.4	6.8	5.1	5.6	2.2
Ala	7.0	4.2	6.2	4.5	3.7	2.1
(Cys-)	3.9	7.0		4.0	3.3	3.1
Val	5.2	4.1	5.4	4.3	4.3	2.7
Met	1.3	1.1		0.8	1.7	0.7
Ile	1.6	3.0	2.9	2.0	2.6	2.8
Leu	4.9	4.8	6.3	4.1	6.4	4.2
Tyr	2.3	1.5	2.6	1.6	2.9	0.8
Trp	2.9	2.2				0.4
Phe	1.1	1.2	1.8	2.0	1.7	3.5

[1]Composition expressed as amino acid N as percentage of the total amino acid N.

Kelley's globulin (29) was prepared by fractional precipitation in phosphate buffer and chromatography on DEAE-cellulose. The gamma gliadin preparation was obtained by the fractionation of gluten on CMC. A comparison of the values for the protein isolated in this investigation (Table II, column 3) with the values in Table III reveals the similarity between this protein and other soluble protein preparations. All of these preparations differ considerably from the γ-gliadin composition.

An interesting similarity is noted between the amino acid composition of the isolated protein and fraction 5 of Nimmo et al. The major protein component in their fraction migrated slowly toward the cathode in cacodylate buffer, pH 6, when subjected to electrophoresis in polyacrylamide gel. This was similar to the behavior of the isolated protein under the same conditions (see Figure 12).

Spectrophotometric Determination of Free Sulfhydryl Groups

The spectrophotometric procedure for free sulfhydryl groups (67) takes advantage of the shift in the absorption maximum of p-chloromercuribenzoate when it reacts with sulfhydryl groups to form a mercaptide. The isolated protein was assayed for free sulfhydryl content by this method with egg albumin being assayed at the same time as a check for the procedure. In buffer, the assay showed egg albumin to contain 1.9 moles of -SH per mole of protein while in buffer containing 0.6 percent sodium lauryl sulfate, it gave a value of 3.8 -SH groups per mole of protein. The number of free sulfhydryls reported in native egg albumin is two or three, whereas four moles of -SH per mole of protein are observed when the assay is carried out in the presence of a denaturing agent such as detergent or urea (67). The isolated protein exhibited only 0.1 moles of sulfhydryl per mole of protein both in the

presence and absence of the detergent. These data indicate the absence of free sulfhydryl groups in the isolated protein and are compatible with the amino acid data showing ten moles of half-cystine (equivalent to five disulfide bonds) per mole of protein.

N-terminal Amino Acid Determination

The determination of the N-terminal amino acid of the isolated protein utilized the procedure for the preparation of the Dnp-derivative of the N-terminal amino acid as outlined by Fraenkel-Conrat (68). Seven mg of protein were reacted with 1-fluoro-2,4-dinitrobenzene. The resulting Dnp-protein was hydrolyzed in six N HCl and the hydrolysate extracted with ether. The ether phase components were separated by two dimensional paper chromatography. Four yellow-colored areas were visible on the developed chromatogram. These four spots were present whether the Dnp-protein was hydrolyzed four hours or sixteen hours at 100° C. Exposure of the largest spot to HCl gas resulted in its disappearance indicating that it was 2,4-dinitrophenol, a reaction by-product. Two of the remaining spots were tentatively identified by their location on the chromatogram relative to the dinitrophenol spot. They were 2,4-dinitroaniline, also a reaction by-product, and Dnp-serine. The R_f of the suspected Dnp-serine spot was identical to that of authentic Dnp-serine when the two samples were chromatographed side by side. The free amino acid, serine, could not be regenerated since base hydrolysis of the Dnp-derivative destroys serine. The fourth spot was as intense as the Dnp-serine spot and was located in an area on the chromatogram corresponding to the degradation products of Dnp-proline. Since Scanes (82) has shown that Dnp-proline is very rapidly destroyed upon acid hydrolysis, shorter hydrolysis times would not add

further information.

Because Dnp-histidine, Dnp-arginine, and Dnp-cysteic acid are soluble in the aqueous phase of the hydrolysate, the aqueous phase was chromatographed in a different solvent system. Only one yellow spot was observed, and its R_f suggested it to be either ϵ-Dnp-lysine or Dnp-arginine. Upon spraying the chromatogram with ninhydrin, the yellow spot turned brown; this indicated the spot was ϵ-Dnp-lysine. This fact was further substantiated by spraying a similar chromatogram with Sakaguchi's reagent. The spot remained yellow; by comparison, authentic Dnp-arginine turned orange and ϵ-Dnp-lysine remained yellow (83).

Results from the fluorodinitrobenzene procedure indicated that serine and perhaps proline were present in the protein as N-terminal amino acids. Because acid hydrolysis of the Dnp-protein destroys Dnp-proline, the phenylisothiocyanate method was employed for the N-terminal amino acid determination. The PTH-derivative of the N-terminal amino acid of egg white lysozyme was determined simultaneously to check the procedures. The isolated protein was precipitated with five percent trichloroacetic acid before treatment with the phenylisothiocyanate to remove any small amino-containing compounds. After reaction, cyclization, and extraction, the only PTH-derivative isolated from the soluble wheat protein was PTH-serine. In agreement with the known data for lysozyme, PTH-lysine was the only derivative obtained. The unknown derivatives were compared with synthesized standards by one dimensional chromatography in two different solvent systems (77). A second treatment of the isolated protein with phenylisothiocyanate released a single PTH-amino acid derivative which had an absorption spectra and a chromatography R_f value similar to literature values for PTH-glycine. This

derivative was not compared with a standard, and its identification is only tentative. Neither the PTH-amino acids nor the Dnp-amino acids were quantitated.

A possible explanation for the extraneous spots in the Dnp-preparation is the presence of contaminating amines in the protein preparation. These may have contaminated the protein during dialysis of the protein against steam condensate-distilled water. This possibility was not checked by precipitation of the protein before treatment with the 1-fluoro-2,4-dinitrobenzene.

C-Terminal Amino Acid Determination

Efforts to determine the C-terminal amino acid of the isolated protein employed the method of enzymatic C-terminal group analysis by carboxypeptidase A (70). Lysozyme was also subjected to the procedure as a check. The substrate was denatured before incubation with carboxypeptidase A, thus minimizing the possibility that the C-terminal residue would be inaccessible to the enzyme due to the native configuration of the protein substrate. The C-terminal amino acid of the isolated protein was not susceptible to hydrolysis by carboxypeptidase A at molar ratios of enzyme to protein as great as one to fifteen and incubation periods up to eight hours at 25° C, pH 8. On the other hand, the C-terminal amino acid of lysozyme, leucine, was released after two hours of incubation at a molar ratio of enzyme to substrate of 1:50 under the same conditions of temperature and pH.

The resistance of the C-terminal amino acid to attack by carboxypeptidase A under the conditions mentioned above may offer a clue to the chemical nature of this amino acid. Neurath (84) has stated that carboxypeptidase A is inactive toward protein substrates in three cases:

when the carboxyl group of the C-terminal amino acid is substituted, when the C-terminal amino acid is lysine or arginine, and when proline (or hydroxyproline) is the C-terminal or penultimate amino acid. The possibility that the C-terminal amino acid of the isolated protein may fall into one of the categories mentioned above suggests that the next approach for determination of the C-terminal amino acid be either treatment of the protein with carboxypeptidase B, which is specific for C-terminal arginine or lysine, or a chemical determination such as hydrazinolysis.

Reduction and Alkylation of Disulfide Bonds

Changes in the elution pattern from Sephadex G-75 and the electrophoretic pattern in starch gel were studied after reduction and alkylation of the disulfide bonds of the isolated protein. Figure 18 A shows the G-75 elution pattern of the native and reduced protein, while Figure 18 B shows the starch gel electrophoresis pattern of each. It is apparent that the native protein was eluted from the column as a single symmetrical peak, whereas the reduced and alkylated protein was eluted in a broad, unsymmetrical peak. The results suggest an array of randomly coiled configurations for the reduced protein polypeptide chain as opposed to a more ordered, globular structure in the native protein. When lysozyme, a single polypeptide chain with five intramolecular disulfide bonds, was subjected to the same treatment, an elution pattern similar to that for the reduced and alkylated isolated protein was obtained.

