STUDIES ON WHEAT FLOUR PROTEINS

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Bachelor of Science

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1961

Submitted to the faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
August, 1963

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ACKNOWLEDGMENTS

The author gratefully acknowledges the guidance and counseling of Mr. Donald C. Abbott throughout this investigation. He also wishes to express appreciation to Dr. James E. Webster and Dr. David R. Wilken for reading the manuscript and giving helpful suggestions. Thanks are also due to Mr. Donald C. Sanders for photographing the starch gels.

The author is grateful to the Biochemistry Department for the provision of laboratory facilities and for financial aid.

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INTRODUCTION

It has been known for many years that wheat flours vary widely in their baking characteristics. It is believed that the flour proteins influence many of these characteristics evident in the baked products. Experimental evidence indicates that these proteins impart to the dough its characteristic cohesive properties and that they are responsible for the gas retention properties in the dough which determine the loaf size and the texture of the baked products.

There are four main classes of proteins in wheat flour as determined by their solubility characteristics. These are: 1) the albumins, which are water soluble; 2) the globulins, which are soluble in neutral salts; 3) the gliadins, corresponding to prolamines which are soluble in 70 percent ethanol; and 4) the glutenins, corresponding to the glutelins which are soluble in acidic or basic solutions. The gliadin and glutenin fractions of wheat flour grouped together comprise a protein complex termed gluten. This classification of the wheat flour proteins based on their solubility characteristics has been shown to be inadequate by electrophoretic and sedimentation studies; however, this is the classification used most often in the literature.

To definitely correlate many of the characteristics of baked products to the proteins contained within the dough, it will be necessary to separate and characterize the proteins as individual components. A chemical and physical characterization of the individual proteins would greatly facilitate assigning their role in the baking process. The

present investigation was undertaken to study the gluten proteins of wheat flour as individual components. Molecular sieve processes, pH fractionation, and alcoholic fractionations were investigated as methods for separating major fractions, and zone electrophoresis was employed to determine the number of components present in the fractions. The electrophoretic composition of the water soluble proteins was also investigated and compared with the electrophoretic composition of gliadin fraction of gluten.

HISTORICAL BACKGROUND

One of the basic needs in the investigation of flour proteins is a suitable method with which to separate and identify individual components. Because of the lack of such a method, much research has been conducted on mixtures. This has resulted in conflicting information concerning the flour proteins. This work, however, has been useful in supporting the concept that flour proteins are directly related to baking quality. Nevertheless, this concept is based on inference and will continue to be until these proteins are separated and isolated and until more information is obtained about their molecular structure. Realizing this basic need, many investigators have attempted to separate the flour proteins and characterize individual components.

Moving-boundary electrophoresis has been employed extensively in investigations of gluten. One of the most serious problems confronting workers using this technique was the lack of a suitable solvent. The development of a buffer system which would give symmetrical patterns in both limbs of the Tiselius apparatus was necessary, since symmetry is required for determining the number of distinct non-interacting components. This was particularly difficult, since gluten and its components are soluble in aqueous media only at very low ionic strengths or in buffers which tend to combine with the protein. This problem was satisfactorily resolved by Jones et al. (1). They demonstrated that symmetrical patterns for ascending and descending boundaries in moving boundary electrophoresis could be obtained by use of relatively low protein concentrations and low

ionic strength buffers. A variety of buffers were used; these were sodium lactate, sodium phosphate, sodium chloroacetate, sodium acetate-hydrochloric acid, aluminum chloroacetate, and aluminum lactate. The pH and ionic strength of all the buffers were 3.1 and 0.03, respectively, while the protein concentration used was 0.3 percent. Four peaks were noted in the sodium lactate and sodium phosphate buffers and these were termed the alpha, beta, gamma, and omega peaks. Using sodium chloroacetate and sodium acetate-hydrochloric acid buffers, these investigators were able to resolve the alpha peak into two components which were designated $alpha_1$ and $alpha_2$. The aluminum lactate buffer proved to be the most useful since symmetrical patterns could be obtained in it using higher concentrations of proteins than could be used with the other buffers. Also, the results from electrophoresis in this buffer indicated that the beta peak contained more than one component. Since the development of this buffer for use in moving boundary electrophoresis, it has been used extensively as a solvent in studies of wheat proteins.

Clusky et al. (2), employed moving boundary electrophoresis to compare the gluten from hard wheat and soft wheat flours. They reported that the gluten of the hard wheat flours contained more alpha and less beta than did the gluten of the soft wheat flours; the amounts of the gamma and omega components were nearly equal. Jones and Dimler (3), using this technique reported that the electrophoretic composition of the gluten from whole flour and from high- and low-protein fractions of air-classified flours were identical. Recently, Koenig (4) made a study of the electrophoretic composition of gluten proteins in acidic and basic buffers and concluded that more satisfactory electrophoretic patterns were obtained from moving boundary electrophoresis in acidic buffers.

Kelley and Koenig (5) analyzed the gluten fraction of various wheat varieties by moving boundary electrophoresis and observed from seven to eleven components in gluten, the number depending upon the variety. They also observed that the Hard Red Winter, the Hard Red Spring, and the Durum wheats showed electrophoretic patterns which are characteristic of their respective classes, while the White wheats showed some electrophoretic differences within the class.

Continuous paper electrophoresis has also been employed to separate gluten proteins. Zentner (6) reported that he was able to separate gluten into seven protein fractions. The second fraction, which contained 54 percent of the total protein, was a lipoprotein fraction, as was indicated by its ability to be stained by a lipid soluble dye.

The most successful technique used thus far for determinating the number of components in the gluten fraction is starch-gel electrophoresis. Woychik et al. (7) made a comparison of the patterns obtained by starch-gel electrophoresis and the peaks obtained in moving boundary electrophoresis. Aluminum lactate buffer, pH 3.1, 0.05 ionic strength containing 3 M urea was used for the starch-gel technique; the same buffer without the urea was used for moving boundary electrophoresis. They reported that there were eight readily observable components which moved into the gel and one component which remained at the origin, as compared with four major peaks observed from moving boundary electrophoresis. Elution of the immobile component from the origin of the gel and subsequent electrophoresis by the moving boundary technique yielded a single peak with a mobility corresponding to that of the alpha component previously noted by Jones et al. (1). This component was assumed to be glutenin because it exhibited the elastic and cohesive properties of whole gluten.

These properties have previously been attributed to the presence of the glutenin fraction (1). The proteins moving into the gel were termed $alpha_1$, $alpha_2$, $beta_{1-4}$, gamma, and omega gliadins in order of decreasing mobility. Due to the additional heterogeneity in wheat gluten demonstrated by starch-gel electrophoresis the nomenclature of the wheat gluten complex was changed from that used by Jones et al. (1). This revision is presented in Table I.

Woychik et al. (7) also made a comparison between starch-gel patterns obtained when electrophoresis was carried out in the presence or absence of urea. The proteins were better resolved and the gel patterns were more distinct when electrophoresis was conducted in the buffer containing the urea. They suggested that the improved resolution might be attributed either to slower diffusion of the proteins or to reduced inter-molecular attractions of the components in the presence of urea. The use of urea in the aluminum lactate buffer was also found to be advantageous in that higher concentrations of gluten solutions could be used, and starch-gels of superior physical qualities could be obtained.

Kaminski (8) reported that gluten contains 21 components as determined by starch-gel electrophoresis. These results, however, were obtained from hand-kneaded dough which had been washed with distilled water and then dispersed in aluminum lactate buffer. When the gluten was prepared from flour which had been extracted with water and dilute salt solutions, 12 main bands were noted as well as five weaker ones. It is possible that some of the faster moving components in the hand-kneaded dough prepared by washing with distilled water were albumins and globulins trapped within the gluten.

Elton and Ewart (9) compared starch-gel electrophoresis patterns of

glutens from wheat varieties of widely differing baking characteristics. The four varieties examined exhibited patterns of similar electrophoretic mobilities. The patterns differed only in the distribution of intensities of the bands. Later work by the same investigators (10) indicated that there was great variation in the leading gluten band among varieties. They suggested that it is this component which corresponds to the alpha component reported by Jones et al. (1). They also suggested that if this component were associated with the gluten-like properties of dough, it is significant that the greatest variation occurred in this band. It is questionable, however, whether this component is associated with gluten-like properties of dough, since Woychik et al. (7) demonstrated that the component exhibiting these properties does not move into the gel. Possibly, these workers were referring to the $alpha_2$ peak which is observed in moving-boundary electrophoresis but which is not believed to be responsible for these properties.

Coulson and Sim (11) reported the presence of 20 components in wheat gluten as shown by starch-gel electrophoresis. These investigators also reported that when a solution of gluten in 0.1 N acetic acid was allowed to stand for several days, changes occurred in the concentrations of the individual proteins. The electrophoretic patterns were qualitatively constant, but the faster moving components became more concentrated at the expense of those of lower mobility. They stated that this may be due to protein dissociation into genetic units and that some of these units may be basic components of gluten.

End group analysis has been used in an effort to determine the minimum number of proteins present in wheat gluten. Winzor and Zentner (12) reported the presence of eleven N-terminal amino acids in wheat gluten.

These were glutamic acid, aspartic acid, serine, threonine, glycine, alanine, valine, leucine, phenylalanine, methionine, and histidine. These results indicate that the minimum number of polypeptides present in gluten is eleven. Other workers have reported detection of some of the amino acids listed above as end groups in gluten, but the results show considerable variation (13, 14, 15, 16). The reasons for the diversity of results are not apparent. There is, however, a possibility of differences in protein structure of the various wheats which were studied.

There have been some attempts to separate the gluten proteins by ion-exchange chromatography. Woychik et al. (17) reported that a chromatographic separation of wheat gluten proteins could be accomplished by stepwise elution from carboxymethylcellulose employing buffers with increasing hydrochloric acid concentration, 0.01 N acetic acid being the starting buffer. The alpha, beta, gamma, and omega gliadins were separated in this manner and were identified by moving boundary electrophoresis. The glutenin fraction was not obtained by this procedure and was believed to have been retained by the carboxymethylcellulose. The relative concentrations of the fractions eluted from the column were as follows: alpha, 60 percent; beta, 15 percent; gamma, 15 percent; and omega, 3 percent. The remaining seven percent was assumed to be albumin and globulin contaminants.

Simmonds and Winzor (18) reported the separation of the gluten proteins into 11 fractions on carboxymethylcellulose equilibrated with 0.05 M acetate buffer, pH 4.1. Under these conditions all the proteins were adsorbed except one which passed through the column unretarded. By employing a gradient to 0.2 M NaCl at pH 4.1, in the presence of 1 M

dimethylformamide to prevent protein precipitation, eight fractions were eluted. The tenth fraction was eluted from the column with 0.005 M acetate buffer containing 1.0 M dimethylformamide and 0.5 M NaCl; the eleventh fraction was eluted with a phosphate solution, pH 12 containing 0.005 M trisodium phosphate, 1.0 M dimethylformamide and 0.5 M NaCl. Rechromatography of fractions three, four, six and ten on carboxymethylcellulose indicated these were distinct protein entities.

Recently, Graham (19) has employed starch-gel electrophoresis to investigate the fractions obtained by Simmonds and Winzor (18) from flour gluten on carboxymethylcellulose. She noted that these fractions were not homogeneous and that similar electrophoretic components were present in several of chromatographic fractions. Similar results were obtained by starch-gel electrophoresis of the fractions separated by Coates and Simmonds (20) and Simmonds (21) by fractionation of the pyrophosphate soluble flour proteins on diethylaminoethyl cellulose (DEAE-cellulose). The overall protein patterns on the gels, however, were different from those for the gluten proteins. Graham (19) also compared the electrophoretic composition of the extracts of wheat flour obtained by use of different solvents. Sodium pyrophosphate, water, ethanol, and acetic acid were the solvents employed. The water extract contained a larger proportion of the slower-moving gliadin proteins, but lacked some of the faster-moving globulin proteins, as compared with the pyrophosphate extract. The electrophoretic patterns of the ethanol and acid extracts were similar to each other but differed from the patterns of the pyrophosphate and water extracts in that many of the faster moving components were absent and the gliadin proteins were present in higher quantities. These results substantiated the inadequacy of classification

of the proteins of flour by solubility characteristics.

A combination of ion-exchange chromatography and starch-gel electrophoresis has also been employed by Graham and Morton (22) in studies of the proteins of developing wheat endosperm. No marked changes were found to occur in the types of proteins present in the endosperm between the twelfth day after flowering and maturity.

This brief survey covers only the investigations of wheat proteins reported in recent years and is confined primarily to techniques employed to separate these proteins. Several reviews (23, 24, 25, 26, 27) have been written which survey the studies of wheat proteins more extensively.

TABLE I

NOMENCLATURE REVISION OF THE PROTEINS OF THE WHEAT GLUTEN COMPLEX[a]

Jones et al. (1)	Woychik et al. (7)
$Alpha_1$ gluten	Glutenin
$Alpha_2$ gluten	$Alpha_1$ and $alpha_2$ gliadin
Beta gluten	$Beta_{1-4}$ gliadin
Gamma gluten	Gamma gliadin
Omega gluten	Omega gliadin

[a] Woychik, J. H., Boundy, J. A. and Dimler, R. J., Arch. Biochem. Biophys., 94, 477 (1961).

EXPERIMENTAL PROCEDURE

A. Materials

The electrophoresis apparatus employed was a commercial unit, Model EC305, manufactured by the EC Apparatus Company, New York, New York. Starch used in electrophoretic studies was obtained from Connaught Medical Research Laboratories, Toronto, Canada. The aluminum lactate used was a commercial product from K and K Laboratories, Inc., Jamaica, New York.

The dextran gels employed were commercial Sephadex preparations, G-100 and G-200, obtained from Pharmacia, Uppsala, Sweden.

The wheat flours were milled on a Buhler experimental mill and were supplied by the Small Grains Laboratory, Agriculture Experiment Station, Oklahoma State University. The flours used were milled from three Hard Red Winter wheat varieties, Superking, Comanche, and Ponca. These flours contained 14.7 percent, 12.9 percent and 13.7 percent protein and 7.6 percent, 9.3 percent, and 9.8 percent moisture, respectively.

The starch-gels were photographed by transmitted light on Panatomic-X film through a type A (red) filter. The films were developed for seven minutes at 25° C in D-76 developer diluted one to one. Enlargements were made on Kodabromide paper (F-4) developed in Dektol.

B. Methods

Preparation of aluminum lactate buffer. A solution of 0.00835 M aluminum lactate was prepared and the pH adjusted to 3.1 with lactic acid. The calculated ionic strength of a buffer prepared in this manner is approximately 0.05, but this may not be the actual ionic strength. The

actual ionic strength is best determined by conductivity measurements. The buffer containing 3 M urea was prepared by adding the urea to the aluminum lactate solution before adjusting the pH.

Preparation of the gluten proteins. The gluten proteins were prepared by the method of Jones et al. (1). The flow diagram in Figure 1 briefly outlines this procedure. Fifty grams of flour were extracted with 120 ml of water-saturated butanol for one hour with stirring and were then centrifuged at low speed for five minutes. The butanol supernatant was decanted and the flour remaining in the centrifuge cup was washed two to three times with ether to remove any remaining butanol. The defatted flour was air dried and then mixed with water until a dough was formed. The dough was hand-kneaded under a stream of 0.1 percent NaCl solution to remove starch as well as proteins soluble in water and dilute salt solutions. The gluten ball remaining was dispersed in 0.01 N acetic acid at about 5 percent protein concentration by stirring overnight; it was then centrifuged at 2,000 x g for 20 minutes to remove remaining starch. The opaque dispersion that resulted was centrifuged at 20,000 x g for 20 minutes to give a clear supernatant solution. This solution was heated quickly to 99° C to inactivate proteolytic enzymes and cooled immediately. It was then freeze-dried, yielding a fluffy white product.

Preparation of water soluble proteins of flour. Figure 2 is a flow chart outlining the preparation of the water soluble proteins of flour. Ten grams of flour were extracted with 15 ml of water for one hour with occasional stirring. The flour-water slurry was centrifuged at 10,000 x g for 20 minutes and the supernatant decanted. The supernatant containing the water-soluble proteins was frozen and stored until used for

electrophoresis experiments. The experiments were carried out within four days from the time the protein solutions were prepared.

Preparation of the glutenin proteins. The glutenin proteins were prepared by two techniques. The first technique used is outlined in Figure 3. Whole gluten was extracted with 70 percent ethanol for 20 minutes at room temperature. The extraction mixture was centrifuged at 20,000 x g for 20 minutes at room temperature. The residue containing the glutenin was then dissolved, at a concentration of approximately 100 mg per ml in aluminum lactate buffer, pH 3.1, 0.05 ionic strength containing 3 M urea. This solution was then used for electrophoretic studies. The second technique for preparation of glutenin was by molecular sieve filtration. This procedure is described in a later section.

Preparation of the gliadin proteins. The gliadin proteins were prepared by several methods.

1. Direct extract of flour. The procedure for the alcoholic extraction of flour is outlined in Figure 4. Four grams of flour were extracted in 10 ml of 70 percent ethanol for 20 minutes with rapid stirring. The flour-ethanol mixture was then centrifuged for 20 minutes at 20,000 x g at room temperature. The supernatant containing the gliadin proteins was decanted and used immediately for electrophoretic investigations. A portion of this solution was also diluted to twice its volume with water and chilled for six hours to precipitate the gliadin proteins. The chilled mixture was then centrifuged at 20,000 x g for 20 minutes at 2° C. The gliadin residue was suspended in 2 ml of aluminum lactate buffer and used for electrophoretic investigations.