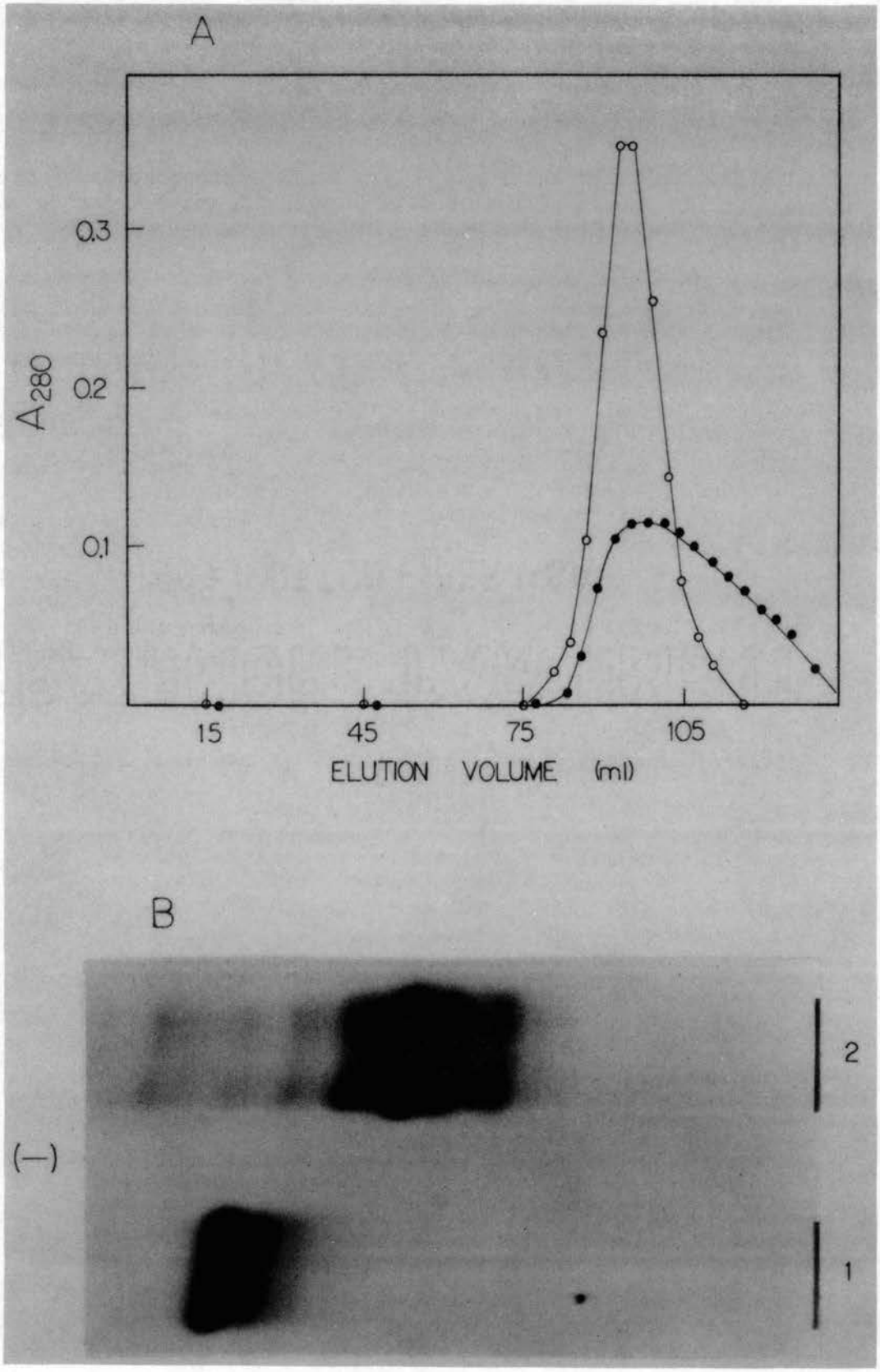
The reduced and alkylated protein migrated essentially as one band in starch gel and exhibited about 70 percent of the mobility of the native protein. Two explanations may be offered for the presence of

Figure 18. Gel Filtration and Starch Gel Electrophoresis of the Isolated Protein After Reduction and Alkylation of the Disulfide Bonds

A. Chromatography on a Sephadex G-75 column (1.3 x 100 cm) of the isolated protein before and after reduction. O, five mg of protein treated with five M guanidine hydrochloride. ●, five mg of protein after reduction and alkylation of the disulfide bonds.

B. Starch gel electrophoresis for 14 hours at five V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B. The cathode end was toward the left of the gel.

1. Native protein
2. Protein after reduction and alkylation of disulfide bonds


A
0.3
0.2
0.1
A280
15
45
75
105
ELUTION VOLUME (ml)
B
(—)
2
1

about eight minor bands in the reduced and alkylated protein preparation: (1) reduction and/or alkylation of all ten cysteine residues involved in the disulfide bonds may not have been complete, thus leaving five different protein species, each with different degrees of unfolding; (2) some of the free amino groups in the protein may have been alkylated by the iodoacetamide, thereby changing the net positive charge on the protein at low pH. It has been shown that a histidine residue of ribonuclease is attacked by some so-called sulfhydryl reagents such as iodoacetate and bromoacetate (85). Although the nitrogens in the ϵ-amino group of lysine and the guanido group of arginine are in an electron environment different from the nitrogens in the imidazole ring of histidine, it may be possible that certain of these amino groups would be in an environment within the protein molecule which would permit alkylation.

General Discussion and Conclusions

The wheat protein isolated in this investigation appears to belong to the class of wheat flour albumins by virtue of its solubility, molecular weight, and starch gel electrophoretic properties. Several of the chemical and physical properties of this protein have been established, but other work of this nature will be required to complete its characterization. Future physical property studies should include a determination of the isoelectric point, and the evaluation of the optical rotatory dispersion properties of this protein. The determination of the C-terminal amino acid and fingerprinting after treatment with proteolytic enzymes are two additional chemical characterization studies which should be included.

The next avenue of investigation on this protein is the investiga-

tion of its biological role in the wheat kernel. The role generally ascribed to the soluble wheat proteins is enzymatic in nature. It is believed that at some time during kernel development some of the soluble proteins serve as enzymes, or subunits of enzymes, involved in the synthesis of cell constituents. Others are hydrolytic enzymes such as proteinases, lipases, or amylases, which degrade the seed storage components to furnish metabolites to the embryo of the germinating seed. If the biological role of the protein isolated in this investigation is that of an enzyme, the discovery of this fact may come from a direct, systematic screening of possible enzymatic activities. Or, incidental to these isolation and characterization studies, an enzyme (or its subunit) which may be purified from wheat flour in the future may be shown to have chemical and physical properties corresponding to those of the protein isolated in this study.

The fact that the isolated protein appears to be the most abundant protein in a water extract of hard red winter wheat flour suggests that it might play a role in the structure of some of the gluten constituents. Preliminary evidence from starch gel electrophoresis of reduced glutenin proteins reported by Woychik (86) indicated that some of the water soluble proteins may be incorporated into the glutenin structure. This possibility might be answered by the isolation of these albumin-like proteins from reduced glutenin and comparison of their properties with those of the protein isolated in this study.

Although subsequent, perhaps, to the determination of its biological role, a study of the distribution of the isolated protein in the kernel components, in the various botanical groups of wheat, and in the other cereal grains may add further insight into some of the

relationships among these groups.

The present work has resulted in the isolation and partial characterization of one of the components of the water soluble flour proteins. The biological role of this protein and of other water soluble components is yet to be established. Only after other soluble flour proteins and gluten proteins have been isolated and characterized, will there emerge a clearer perspective of their relationships to one another and of their contributions to the properties of wheat flour and dough.

CHAPTER IV

THE MOLECULAR WEIGHT DISTRIBUTION OF LACTIC ACID EXTRACTABLE PROTEINS FROM MATURING WHEAT

Experimental Procedure

Endosperm Samples

Two varieties of Hard Red Winter wheat, Triumph and Kaw, were used in this experiment. Samples of Kaw were collected 8, 11, 25, and 35 (mature) days after heading. Triumph samples were collected 11, 13, 21, 28, and 38 (mature) days after heading. Flowering dates for these samples would be one to two days later.

The two most immature samples from each variety were collected by removing the heads in the field and immediately placing them in dry ice. The samples were stored at -20° C until the endosperm was isolated. The endosperm was collected by excising the germ with a razor blade and squeezing the endosperm out of the testa. Endosperms were lyophilized and then ground in a Wiley mill to pass through a # 30 screen. The ground samples were stored at -20° C.

The more mature sample heads were threshed by hand, and the grain was allowed to dry at room temperature under a stream of air. These samples were then milled on a micromill designed by the Departments of Biochemistry and Agriculture Engineering (87). Flour samples were stored at -20° C.

Samples for gel filtration experiments were prepared by stirring

one g of finely ground, dry endosperm with six ml of 0.5 N lactic acid for 16 hours at 4° C. The suspension was then centrifuged at 95,000 x g for 30 minutes in a Spinco Model L preparative ultracentrifuge. An aliquot of the supernatant solution equal to a given amount of protein for each variety was made up to two ml and placed on the column. Protein in the supernatants was determined by the Biuret method (60), by a microkjeldahl procedure, or by the method of Lowry et al. (61). The protein remaining in the residue was assayed by macrokjeldahl analysis or by a modified Biuret procedure for flours (88).

Gel Filtration Columns

Sephadex G-200 equilibrated in 0.5 N lactic acid was used as the gel filtration medium for all experiments. Column dimensions were 2 cm x 72 cm. Conditions were kept as uniform as possible for each series of samples of a given variety. The columns were calibrated with the following proteins: Catalase (Worthington Biochemical Corporation, Freehold, N. J.), Bovine Serum Albumin (Mann Research Laboratories, Inc., New York), Ovalbumin (Sigma Chemical Co., St. Louis, Mo.), and lysozyme (Nutritional Biochemicals Corporation, Cleveland, Ohio).

Fractions from the columns were analyzed by one or more of the methods previously described and will be discussed in more detail in the results. The protein recovery from the columns was 93 $\pm$ six percent as estimated by Lowry protein assays.

Isolation of the Free Amino Acids

The free amino acids were isolated by a method similar to that described by Jennings and Morton (46). Two grams of endosperm were extracted in ten ml of deionized water for 30 minutes with stirring. The mixture was then centrifuged at 10,000 x g for 30 minutes. The

supernatant solution was saved and the precipitate re-extracted twice in the same manner. The pooled supernatant solutions were dialyzed against seven volumes of deionized water. The dialysis water was changed every six hours over a period of 18 hours. The three dialysates were pooled (about 450 ml total) and lyophilized. The dialyzable materials thus obtained were dissolved in citrate buffer, filtered through a fritted glass filter, and assayed by a quantitative ninhydrin procedure (89). Aliquots were analyzed for amino acids on a Beckman model 120 C automatic amino acid analyzer using the analysis system for physiological samples. Recovery of ninhydrin positive material from the columns was 95 $\pm$ five percent.

Results and Discussion

Protein Extraction

Due to the different solubility characteristics of the various flour proteins, a preliminary study was undertaken to find a solvent system most suitable for extraction of the proteins from the endosperm samples. Many solvent systems have been employed by different workers for the extraction of flour proteins, but the system of Meredith and Wren (31) appeared to give superior yields. The solvent which they employed was three M urea and 0.01 M cetyltrimethylammonium bromide in 0.1 N acetic acid. This solvent system, which they termed AUC, extracted about 95 percent of the total flour proteins under the conditions employed in their studies.

Since it was desirable to solubilize as much of the endosperm protein as possible, the AUC system as well as several other solvents were examined for comparative purposes. These preliminary experiments

were carried out on a composite flour made up of several hard red winter wheat varieties. Six ml of solvent and one g of flour were stirred in the cold over night and centrifuged at 90,000 x g for 30 minutes. The supernatant volumes were measured and biuret protein determinations were made on aliquots of the supernatants. The percent of the total flour proteins extracted by each solvent is shown in Table IV.