2. Alcoholic extract of water extracted flour. The flow chart

in Figure 5 summarizes the preparation of gliadin from wheat flour by the method of Elton and Ewart (10). Two hundred grams of flour were extracted with stirring for one hour at room temperature with 800 ml of water and then centrifuged at 6500 x g. The supernatant containing the soluble proteins was discarded. The residue containing the gluten, starch, and other particulate matter was extracted with 500 ml of 70 percent ethanol overnight with continuous stirring and then centrifuged at 20,000 x g for 20 minutes at room temperature. The supernatant containing the gliadin proteins was dialyzed in the cold against 0.01 N acetic acid for 24 hours to remove the ethanol. The dialysate was then freeze-dried to yield a fluffy, white product.

3. Alcoholic extraction of whole gluten. Figure 3 includes a flow chart outlining the preparation of gliadin from whole gluten. Fifty milligrams of gluten were extracted for 20 minutes in one ml of 70 percent ethanol with stirring and then centrifuged at 20,000 x g for 20 minutes at room temperature. The supernatant containing the gliadin proteins was decanted and employed in electrophoretic studies.

4. pH fractionation of whole gluten. The preparation of gliadin proteins by pH fractionation according to Jones et al. (1) is summarized in Figure 6. One hundred milliliters of a 0.3 percent solution of wheat gluten in 0.017 M aluminum lactate, pH 3.4, were adjusted to pH 4.8 with 0.2 M NaOH. The fractionated mixture containing precipitated glutenin was left overnight in the cold and then centrifuged at 20,000 x g for 20 minutes at 2° C. The supernatant fraction was dialyzed against 0.01 N acetic acid and then freeze-dried. A second gliadin preparation was obtained by a modification of this procedure. This involved using 0.01 N acetic acid as the protein solvent instead of aluminum lactate buffer and

omitting the dialysis step. The gliadin proteins were also prepared by a molecular sieve fractionation to be described in the following section.

Preparation of the glutenin and gliadin fractions by molecular sieve process employing Sephadex G-200.

Preparation of the column. After the Sephadex gel had been allowed to swell and the fines removed by decantation, the column was prepared by pouring a thick slurry of the gel through a tube inserted to the bottom of the column. Glass wool layered with small glass beads was substituted for a glass frit in the columns used for whole gluten separation, because columns fitted with frits ceased to flow when the glutenin fraction reached the frit. Although the flow rate decreased appreciably at this point in columns containing the glass wool, the column continued to flow. The length to diameter ratio and the volume of the columns prepared in this manner were ten to one and 880 ml, respectively.

Fractionation procedure. Two hundred fifty milligrams of gluten protein in 20 ml of solution were added to the column and eluted by the appropriate buffer. The eluate was collected by an automatic fraction collector and the absorbance of the individual tubes was measured at 280 mμ to determine the elution patterns. The tubes comprising an elution peak were pooled, dialyzed if necessary, and freeze-dried. All fractions were qualitatively analyzed by starch-gel electrophoresis.

Fractionation of gliadin preparations by molecular sieve action. Gliadin obtained both by fractionation of gluten on Sephadex G-200 and by pH fractionation procedures were further fractionated on Sephadex G-100. The column was prepared as described by Flodin (28). The height to diameter ratio and the volume of the column were ten to one and 590 ml, respectively. In these experiments a column containing a frit was

used since the flow rate was not appreciably altered by gliadin. The flow rate was maintained at approximately 20 ml per hour. The eluate was subjected to the procedures described for the eluates from the Sephadex G-200 columns.

Quantitative protein estimations. Total nitrogen was determined on gluten and gliadin preparations by a microkjeldahl procedures (29). Conversion of nitrogen to protein was made by use of a factor of 5.7. Estimations of protein in the fractions from the column were carried out using the method of Lowry et al. (30).

Starch-gel electrophoresis. The basic procedure followed for starch-gel electrophoresis was essentially as described by Smithies (31), but modified for use with wheat flour proteins. The method used in this investigation is similar to that described by Woychik et al. (7). The buffer used was aluminum lactate, pH 3.1, 0.05 ionic strength, and containing 3 M urea. Starch gels were prepared by heating 60 g starch in 400 ml buffer over a Bunsen burner with continuous swirling until the starch gelatinized. Heating was continued until the solution boiled slightly, then the flask was evacuated for 30 seconds to remove air bubbles and the solution poured into six plexiglas trays (25 x 1.9 x 0.6 cm). The trays were covered with plastic strips and refrigerated for several hours. After refrigeration excess gel was removed from the trays by slicing with a thin wire. The protein was applied to the gels by saturating a filter paper wick with the protein solution and inserting the wick into a slit previously cut near the anode end of the gel. The gels were covered with thin plastic strips and placed in the electrophoresis apparatus. Paper wicks extending from each end of the gel tray were placed into the buffer compartments. A potential difference of approximately

six volts per cm was applied across the gels for a specified time. After electrophoresis, the gels were sliced into four one-sixteenth inch sections. The two center sections were stained in a saturated solution of Amido Black 10B in five percent acetic acid for at least ten minutes. The excess dye was removed by repeated washing of the stained strips with five percent acetic acid. The details concerning the method used are summarized in Table II.

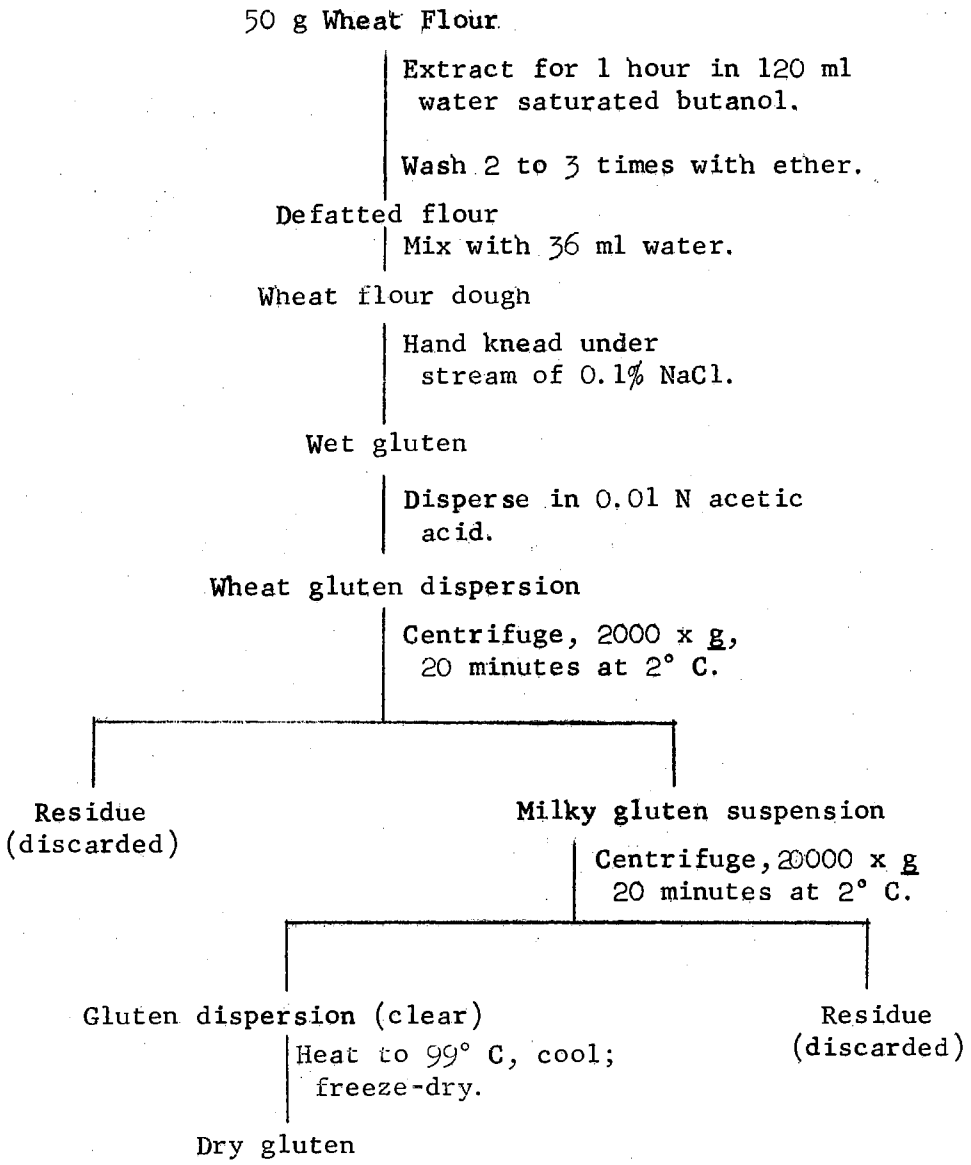


Figure 1. Flow diagram outlining gluten preparation.

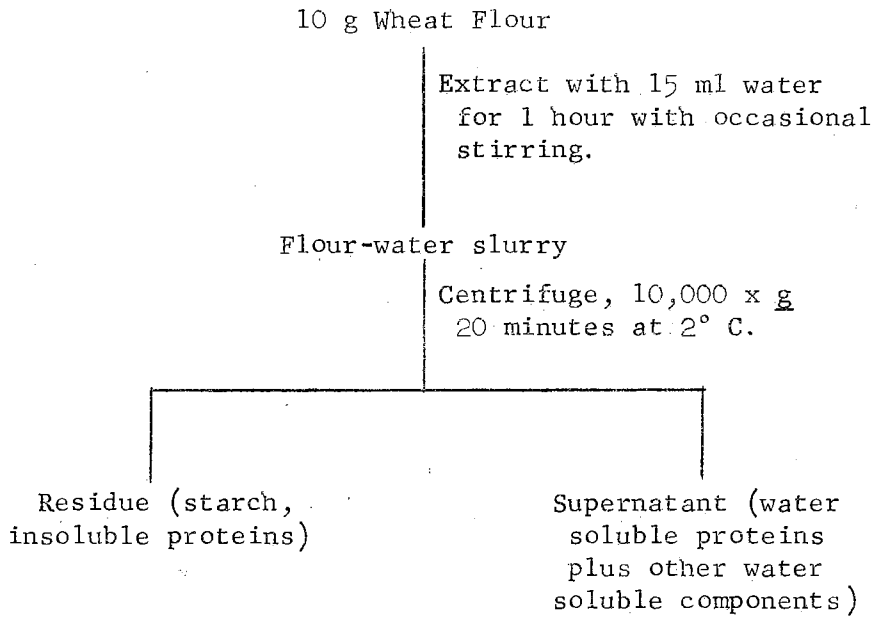


Figure 2. Flow diagram outlining procedure for preparation of water soluble proteins of flour.

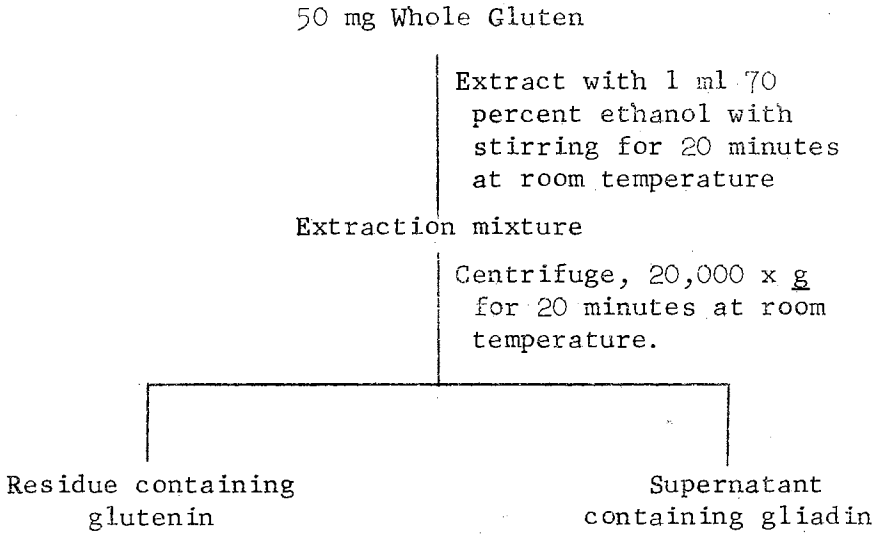


Figure 3. Flow diagram outlining the preparation of glutenin and gliadin by alcoholic extraction of gluten.

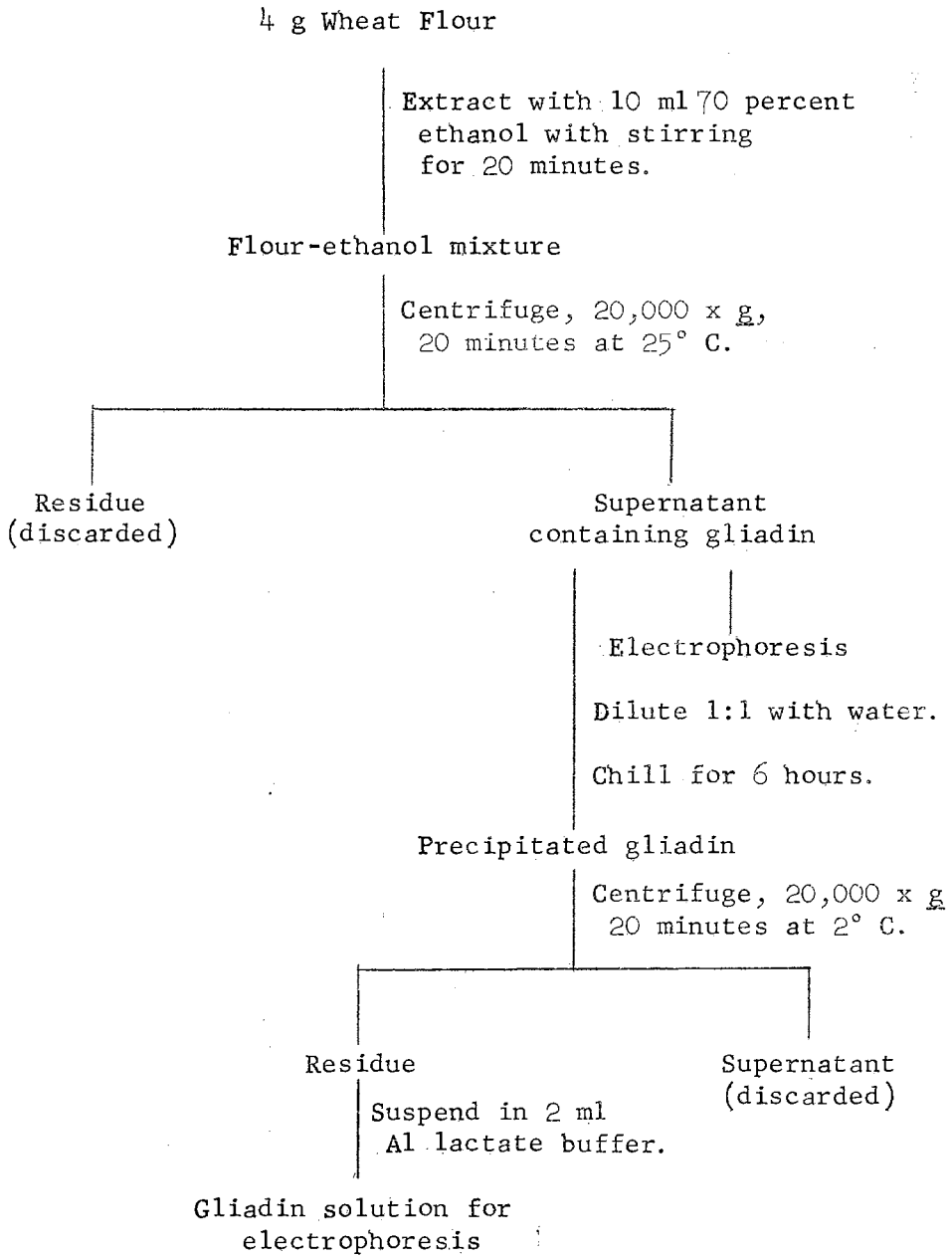


Figure 4. Flow diagram outlining preparation of gliadin by alcoholic extraction of flour.

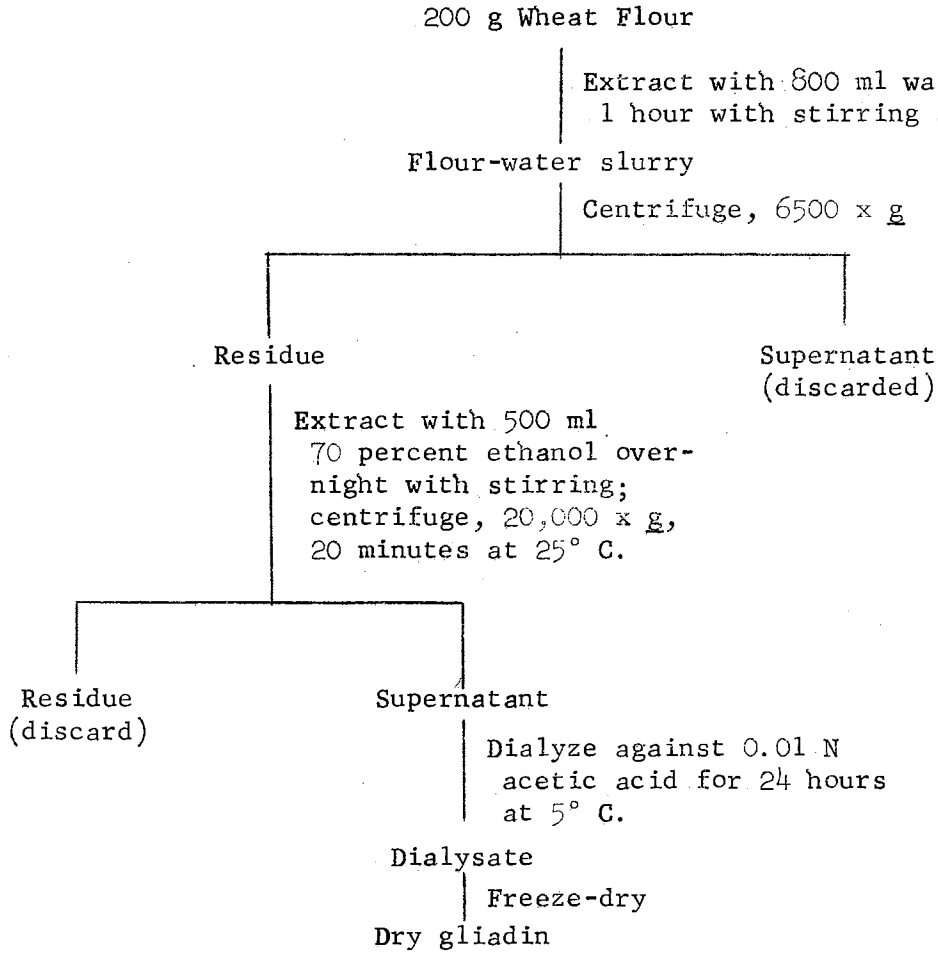


Figure 5. Flow diagram outlining preparation of gliadin by alcoholic extraction of water-extracted flour.