TABLE IV

EXTRACTION OF FLOUR PROTEINS BY VARIOUS SOLVENTS

Solvent	Percent of Total Flour Protein Extracted
AUC	76
LUC[1]	72
0.5 N lactic acid	63
8 mM aluminum lactate buffer, pH 3.3	60
0.1 N acetic acid	51

[1]0.5 N lactic acid + three M urea + 0.01 M cetyltrimethylammonium bromide.

The AUC and LUC systems gave the best extractions, and they were next tried as eluants for the gel filtration columns. Both solvent systems gave similar results when used to extract the flour proteins and as eluant for a Sephadex G-200 column. Three protein peaks, as indicated by the absorbance at 280 mμ, were obtained. The results were comparable to those obtained by Meredith and Wren (31) who used the AUC solvent. The fractions from each peak were pooled, dialyzed, lyophilized, and the dried material was examined by starch gel electrophoresis

at pH 3.3. The electrophoresis patterns of all three protein peaks were characterized by heavily streaked areas showing only a few very faint bands. The presence of precipitated proteins was suggested by heavily impacted areas at the sample origins of the gels. Thus, it was decided that the AUC and LUC systems were unsatisfactory solvent systems for this investigation, since starch gel electrophoresis was to be used for a qualitative evaluation of the proteins found in the fractions of different molecular weight. Because its extracting capabilities were the next best, 0.5 N lactic acid was tried as the protein extracting solvent and column eluant. Three protein peaks were observed when a 0.5 N lactic acid extract was chromatographed on G-200. Starch gel electrophoresis patterns of the protein peaks were very satisfactory and showed that each peak contained a different group of proteins. Therefore, 0.5 N lactic acid was employed in the subsequent studies on the maturing wheat endosperm as both the extracting and the eluting solvent.

Extracts of the four Kaw samples and five Triumph samples were prepared using lactic acid as described above. Table V shows the protein contents of the various endosperm samples, the amount of nitrogenous materials extracted into the 0.5 N lactic acid solvent, and the amount of nitrogenous materials left in the residue. Column 1 shows that the nitrogen content of the various endosperm samples is of the same magnitude, but direct comparisons are not feasible since the two most immature samples from each variety were prepared by squeezing the kernel while the other samples were milled. Columns 2 and 3 or 6 and 7 suggest that the immature samples contained a larger percentage of non-protein nitrogen than did the more mature samples. This is shown by

TABLE V

EXTRACTION OF PROTEIN FROM KAW AND TRIUMPH ENDOSPERM SAMPLES

Sample	mg Endosperm Protein / g Flour (1) Kjeldahl	mg Extract Protein[1] / g Flour (2) Biuret	(3) Kjeldahl	mg Residue Protein[1] / g Flour (4) Kjeldahl	(5) Biuret[2]	% Protein Extracted (6) Biuret	(7) Kjeldahl
Kaw, 8 days after heading	116.5	45.0	66.4	51.3	46.5	38.6	57.0
Kaw, 11 days after heading	121.0	62.5	78.4	40.9		51.7	64.8
Kaw, 25 days after heading	108.1	67.2	90.6	21.0		62.2	83.8
Kaw, mature	111.0	72.3	77.2	35.0	33.3	65.1	69.2
Triumph, 11 days after heading	121.25	63.0	80.1	39.9		52.0	66.1
Triumph, 13 days after heading	115.1	64.0	81.2	37.6		55.6	70.5
Triumph, 21 days after heading	108.9	66.5	88.1	23.1		61.1	80.9
Triumph, 28 days after heading	113.4	79.5	89.8	23.7		70.1	79.2
Triumph, mature	122.5	85.0	96.9	27.1		69.4	79.1

[1] From one g flour extracted with six ml of 0.5 N lactic acid.

[2] Biuret assay modified for flour by Jennings (88).

the greater discrepancies between the biuret assay of extracted proteins and the Kjeldahl assay of extracted nitrogen containing compounds. Column 4 indicates that certainly not all of the nitrogenous material is extracted, while column 5 shows that this material in the residue is indeed protein. In fact, the immature sample appeared to have more non-extractable protein than the mature sample. These data limit the quantitative interpretations that can be derived from the gel filtration experiments and will be discussed in greater detail in conjunction with those experiments.

Gel Filtration Studies

For each variety, the elution patterns of the proteins from samples at various stages of maturity were compared on the same G-200 column. The two columns used were calibrated with five proteins of known molecular weights so that the molecular weights of the protein fractions could be estimated. Duplicate column runs were made on each sample of both varieties. The amount of protein placed on the column was the same for each sample of a variety. This was accomplished by diluting an aliquot of each sample to the same protein concentration (by the Biuret assay) as that of the most dilute sample. For both Kaw and Triumph, the most immature sample yielded the extract of lowest protein concentration. Two milliliters of extract were applied to the column; this represented 19 mg of protein for the Kaw samples and 25 mg of protein for the Triumph samples. The eluate was collected in three ml fractions, and the absorbance of each fraction was determined at 280 and 260 mμ. Figure 19 shows the elution patterns of the four Kaw samples, and Figure 20 shows the elution patterns of the five Triumph samples. The material in the first peak was eluted in the column void volume. The possibility

Figure 19. Gel Filtration on Sephadex G-200 of Kaw Endosperm Proteins

Two ml of a 0.5 N lactic acid extract that contained 19 mg of protein were placed on a 1.85 x 71.5 cm column of Sephadex G-200. The sample was eluted with 0.5 N lactic acid. (———), absorbance at 280 mμ. (-----), absorbance at 260 mμ.

A. Kaw sample collected May 11, 1965, eight days after heading. The elution volume of the last peak was 201 ml, $A_{260} = 1.70$, $A_{280} = 0.640$.

B. Kaw sample collected May 14, 1965, 11 days after heading. The elution volume of the last peak was 201 ml, $A_{260} = 0.820$.

C. Kaw sample collected May 28, 1965, 25 days after heading.

D. Kaw sample collected June 17, 1965, mature grain.

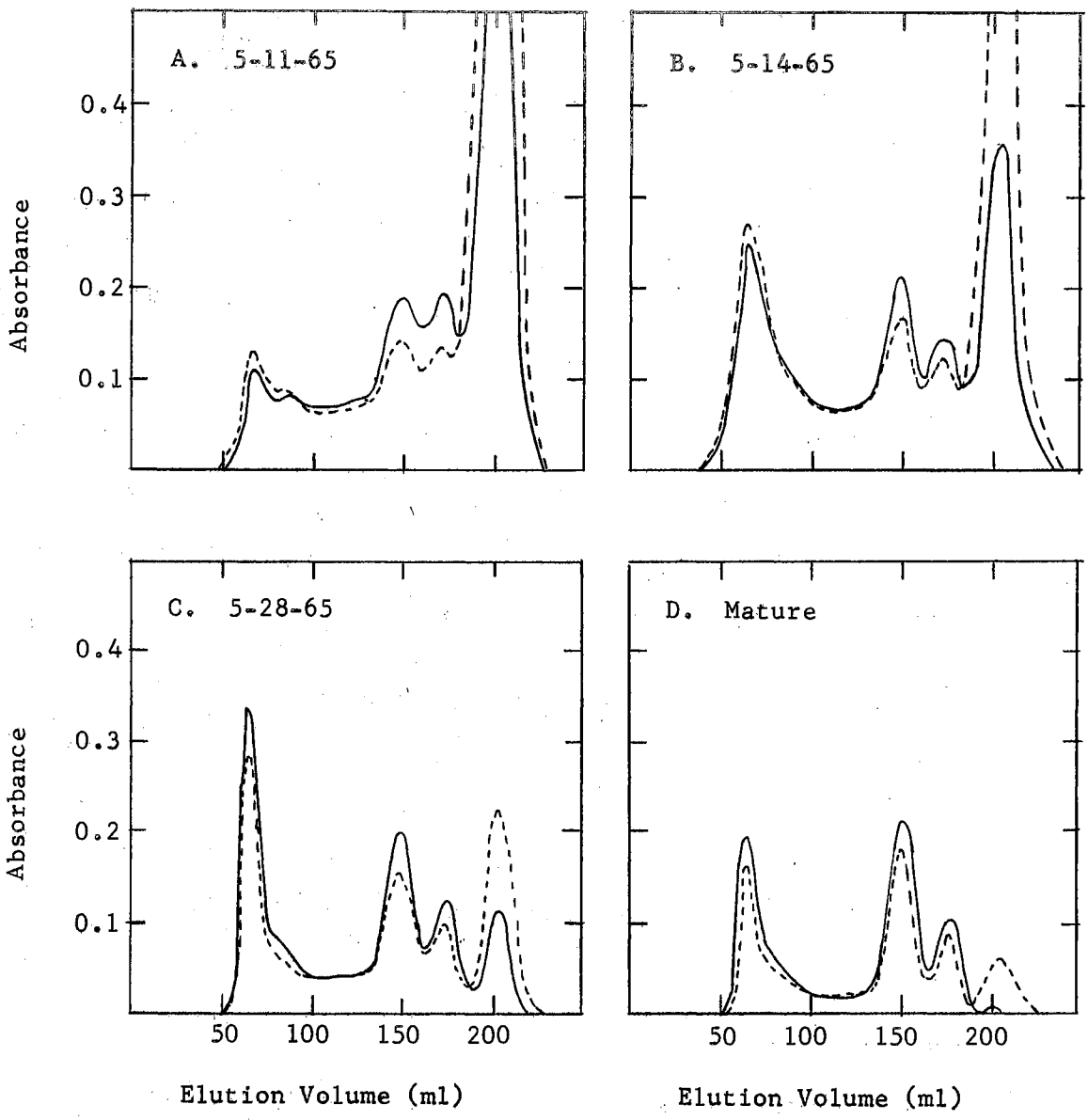

A. 5-11-65
B. 5-14-65
C. 5-28-65
D. Mature
Absorbance
0.4
0.3
0.2
0.1
50
100
150
200
Elution Volume (ml)

Figure 20. Gel Filtration on Sephadex G-200 of Triumph Endosperm Proteins

Two ml of a 0.5 N lactic acid extract that contained 25 mg of protein were placed on a 2.0 x 73.5 cm column of Sephadex G-200. The sample was eluted with 0.5 N lactic acid. (———), absorbance at 280 mμ. (- - - - -), absorbance at 260 mμ.