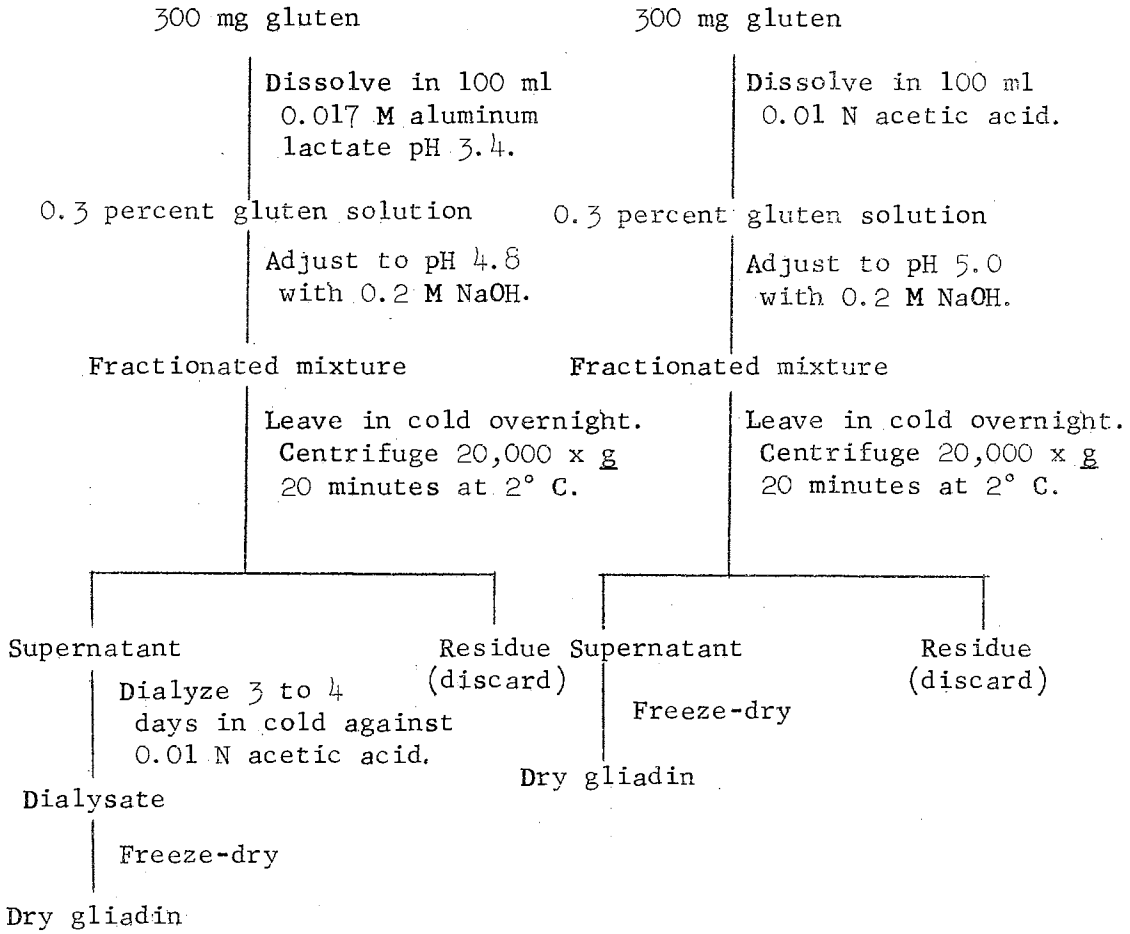


Figure 6. Flow diagram summarizing gliadin preparations by pH fractionation.

TABLE II

DETAILS IN STARCH-GEL ELECTROPHORESIS PROCEDURE[a]

Buffer	Al lactate, pH 3.1, 0.05 μ containing 3 M urea
Conc. of samples	
Gluten	10 mg/gel
Gliadin	4-5 mg/gel
Glutenin	4-5 mg/gel
Water extract	3-4 mg/gel
Conc. of gel	15 g starch/100 ml buffer
Size of sample wick	1.9 X 0.6 cm of No. 470 Schleicher and Schuell filter paper; water extracts - 2 wick thicknesses; all other samples 1 wick thickness
Size of gel trays	25 X 1.9 X 0.6 cm
Voltage	6 volts/cm
Length of electrophoresis	
Gluten, gliadin, glutenin	36 hours
Water extracts	12 hours
Protein stain	Saturated solution of amido black 10B in 5 percent acetic acid
Wash solution	5 percent acetic acid

[a] Any deviations from these details are noted in the text.

RESULTS AND DISCUSSION

As was noted in the literature review, there has been much starch-gel electrophoresis work with gluten proteins on an analytical scale with seemingly good resolution of proteins. It was thought that this procedure might be adapted as a small scale preparative method for isolating the proteins of the gluten complex, but preliminary investigations indicated that it was not suitable. As shown on Plate I, gel 1, some protein components are not well resolved and are very close together on the gel; thus, the physical removal of individual proteins from the gel would be very difficult. Although location of the bands can be accomplished by staining only a narrow strip cut from the gel, determining the exact position of each band on the main part of the gel is very difficult when the bands are so close together since the length of the stained gel may change during the staining process leading to a mislocation of the protein bands. The separation is further complicated by the curvature of the bands which causes some overlapping of proteins on the gel.

While starch-gel electrophoresis could not be used as a preparative method under the condition described, it appeared to have possibilities as an analytical tool to assay preparations from other isolation and purification procedures. To be employed in this manner, however, improved starch gel patterns were desirable. The need for improved patterns is illustrated by gel 1, Plate I. In this gel the resolution of some bands is poor, and the determination of the number of protein bands is difficult

because of streaking from the origin. It was thought that these effects might be due to the presence of glutenin, the high molecular weight component of gluten. This was substantiated by electrophoresis of a glutenin preparation. This preparation produced the same effects, the streaking and hardening at the origin, as were noted in the gliadin preparation described above. For this reason, removal of the glutenin proteins seemed desirable. This was first attempted by alcoholic extraction of flours and gluten samples. Typical results of electrophoretic analysis of gliadin prepared in this manner are shown in Plate I, gel 2. The starch gels were usually streaked and were deeply stained at the origin while the protein bands were smeared and unclear. The gel at the origin appeared to contain much of the applied protein and was always hardened at completion of electrophoresis. Because of the difficulty slicing through the hardened material the ends of the gels were removed. The hardening of the gels at the origin indicated that glutenin was present in the alcoholic extracts of flour and gluten. Thus, classical methods for gliadin preparation based on its solubility in 70 percent ethanol are unsatisfactory because such gliadin is contaminated with glutenin. It was evident that other methods would be required to remove the glutenin proteins from gliadin.

In addition to a method for the preparation of gliadin suitable for electrophoretic work, a technique to separate the proteins of the gluten complex on a preparative scale was desired. Based on reports that gluten is composed of proteins of widely varying molecular weight fractions, it was thought that Sephadex G-100 and G-200 might be used to facilitate a separation of components of the gluten complex. Preliminary experiments with Sephadex G-200 on small columns (2.4 x 24 cm) resulted in a separation

both of gluten and of gliadin prepared by alcoholic extraction into two fractions. Because only small quantities of protein could be used with these columns; fractions obtained could not be assayed by starch-gel electrophoresis. Therefore, a larger Sephadex G-200 column was prepared as described in the methods section, in order to facilitate fractionation of larger quantities of protein. It had been observed with the small columns that there was a marked reduction in the flow rate when the first protein component, presumably glutenin, was eluted. For this reason gliadin prepared by alcoholic extraction of flour was used first on the large column since this preparation was presumed to contain less glutenin than whole gluten. Presumably this preparation would slow the flow rate less than whole gluten. The elution pattern for 250 mg of Comanche gliadin is shown in Figure 7. The four fractions present were subjected to analysis by starch-gel electrophoresis. The results of electrophoresis of the fractions, shown in Plate II, indicate that the first three fractions, comprising approximately one-half of the protein recovered, contained glutenin proteins since the protein remained at the origin as is noted in gels 1, 2 and 3. The fourth fraction, containing the remaining protein, was comprised of components, shown in gel 4, which migrated at a mobility similar to that of the gliadin proteins and is, therefore, assumed to be the gliadin fraction of gluten. These results substantiate the earlier conclusion that alcoholic extraction is not a satisfactory preparation for gliadin. Apparently both the glutenin and gliadin are dissolved in the alcohol. On the basis of these results, gel filtration on Sephadex G-200 appeared to be superior to other methods for fractionating gluten-like samples into the component fractions, glutenin and gliadin. The resolution and clarity of starch gel patterns for the gliadin from the

column were much better than had been noted before from the gluten and from the gliadin prepared by alcoholic extraction, and there was no evidence of streaking or hardening of the gel. Sephadex G-200 appears to be useful for the separation of glutenin from gliadin but not for the fractionation of the proteins of the gliadin group.

From the results obtained from gel filtration of alcoholic extracts on Sephadex G-200, it was concluded that the alcoholic extract was not superior to whole gluten as starting material for this type fractionation since this extract contained glutenin which slowed the column appreciably. For this reason gluten was used in subsequent fractionations employing Sephadex G-200.

The elution pattern of Comanche gluten fractionated by molecular sieve action on Sephadex G-200 is shown in Figure 8. The results of starch-gel electrophoresis of the elution peaks from the fractionation are shown in Plate III. Electrophoretic analysis of the first two peaks, shown in gels 1 and 2, indicated that they are glutenin since most of the protein remained at the origin, and the gels were somewhat streaked. The next large peak, which was divided into three portions for electrophoretic analysis, shown in gels 3, 4 and 5, yielded 17 bands. The four slowest moving components in the gels were more concentrated in the portion of the peak eluted first from the column. This indicates that these proteins are of higher molecular weight than other components in this peak. This conclusion assumes no adsorption or other charge effects which would affect the elution characteristics of the proteins.

The gliadin peaks resulting from fractionation of both the gliadin and gluten from Comanche wheat flour on Sephadex G-200 were further fractionated on Sephadex G-100. Approximately 90 mg of protein were

applied to the column. A typical elution pattern is shown in Figure 9.

Because of the small quantities of protein recovered in the peaks from the Sephadex G-100 column it was not possible to determine the number of protein components present by application of starch-gel electrophoresis. Therefore, a method was sought to prepare gliadin of the same purity as that from Sephadex fractionations but in greater quantities than possible with Sephadex. The pH fractionation of gluten as employed by Jones et al. (1), appeared to be applicable since these workers reported suitable separation of glutenin from gliadin by a precipitation of the glutenin proteins. Since gliadin prepared by alcoholic extraction of flour was available in sufficient quantity, it was subjected to pH fractionation using aluminum lactate as a solvent. Electrophoretic analysis of the gliadin preparation, shown in gel 4, Plate IV, indicated that the glutenin had been removed. The gliadin proteins were well resolved and the bands on the gels were easily distinguished. A comparison of the gel pattern, shown in gel 4, Plate IV, with those from peak three of the eluate from the gluten fractionation of Sephadex G-200, shown in gel 1, 2 and 3, Plate IV, revealed that there were not as many proteins present in the gliadin prepared by pH fractionation as in the gliadin prepared by the molecular sieve process; however, the overall gel patterns were similar. It is possible that the proteins absent from the gliadin prepared by the pH fractionation may have been lost before this fractionation was carried out since whole gluten was not used as a starting material.

Since the overall patterns were similar to those of gliadin prepared by Sephadex G-200 fractionation, the preparation was used for further fractionation on a Sephadex column. Two hundred fifty milligrams of the

Comanche gliadin prepared by pH fractionation of an alcoholic extract were subjected to fractionation on Sephadex G-100. The elution pattern was similar to that in Figure 9. Peaks one, two, three and four contained 15.5, 59, 21.5 and 4 percent of total protein eluted, respectively. Approximately 80 percent of the total protein applied to the column was recovered in the eluate. The protein in peaks one, two and three were subjected to starch-gel electrophoresis for 36 hours. The results are shown in gels one, two and three in Plate V. Peak one is a glutenin contaminant of the gliadin fraction as evidenced by its lack of mobility. Peak two is composed of gliadin proteins and one faster moving component. Only four faint bands were noted on the gel containing a sample from the third peak. Two of these were of the same mobility as the gliadin proteins; the other two bands were faster moving. It was suspected that this peak might contain highly mobile water soluble proteins as contaminants of gliadin and that nearly all of these had migrated off the gel in 36 hours. Therefore, electrophoresis was applied to this peak for 12 hours with the results shown in gel 4, Plate V. It is evident that this third peak contains substantial quantities of rapidly-moving proteins. The nine faster moving components were shown by later experiments to correspond in mobility to the water soluble proteins of flour, while the two slower moving components corresponded to the gliadin proteins. The results of starch-gel electrophoresis of the eluted peaks from gliadin on Sephadex G-100 indicate that this Sephadex gel is not suitable for fractionation of the gliadin proteins under the conditions employed. It should be useful, however, for separating flour proteins into the glutenin, gliadin, and water- and dilute salt-soluble proteins on the basis of their differing molecular weights.

Using a gluten preparation from Ponca wheat flour, Woychik et al. (7) reported only eight protein bands moving into the gel. It was of interest, therefore, to determine whether the increased resolution of gliadin proteins from Comanche flour in the present study was due to varietal differences or to the molecular sieve process used to prepare the gliadin. In addition, a more rapid method for preparing gliadin in relatively large quantities and free of glutenin was sought. Gluten from a sample of Ponca wheat was prepared and used in experiments designed to accomplish these objectives.

Earlier attempts to prepare gliadin by pH fractionation employed an alcoholic extract of gluten as the starting material. It was found that at least two gliadin components were missing from the gliadin obtained. However, since the pH fractionation procedure is simple and lends itself to the preparation of relatively large quantities of gliadin, it was investigated further. In addition the conditions for gel filtration on Sephadex G-200 were further studied.

Procedures for pH fractionation of dilute solutions of gluten included precipitation of glutenin from both 0.017 M aluminum lactate and o.01 N acetic acid. For gel filtration of gluten on Sephadex G-200, either aluminum lactate, pH 3.1, 0.05 ionic strength or 0.01 N acetic acid, was used as the eluent. The procedures employed are listed in the Experimental Procedure section. By employing dilute acetic acid in either the pH fractionation or the molecular sieve process, it is possible to eliminate the time-consuming dialysis step which is required to remove the aluminum lactate. Most of the acetic acid is removed during the freeze-drying.

The results of electrophoretic analysis of the products obtained by

pH fractionation of gluten in acetic acid and aluminum lactate buffers are shown in gels one and two, respectively (Plate VI). There is a staining of the origin and some streaking of the gels containing samples of the products of fractionation from acetic acid. This observation indicates that some of the glutenin components are still present in the preparation. Their concentration, however, is apparently not great enough to affect the gel patterns. No traces of glutenin are noted in the gels containing the product of fractionation from the aluminum lactate buffer.

The fractionation of Ponca gluten on Sephadex G-200 using acetic acid as eluent was different from that employing aluminum lactate. This difference is apparent in the elution pattern shown in Figure 10. The first peak from the fractionation employing acetic acid is much smaller than the first peak of the aluminum lactate fractionation and the percentage of protein recovered from gluten applied to the column was not as high from the acetic acid fractionation. The recovery from the elution in acetic acid was 80 percent while 95 percent of the original sample was recovered when aluminum lactate was used as the eluent. This indicates that some of the glutenin may have been retained on the column. As shown in Plate VI, the starch-gel pattern of the gliadin fraction (peak two) from the fractionation employing acetic acid revealed that some glutenin protein was eluted from the column with the gliadin proteins. In other respects the starch gel patterns for the gliadin fraction (peak two) from both Sephadex fractionation were similar.

The gel patterns of the gliadin preparations from Sephadex G-200 fractionation contain one more major protein band than the products of pH fractionation. This band exhibits a mobility similar to the omega protein reported by Woychik et al. (7). This protein may have been

precipitated with the glutenin fraction during pH fractionation of gluten.

Of the two pH and the two Sephadex fractionation methods, the fractionation of gluten by Sephadex G-200 using aluminum lactate, pH 3.1, 0.05 ionic strength, appears to be the superior method of preparing gliadin. This conclusion is supported by the starch gel patterns shown in Plate VI.