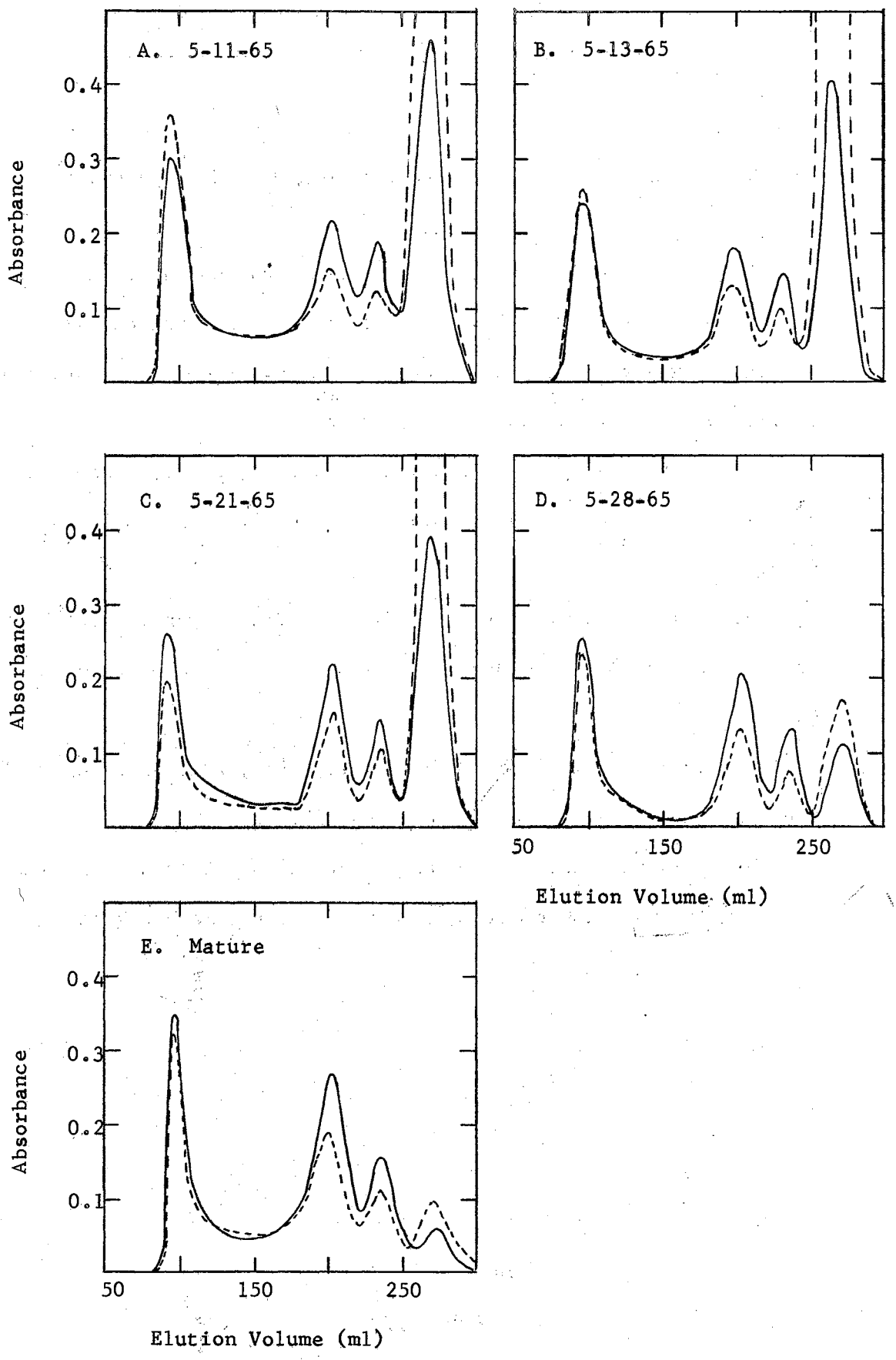
A. Triumph sample collected May 11, 1965, 11 days after heading. The elution volume of the last peak was 270 ml, $A_{260} = 1.10$, $A_{280} = 0.460$.

B. Triumph sample collected May 13, 1965, 13 days after heading. The elution volume of the last peak was 270 ml, $A_{260} = 0.980$, $A_{280} = 0.407$.

C. Triumph sample collected May 21, 1965, 21 days after heading. The elution volume of the last peak was 270 ml, $A_{260} = 0.715$.

D. Triumph sample collected May 28, 1965, 28 days after heading.

E. Triumph sample collected June 17, 1965, mature grain.


A. 5-11-65
B. 5-13-65
C. 5-21-65
D. 5-28-65
E. Mature
Absorbance
0.4
0.3
0.2
0.1
50
150
250
Elution Volume (ml)

that the first absorbance peak from the more immature samples contained large amounts of non-protein materials such as nucleic acids was indicated by its greater absorbance at 260 mμ. A predominance of protein was indicated in the first peak of the more mature samples since the absorbance at 280 mμ surpassed that at 260 mμ. The height of the second and third absorbance peaks varied little among the samples of a variety. The absorbance maximum of the last peak was at 260 mμ for all samples, and the quantity of material in this peak decreased with maturity. Fractions selected from this peak exhibited an absorption spectrum characteristic of the adenosine-containing nucleotides. This would not seem unreasonable since the biosynthesis of kernel constituents is occurring at a very rapid rate in the more immature samples. Enzyme co-factors such as NAD^+, $NADP^+$, and ATP, as well as the various nucleotides, would be present in larger concentrations at this stage of development. The substrate for starch synthesis, ADP-glucose, might also be expected to be present in greater amounts during formation of the kernel.

In order to obtain a more accurate estimate of the protein distribution in the column eluate from each endosperm sample, the column fractions were assayed for protein by the Lowry procedure (61). Duplicate protein assays were made on each Triumph sample, but only a single protein assay was made on each Kaw sample. The results for the Kaw samples are shown in Figure 21, and those for the Triumph samples in Figure 22. The elution pattern of each variety was arbitrarily divided into six groups (Figure 21, D and Figure 22, E). Group 1 eluted in the column void volume and corresponded to a molecular weight of greater than 250,000 to 300,000. Group 2 was in the molecular weight

Figure 21. Molecular Weight Distribution of the Kaw Endosperm Proteins at Various Stages of Kernel Development

Two ml of extract containing 19 mg of protein were placed on a 1.85 cm x 71.5 cm column of Sephadex G-200. Protein (———) in the fractions was measured by the Lowry method.

A. Kaw sample collected May 11, 1965, eight days after heading.

B. Kaw sample collected May 14, 1965, 11 days after heading.

C. Kaw sample collected May 28, 1965, 25 days after heading.

D. Kaw sample collected June 17, 1965, mature grain.

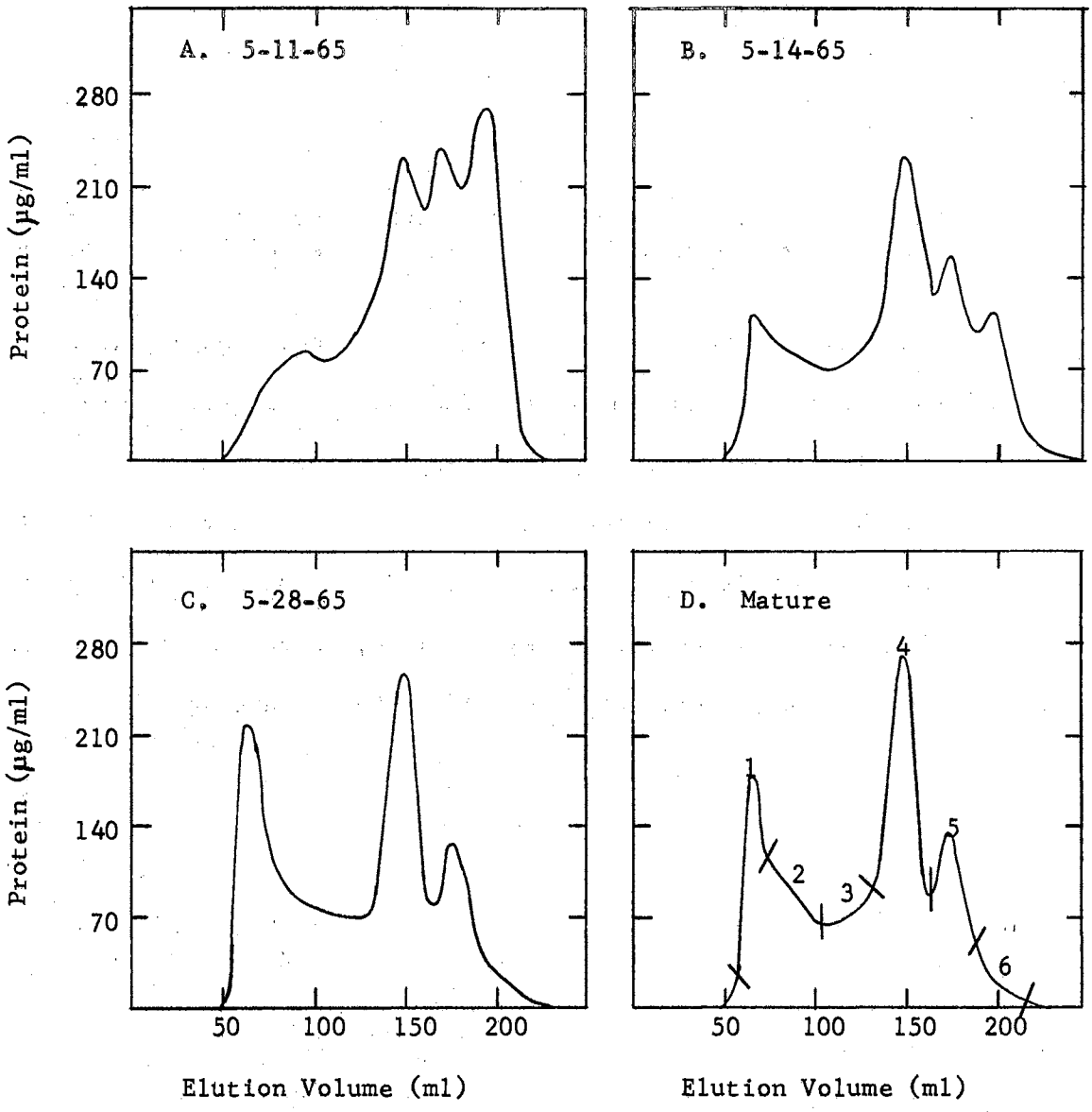

A. 5-11-65
B. 5-14-65
C. 5-28-65
D. Mature
Protein (µg/ml)
280
210
140
70
1
2
3
4
5
6
50
100
150
200
Elution Volume (ml)

Figure 22. Molecular Weight Distribution of the Triumph Endosperm Proteins at Various Stages of Kernel Development

Two ml of extract containing 25 mg of protein were placed on a 2.0 cm x 73.5 cm column of Sephadex G-200. Protein (———) in the fractions was measured by the Lowry method.