The gel patterns obtained from the Ponca gliadin prepared on Sephadex G-200 using aluminum lactate buffer are similar to those reported by Woychik et al. (7) except that in the present study two more bands were noted. One of these is between the $alpha_1$ and $alpha_2$ gliadin bands while the other band is a very faint one appearing between the $alpha_2$ and the $beta_1$ proteins. The starch gel patterns for the beta, gamma and omega proteins are essentially the same as those reported by Woychik et al. (7). The overall electrophoretic patterns for the gliadin of Comanche and Ponca wheat flour, shown in Plate VII, are similar; however, the gliadin of Comanche flour exhibits seven bands not detected in Ponca gliadin. It is possible that the ten protein bands of Ponca gliadin are a minimum number and that increased clarity and resolution of the bands would reveal more proteins. These results indicate, however, that there may be varietal differences between these two wheats.

The data from the fractionation of Ponca gluten on Sephadex G-200 is compatible with reports that the beta and gamma gliadins have molecular weights of 42,000 and 45,000, respectively (32). These results, are not, however consistent with the estimate that impure alpha gliadins have an average molecular weight of about 200,000 (32). If this estimate were correct, it would be expected that the alpha gliadin would be eluted from the column nearer the high molecular weight glutenin components, since the upper molecular weight limit which will partition in Sephadex G-200

is approximately 200,000 (33). The alpha gliadins, however, were eluted from the column within the peak containing the beta and gamma proteins. This indicates that the molecular weights of the alpha gliadins are closer to those of the beta and gamma components than to a molecular weight of 200,000.

Another indication that the alpha gliadins are similar to the beta and gamma gliadins is the observation that for Comanche gliadin the alpha gliadins were eluted from a Sephadex G-100 column in the same peak as the beta and gamma gliadins. The electrophoretic behavior of these gliadins was very similar to that of Ponca gliadins. If it is assumed that the gliadins of Ponca are the same proteins as those of Comanche, then it may be concluded that the molecular weight for the alpha gliadins of Ponca is less than 100,000, since the upper limit for fractionation with Sephadex G-100 is about 100,000 molecular weight (33).

In order to determine whether or not any of the bands on the gel patterns of gluten and gliadin preparations were actually contaminants consisting of water-soluble proteins, the mobilities of the proteins soluble in water were compared to those of gluten. Electrophoresis for both 12 and 36 hour periods was carried out simultaneously on both glutens and water extracts from Comanche, Ponca and Superking flours. The results, shown in Plates VIII and IX indicate that there are some components present in the gluten preparations which correspond to the faster moving proteins of the water extracts. The presence of gliadin proteins in the water extracts shows that the gliadins are slightly water soluble, while the presence of the water soluble components in the gluten patterns may be due to their physical entrapment in the gluten. It is clear that when electrophoresis of gluten or gliadin is carried out for 36 hours the

more mobile water soluble proteins are moved either completely out of the gels or moved above the bands representing the gliadin proteins and do not interfere with the gluten or gliadin bands.

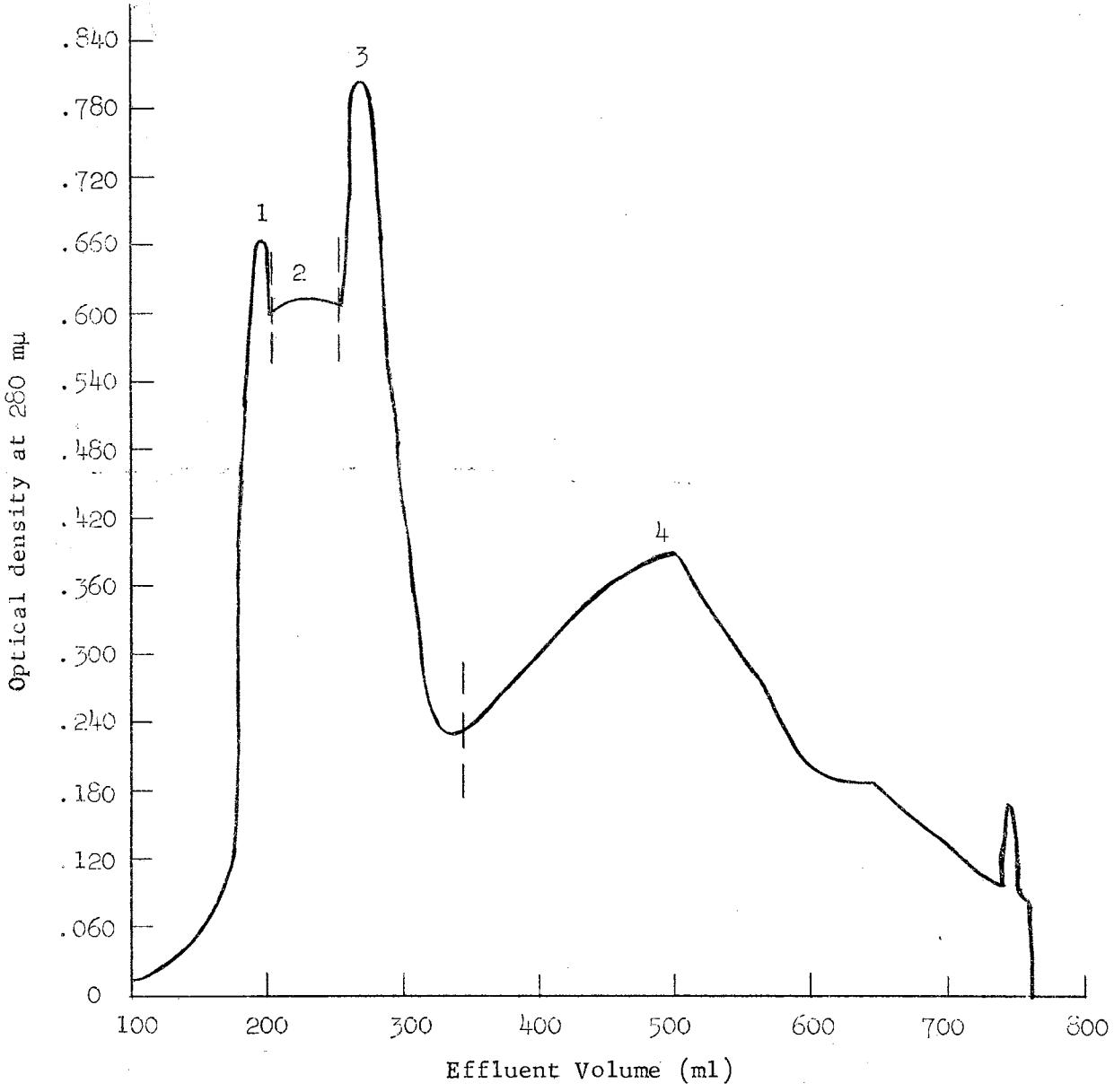


Figure 7. Elution pattern on Sephadex G-200 in Al lactate (pH 3.1, 0.05 μ) of Comanche gliadin prepared by alcoholic extraction of flour.

Note: The eluate was divided at the volumes indicated by the broken lines.