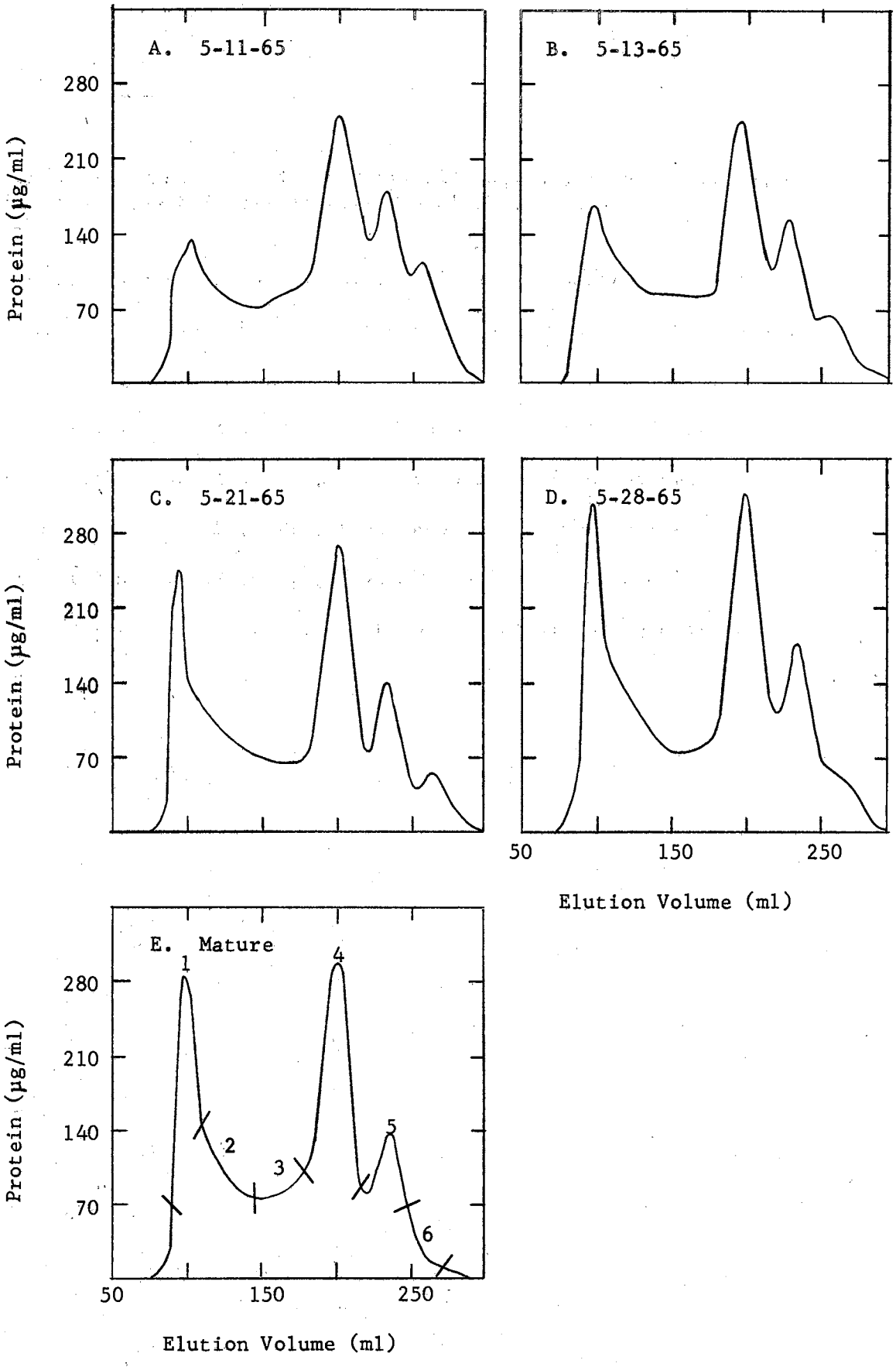
A. Triumph sample collected May 11, 1965, 11 days after heading.

B. Triumph sample collected May 13, 1965, 13 days after heading.

C. Triumph sample collected May 21, 1965, 21 days after heading.

D. Triumph sample collected May 28, 1965, 28 days after heading.

E. Triumph sample collected June 17, 1965, mature grain.


A. 5-11-65
B. 5-13-65
C. 5-21-65
D. 5-28-65
E. Mature
Protein (µg/ml)
280
210
140
70
1
2
3
4
5
6
50
150
250
Elution Volume (ml)

range from 110,000 to 250,000. Group 3 corresponded to a range of 60,000 to 110,000 molecular weight, while the group 4 proteins were in the molecular weight range of 30,000 to 60,000. The proteins of group 5 ranged from 10,000 to 30,000, and the material in group 6 was less than 5,000 molecular weight and had a Kd $\geq$ 1.

For both varieties the proportion of group 1 proteins in the extractable proteins increased with maturation of the kernel. Although difficult to estimate, it appears that the relative amounts of extractable group 2 and group 3 proteins change very little during the development period studied. Figure 21, A indicates that the group 2 proteins made up the first protein peak of the most immature Kaw sample. The relative amounts of the extractable proteins in groups 4 and 5 appear to undergo only slight changes during development. These observations are more distinct with the Kaw samples, probably because the Kaw was sampled at earlier stages of its kernel development than was the Triumph. In fact, the elution patterns of the Kaw harvested 5-14-65 (11 days after heading) and the Triumph harvested 5-11-65 (11 days after heading) correspond rather well.

The material in group 6 gave a very intense color development with Lowry reagent in the immature samples and eluted slightly ahead of the 260 mμ absorbing peak. The large elution volume and the disappearance of this peak with maturity suggest that the materials in this peak are not proteins. It has been reported that tryptophan, tyrosine, most phenols, and guanine give color development with the Lowry reagent (90).

In order to further characterize the material in the last absorbance peak, quantitative ninhydrin assays for amino acids and quantitative anthrone assays for carbohydrates were run on selected fractions from

the G-200 columns of the Kaw samples. The results are shown in Figure 23. The elution volumes of the peak fractions were the same for both assays and were consistently three ml less than the elution volume of the peak fraction of the 260 mμ absorbance peak. There were about 84 mg of carbohydrate material and about 61 μmoles of ninhydrin positive material in the Kaw sample collected eight days after heading. These amounts decreased as the kernel matured to a value of about three mg of carbohydrate material and less than one μmole of ninhydrin positive material in the mature Kaw sample.

Carbohydrate analyses were also run on fractions from the first three absorbance peaks of the most immature Kaw sample and the mature Kaw flour. No carbohydrate was detected in these fractions of the mature flour, while the immature sample showed a small carbohydrate peak with the same elution volume as the first absorbance peak.

This observation supports the possibility of a significant amount of non-protein material in the first absorbance peak of the immature samples.

Starch Gel Electrophoresis Analysis

The fractions in each molecular weight group from the G-200 columns were pooled, dialyzed, and lyophilized. The dry material was then subjected to electrophoresis on starch gel, with the results shown in Figure 24 and Figure 25. A comparison of the starch gel electrophoresis patterns of a given molecular weight group of proteins illustrates the fact that regardless of variety or stage of maturity, there is practically no qualitative variation of the proteins within a group.

The first three molecular weight groups exhibited some impaction at the gel origin and various degrees of streaking through the starch

Figure 23. Distribution of Carbohydrates and Ninhydrin Positive Material in Kaw Endosperm During Maturation

Two ml of extract were placed on a 1.75 cm x 71.5 cm column of Sephadex G-200. (———), carbohydrate measured by the anthrone procedure. (-----), ninhydrin positive material.

A. Kaw sample collected May 11, 1965, eight days after heading.

B. Kaw sample collected May 14, 1965, 11 days after heading.

C. Kaw sample collected May 28, 1965, 25 days after heading.

D. Kaw sample collected June 17, 1965, mature grain.

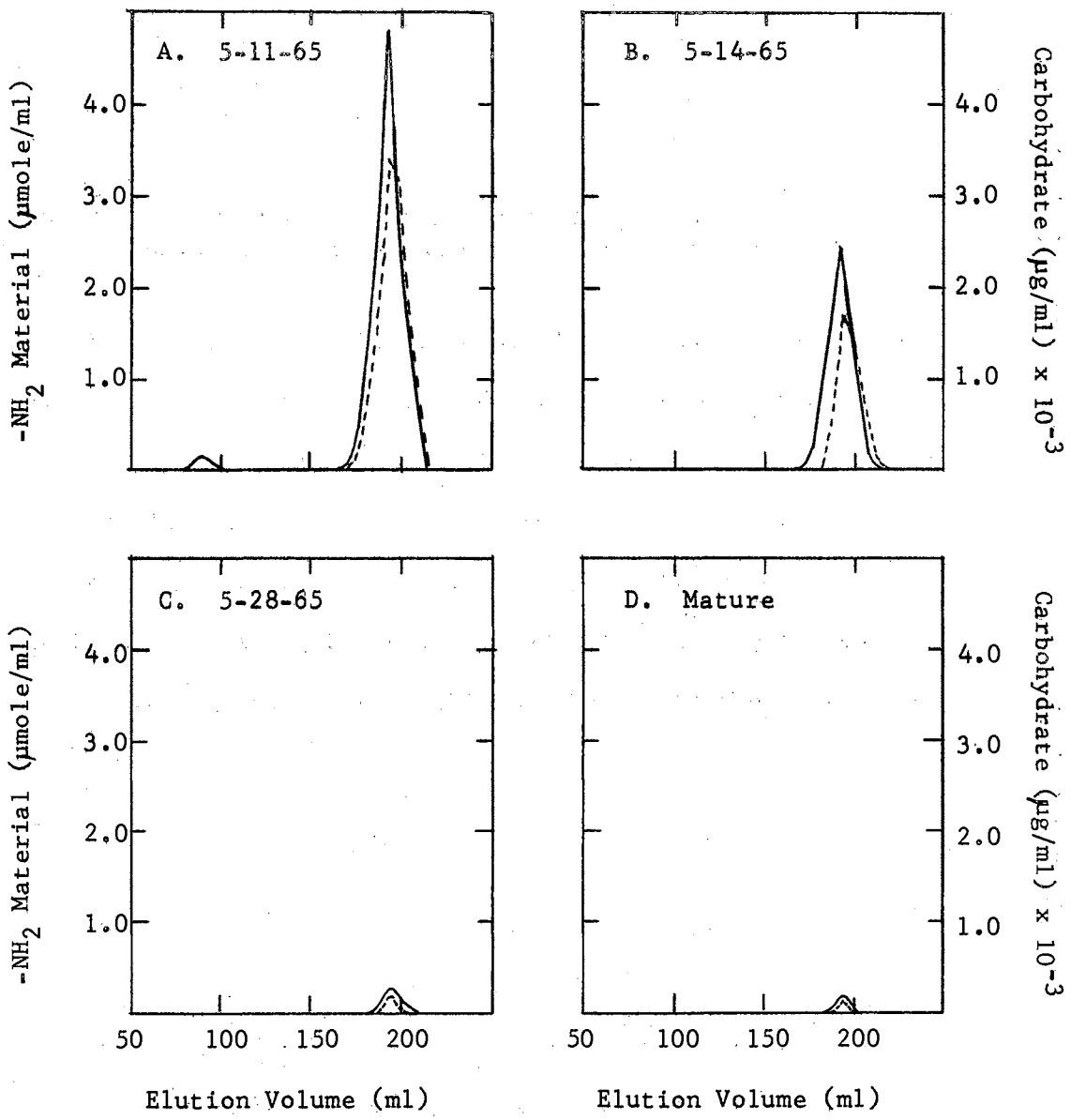

A. 5-11-65
B. 5-14-65
C. 5-28-65
D. Mature
-NH2 Material (μmole/ml)
Carbohydrate (μg/ml) x 10-3
4.0
3.0
2.0
1.0
50
100
150
200
Elution Volume (ml)

Figure 24. Starch Gel Electrophoresis of Fractions From G-200 Gel Filtration of Kaw Samples

Approximately 250 µg of material were applied to each wick. Electrophoresis was for ten hours at 6.5 V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amino Black 10 B in five percent acetic acid. The cathode end is to the left of the gel.

A. Kaw, eight days after heading

1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5
6. Region 6

B. Kaw, 11 days after heading

1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5
6. Region 6, dialyzed
7. Region 7, not dialyzed

C. Kaw, 25 days after heading

1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5
6. Region 6

D. Kaw, mature

1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5
6. Region 6

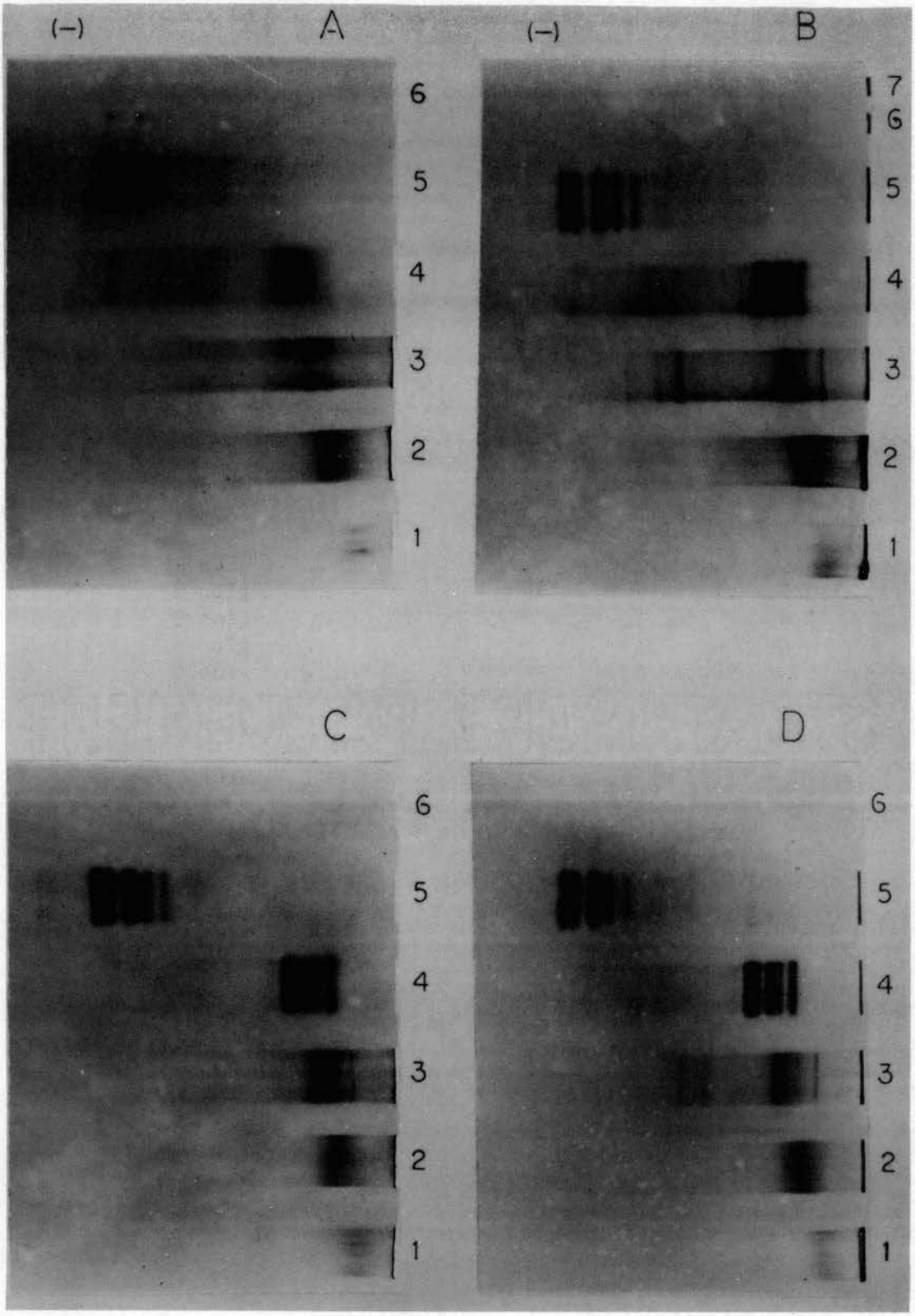

(–)
A
6
5
4
3
2
1
(–)
B
7
6
5
4
3
2
1
C
6
5
4
3
2
1
D
6
5
4
3
2
1

Figure 25. Starch Gel Electrophoresis of Fractions From G-200 Gel Filtration of Triumph Samples

Approximately 300 μg of material were applied to each wick. Electrophoresis was for ten hours at 6.5 V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B in five percent acetic acid. The cathode end is to the left of the gel.

A. Triumph, 11 days after heading

1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5

B. Triumph, 13 days after heading

1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5

C. Triumph, 21 days after heading

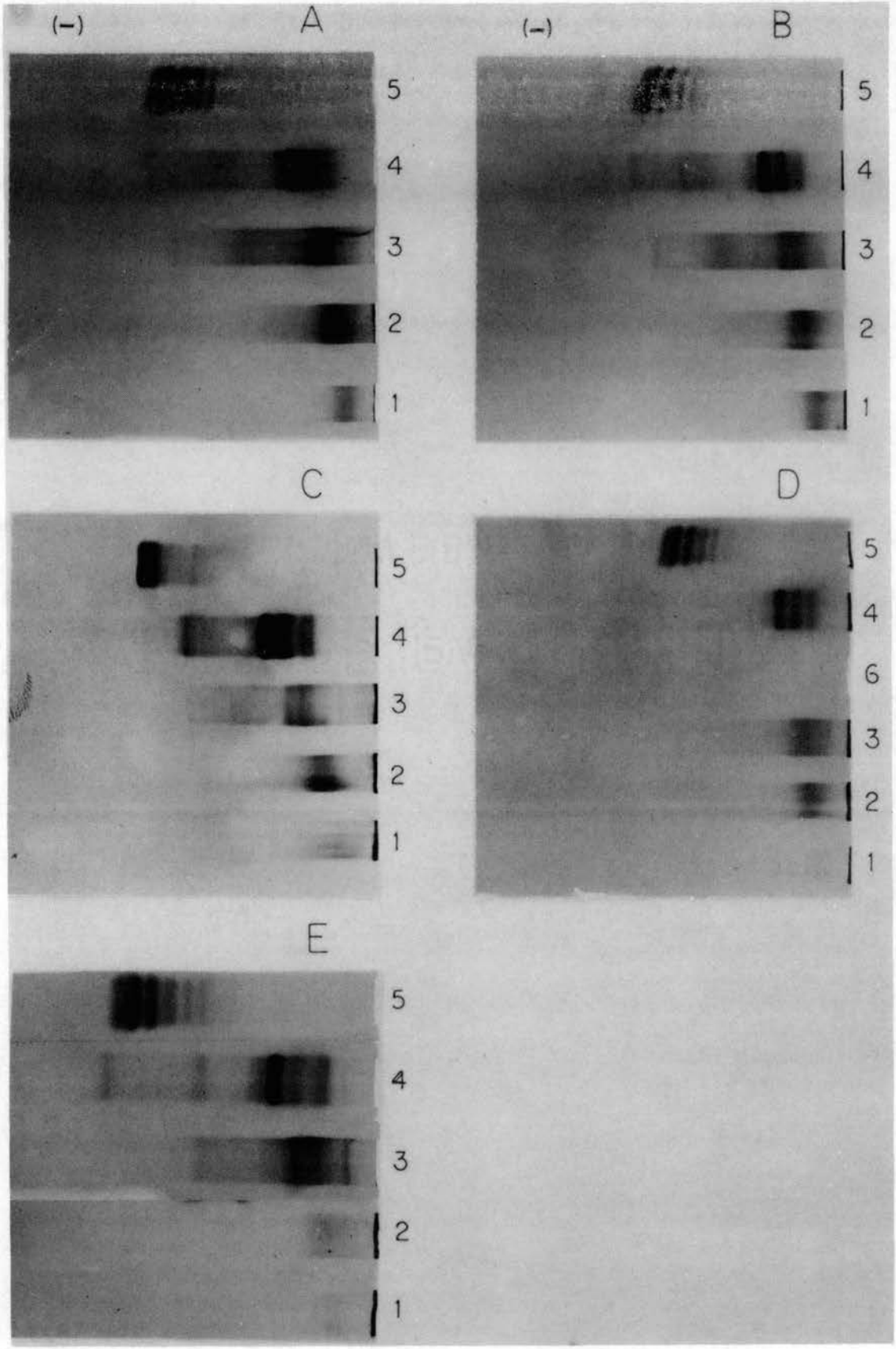
1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5

D. Triumph, 28 days after heading

1. Region 1
2. Region 2
3. Region 3
6. Region 6
4. Region 4
5. Region 5

E. Triumph, mature

1. Region 1
2. Region 2
3. Region 3
4. Region 4
5. Region 5


(–)
A
5
4
3
2
1
(–)
B
5
4
3
2
1
C
5
4
3
2
1
D
5
4
6
3
2
1
E
5
4
3
2
1

gel. This electrophoretic behavior is typical of the glutenins (10) as is also the large molecular weight of 10^5 to 10^6 (53). The group 4 proteins exhibited a migration rate similar to that reported for the gliadins (10), and the molecular weight of about 45,000 from these studies agrees with reports in the literature (31, 74). The albumins, or water solubles, appear to be in the group 5 proteins, as demonstrated by both their starch gel electrophoresis mobilities (10, 26) and their molecular weight of about 20,000 (8, 28, 31). Electrophoresis of both dialyzed and undialyzed samples from the group 6 material failed to reveal any protein bands (Figure 24, B, samples 6 and 7). This further demonstrates the absence of protein in this fraction.

Changes in the relative amounts of some of the endosperm proteins with maturation of the kernel as shown by gel filtration were also demonstrated by the starch gel electrophoresis patterns of the crude extracts. As illustrated in Figure 26, the more mature samples exhibited a darkly stained, impacted area at the origin of the gel. This area was not detectable in the more immature samples. It is probable that this impacted area shows the presence of glutenin proteins since it has been reported by others that the glutenins fail to migrate into the gel matrix, thus causing impaction at the origin (10).

The results of this study indicate that qualitatively, the same proteins are extracted from the endosperm at all stages of maturity examined. However, changes with maturity in the relative amounts of some of the proteins are reflected by changes in the gel filtration elution patterns and the starch gel electrophoresis patterns of the crude extracts.

Figure 26. Starch Gel Electrophoresis of Crude Extracts of Kaw and Triumph Endosperm During Maturation

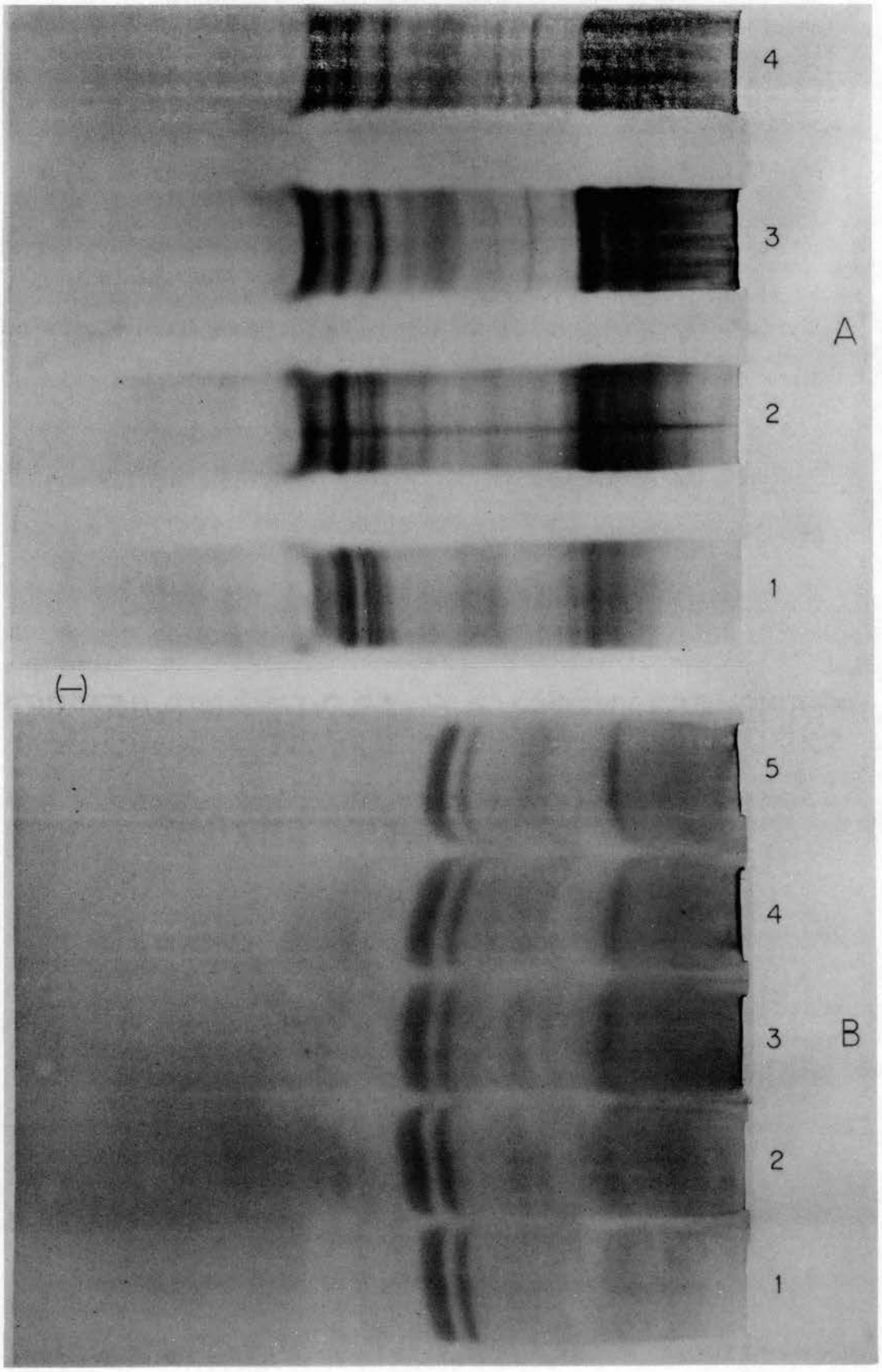
Electrophoresis was for ten hours at 6.5 V/cm in aluminum lactate-urea buffer, pH 3.3. Protein bands were stained with Amido Black 10 B in five percent acetic acid. The cathode end of the gel is to the left side of the figure.

A. Kaw samples, about 400 μg of protein were applied to each wick.

1. eight days after heading
2. 11 days after heading
3. 25 days after heading
4. mature

B. Triumph samples, about 600 μg of protein were applied to each wick.

1. 11 days after heading
2. 13 days after heading
3. 21 days after heading
4. 28 days after heading
5. mature


4
3
A
2
1
(–)
5
4
3
B
2
1

Fluctuation in the Free Amino Acids of the Maturing Endosperm

Ewart (91) has recently reported that the glutenins possess much higher proportions of lysine, glycine, and tryptophan than the gliadins, and somewhat higher proportions of arginine, tyrosine, threonine, aspartic acid + asparagine, serine, and alanine. The gliadins contained more proline, glutamic acid + glutamine, cystine, phenylalanine, and isoleucine.

In an attempt to correlate fluctuations in the levels of these free amino acids with the synthesis of the higher molecular weight proteins, the amount of each amino acid in the various samples was determined. The free amino acids were separated from the proteins and high molecular weight carbohydrate material of a water extract of the endosperm by a dialysis procedure similar to that described by Jennings and Morton (48). Aliquots corresponding to six to eight μmoles of ninhydrin positive material were placed on an automatic amino acid analyzer with the results shown in Table VI. Several unidentified amino-containing compounds are not listed. This isolation and analyses were done only once for each sample. No general trends were observed when the fluctuations of the amino acids present in large amounts in the glutenins were compared with the fluctuations of those abundant in the gliadins. This suggests that earlier and more frequent analyses would be necessary to detect any significant trends. The absolute amount of each amino acid decreased with maturation of the kernel. On the other hand, comparison of the percentages of the amino acids reveals that arginine, aspartic acid, phenylalanine, tyrosine, and isoleucine showed substantial increases in relative amounts, while alanine, serine, proline, and glutamic acid exhibited marked decreases. The relative amounts of the other amino

TABLE VI

CHANGES IN THE FREE AMINO ACID COMPOSITION DURING ENDOSPERM DEVELOPMENT

Amino Acid	Micromoles of Amino Acid per Gram Dry Flour									Percent of Total Free Amino Acids								
	Kaw, Days After Heading				Triumph, Days After Heading					Kaw, Days After Heading				Triumph, Days After Heading				
	8	11	15	Mat[1]	11	13	21	28	Mat	8	11	15	Mat	11	13	21	28	Mat
Ala	42.31	27.50	3.60	0.60	20.41	15.46	13.77	1.86	0.66	19.08	20.73	10.61	8.40	15.82	16.01	24.38	12.58	9.07
Arg	1.92	0.78	0.28	0.12	1.03	0.93	0.25	0.30	0.17	0.86	0.59	0.97	1.68	0.80	0.96	0.44	2.03	2.34
Asn + Gln	17.58	14.22	3.72	0.76	14.23	10.05	4.29	1.67	0.58	7.93	10.72	12.89	10.64	11.03	10.41	7.60	11.30	7.97
Asp	3.30	3.44	2.11	1.11	2.68	2.78	1.98	1.19	1.02	1.48	2.59	7.31	15.55	2.08	2.88	3.51	8.05	14.01
(Cys-)	1.37	0.47	0.17	0.07	0.41	0.16	tr[2]	0.11	0.02	0.61	0.35	0.59	0.98	0.32	0.17	-----	0.74	0.30
Glu	22.25	24.06	2.89	0.43	19.38	11.44	2.31	1.14	0.44	10.03	18.13	10.02	6.02	15.02	11.85	4.09	7.71	6.04
Gly	19.23	10.94	1.50	0.59	16.29	10.98	4.21	1.00	0.52	8.67	8.25	5.20	8.26	12.62	11.37	7.45	6.77	7.14
His	1.37	0.63	0.33	0.12	0.82	0.62	0.33	0.19	0.11	0.61	0.48	1.14	1.68	0.80	0.96	0.44	2.03	2.34
Ile	1.92	0.78	0.44	0.01	0.82	1.08	1.32	0.26	0.13	0.86	0.59	1.53	1.40	0.64	1.12	2.34	1.76	1.79
Leu	2.47	1.09	0.61	0.22	1.24	1.55	1.24	0.41	0.22	1.11	0.82	2.11	3.08	0.96	1.61	2.20	2.77	3.02
Lys	3.85	1.72	0.39	0.14	2.10	2.16	0.42	0.30	0.24	1.73	1.30	1.35	1.96	1.63	2.24	0.74	2.03	3.30
Met	2.20	0.78	0.22	0.06	1.86	1.39	0.66	0.22	0.08	0.99	0.59	0.76	0.84	1.44	1.44	1.17	1.49	1.10
Phe	1.65	0.78	0.39	0.14	1.03	0.93	0.75	0.24	0.14	0.74	0.59	1.36	1.96	0.80	0.96	1.33	1.76	1.92
Pro	8.52	2.50	0.39	0.16	2.68	2.01	1.48	0.22	0.19	3.84	1.88	1.36	2.24	2.08	2.08	2.62	1.49	2.61
Ser	24.73	13.75	2.11	0.73	18.97	13.45	5.36	1.37	0.82	11.15	10.36	7.31	10.22	14.70	14.85	9.49	9.27	11.26
Thr	4.67	2.50	0.78	0.22	2.89	2.63	1.74	0.45	0.24	2.10	2.24	2.11	2.80	2.24	2.72	3.08	3.04	3.30
Trp	-----	-----	0.50	0.11	tr	-----	0.91	0.48	0.10	-----	-----	1.73	1.54	-----	-----	1.61	3.25	1.51
Tyr	1.65	0.78	0.44	0.14	0.82	0.62	2.23	0.26	0.17	0.74	0.59	1.53	1.96	0.64	0.64	3.95	1.76	2.34
Val	4.67	2.50	0.78	0.22	2.06	2.32	1.90	0.48	0.22	2.10	1.88	2.70	3.08	1.60	2.40	3.36	3.25	3.02
Aad	0.27	0.47	0.06	0.01	0.41	0.31	0.42	tr	0.37	0.12	0.35	0.21	0.14	0.32	0.32	0.74	-----	5.08
Abu	0.27	tr	tr	tr	tr	tr	0.16	tr	----	0.12	-----	-----	-----	-----	-----	0.28	-----	-----
γAbu	22.53	8.13	2.56	0.14	7.63	2.94	5.03	1.44	0.52	10.16	6.13	8.87	1.96	5.91	3.04	8.91	9.74	7.14
NH_3	18.68	7.97	1.78	0.49	10.72	6.49	2.97	0.09	0.31	8.42	6.01	6.17	6.86	8.31	6.72	5.26	0.61	4.26
Cit	tr	tr	0.06	0.01	tr	tr	tr	tr	tr	-----	-----	0.17	0.14	-----	-----	-----	-----	-----
Sar	tr	tr	tr	tr	tr	0.31	tr	tr	tr	-----	-----	-----	-----	-----	0.32	-----	-----	-----
Taurine	0.27	tr	0.11	----	tr	tr	tr	tr	tr	0.12	-----	0.38	-----	-----	-----	-----	-----	-----
Urea	8.52	3.75	2.22	----	-----	2.78	-----	0.67	----	3.84	2.82	7.69	-----	-----	2.88	-----	4.53	-----

[1] Mat = mature

[2] tr = trace

acids remained relatively constant. These results are in partial agreement with Jennings and Morton (49) who reported significant increases in aspartic acid and arginine and decreases in lysine and proline.

Ammonia, urea, and 4-amino-butyric acid, although not directly involved in protein synthesis, were present in rather large amounts in the more immature samples.

These results show that the quantity of each amino acid in the kernel decreased with maturation and that the relative proportions of certain amino acids varied with kernel development. Similar results obtained by others (46, 49) were interpreted as indicating variations in the rate of supply of an amino acid to the pool of protein precursors compared with the rate of its incorporation into protein.

Conclusions

Gel filtration and starch gel electrophoresis experiments show an increase in the quantity of high molecular weight protein components in the extractable protein fraction as the wheat endosperm matures. Since appreciable amounts of protein could not be extracted under the conditions employed, the results must be interpreted with reservations. However, several possible explanations for the observed changes may be offered.

The most obvious possibility is that the glutenin proteins are the last storage proteins to be synthesized. Although not fully soluble in the solvent system employed, enough of these proteins were extracted from the more mature samples to change the gel filtration patterns. The occurrence of large amounts of unextracted protein material in the immature samples might be explained by the presence of high proportions of protein-containing organelles such as the endoplasmic reticulum and

the ribosomes which are associated with cell development.

If the protein bodies described by Morton et al. (38, 39) are the sites of gluten protein storage, and if a change occurs in the physical structure of these protein bodies, then the second possible explanation for the observed changes might be that the glutenins became more accessible to the solvent. Jennings has suggested that the protein bodies enlarge with maturity; thus, rupture of some of these bodies or an alteration in the lipo-protein membrane enclosing the storage proteins might allow solvation of the contents. This hypothesis would require that the protein bodies contain essentially only glutenins because the amount of gliadins appeared to remain nearly constant for all dates sampled.

The third possible explanation may be that many of the high molecular weight proteins were synthesized early in the kernel development and remained insoluble throughout. However, in the later stages of development, proteins, perhaps gliadin type proteins, are synthesized and these new proteins are combined with some of the pre-existing gliadins and albumins through intermolecular disulfide bonding or non-covalent associations to form the larger molecular weight proteins observed in these experiments. This behavior has been observed in the case of wheat proteins by several groups (72, 73, 74, 86).

This investigation has answered a few of the questions about changes occurring in the proteins during kernel maturation; however, many questions have been left unanswered. Perhaps the paramount prerequisite to further studies of this type will be the finding of a solvent system which will extract all of the endosperm proteins without grossly disrupting their conformations. Other parameters which must be

more closely controlled in further experiments of this type include earlier and more frequent sampling and further proof of differences among the protein fractions from the gel filtration columns such as amino acid analysis. Separation of the various wheat flour proteins according to their molecular weight by the gel filtration technique might complement some of the other techniques in future work. For example, this technique could be employed to fractionate radioisotope-labeled proteins by a criteria other than their solubility characteristics. Or, together with ion exchange chromatography, it may be useful in further characterizing the storage proteins contained in the isolated protein bodies at various stages of kernel development.

SUMMARY

A combination of ion exchange chromatography on CMC and gel filtration on Sephadex G-75 was employed in the isolation of a water soluble wheat flour protein. The preparation was judged to be 90 percent pure by zone electrophoresis. Sedimentation analysis yielded a single symmetrical peak with an $S^{o}_{20,w}$ of 2.45. Molecular weight estimation by gel filtration yielded a value of 19,300. The diffusion coefficient, as estimated by gel filtration, was 10.47×10^{-7}. The molecular weight calculated from the sedimentation and diffusion coefficients was 20,400. Amino acid analysis showed the protein to have a composition similar to other soluble wheat flour protein preparations. The partial specific volume of the protein, when calculated from the amino acid composition, was 0.727 cc/g. A minimal molecular weight of 9,550 was obtained from the amino acid composition. The ultraviolet absorption spectra showed a maximum at 278 mμ, and from this was calculated an $E^{1\%}_{278}$ of 13.1. No free sulfhydryl groups were detected. Reduction and alkylation of the isolated protein resulted in the appearance of several minor components upon starch gel electrophoresis and a decreased electrophoretic mobility of the main component as compared to the untreated protein. Serine was found to be the only N-terminal amino acid. The C-terminal amino acid was resistant to attack by carboxypeptidase A.

Changes in the lactic acid extractable proteins of developing wheat endosperms were also studied. Gel filtration on Sephadex G-200, as well as starch gel electrophoresis of the crude extracts, demonstrated that

the proportion of higher molecular weight endosperm proteins increased with maturation of the kernel. There appeared to be no qualitative changes in the proteins. No correlation between fluctuations in the levels of the free amino acids and changes in the endosperm proteins could be shown.

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