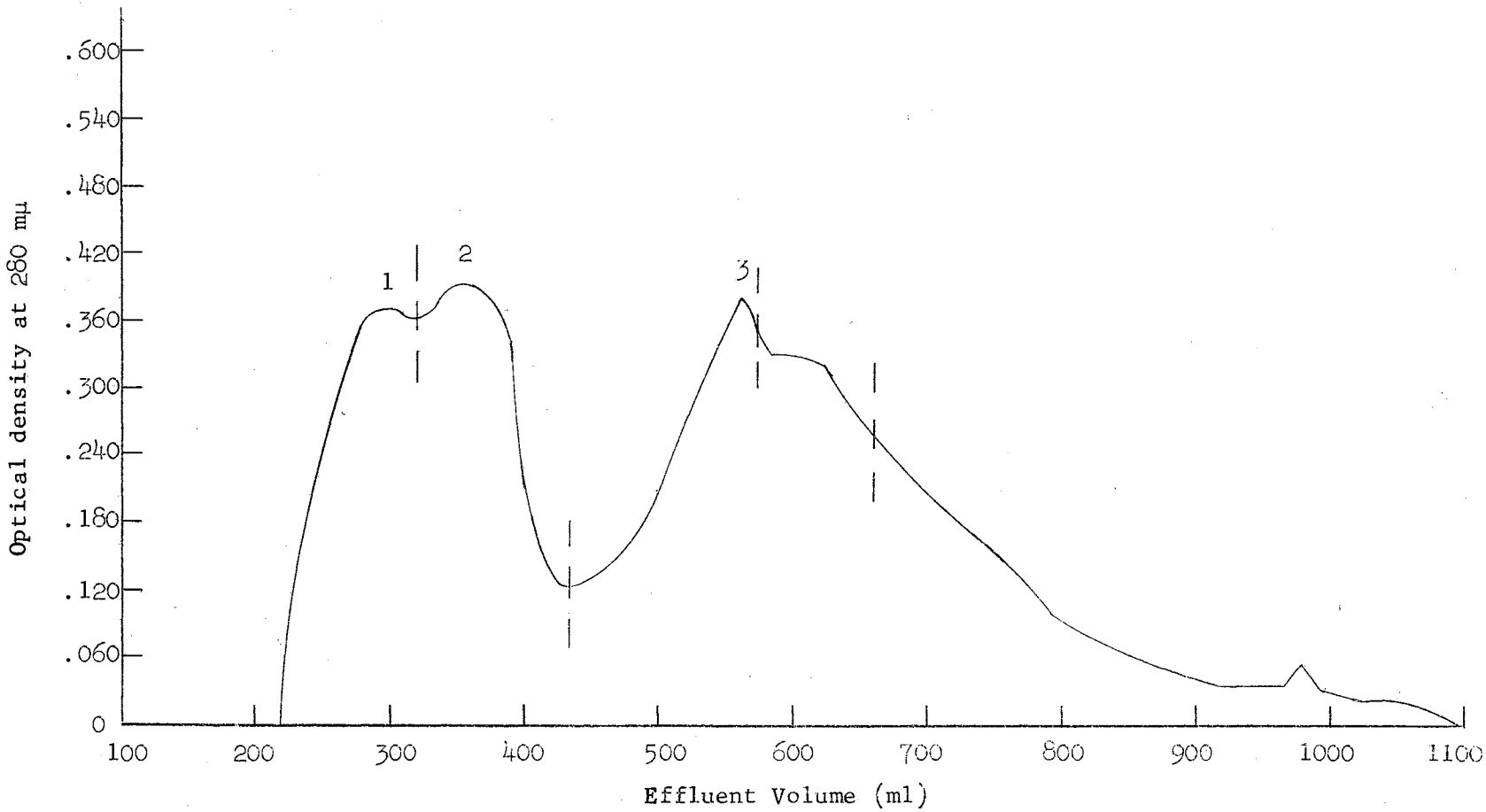


Figure 8. Elution pattern on Sephadex G-200 in Al lactate (pH 3.1, 0.05 μ) of Comanche gluten.

Note: The eluate was divided at the volumes indicated by the broken lines.

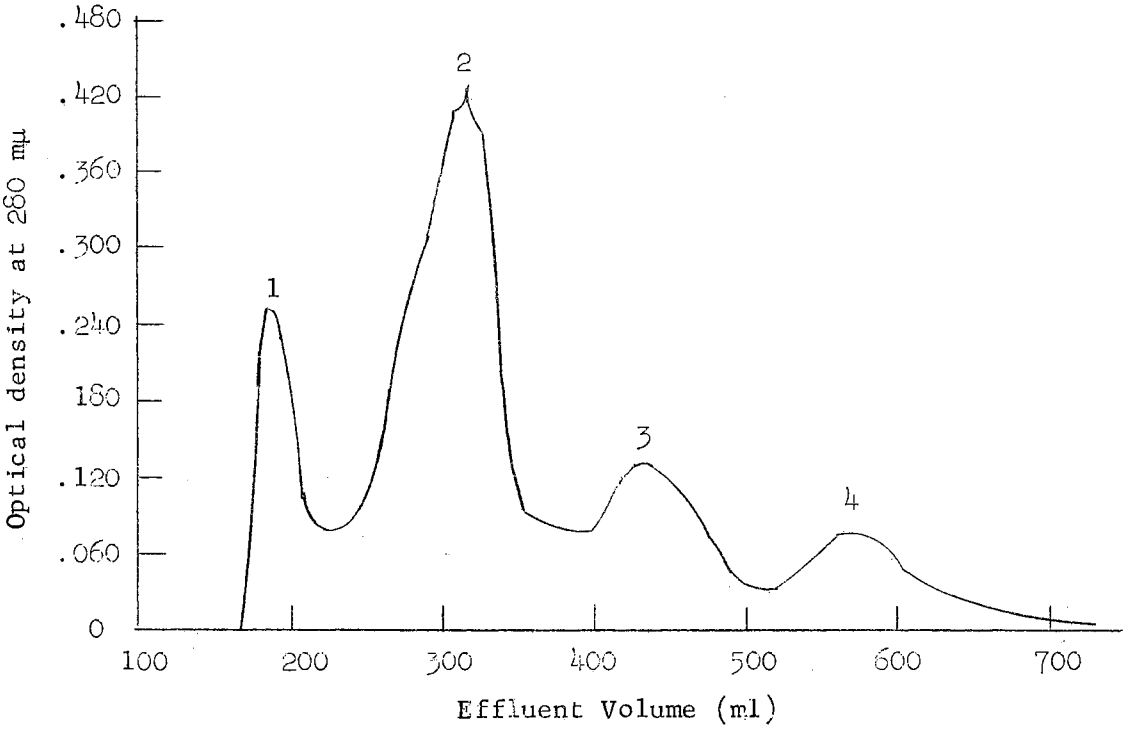


Figure 9. Elution pattern of Comanche gliadin on Sephadex G-100 in Al lactate, pH 3.1, 0.05 μ.

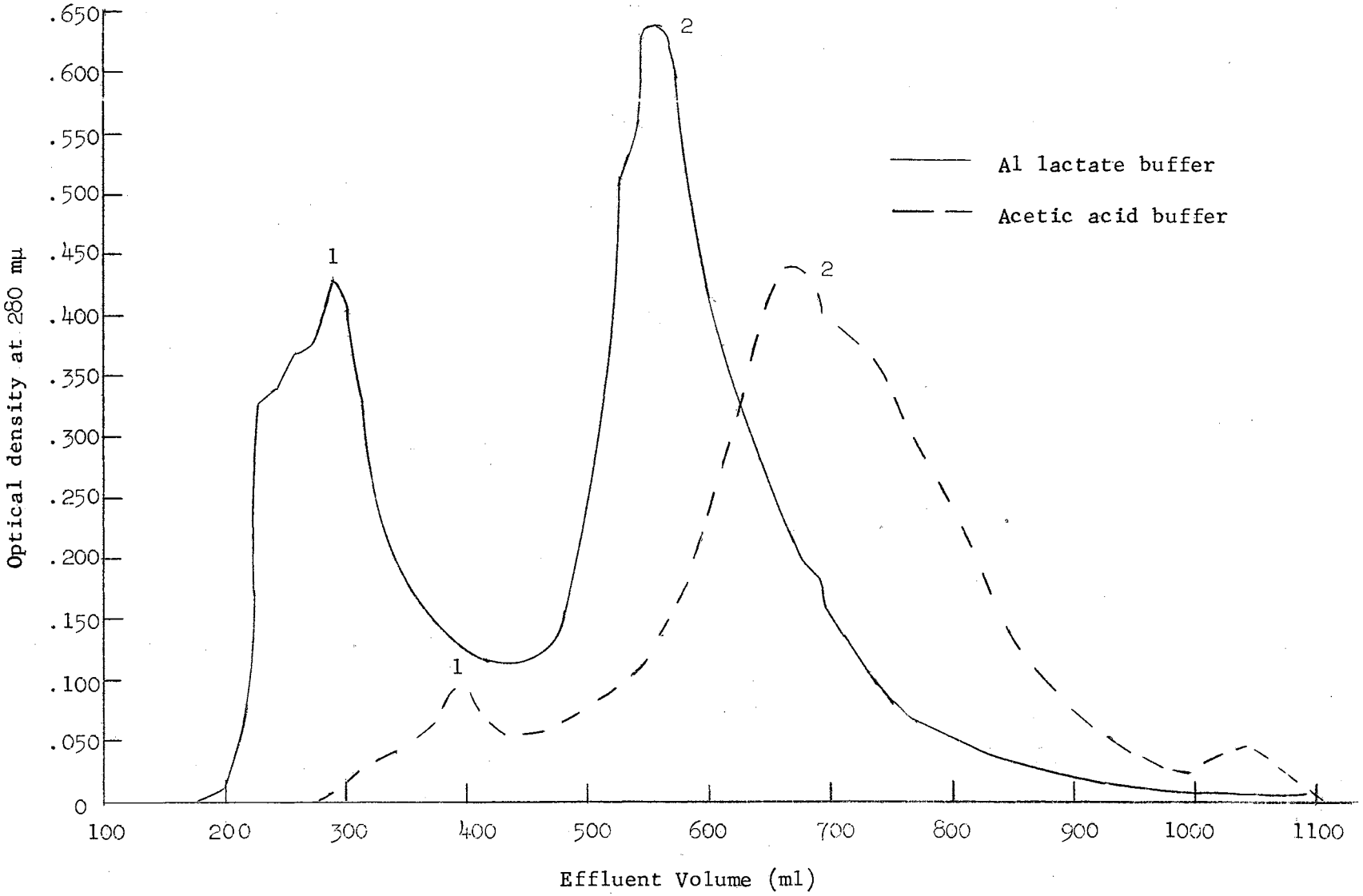


Figure 10. Elution patterns of Ponca gluten on Sephadex G-200 in Al lactate, pH 3.1, 0.05 μ, and acetic acid buffers.

PLATE I

Starch Gel Patterns of Comanche Gluten and Gliadin

1. Gluten
2. Gliadin (Alcoholic extract)

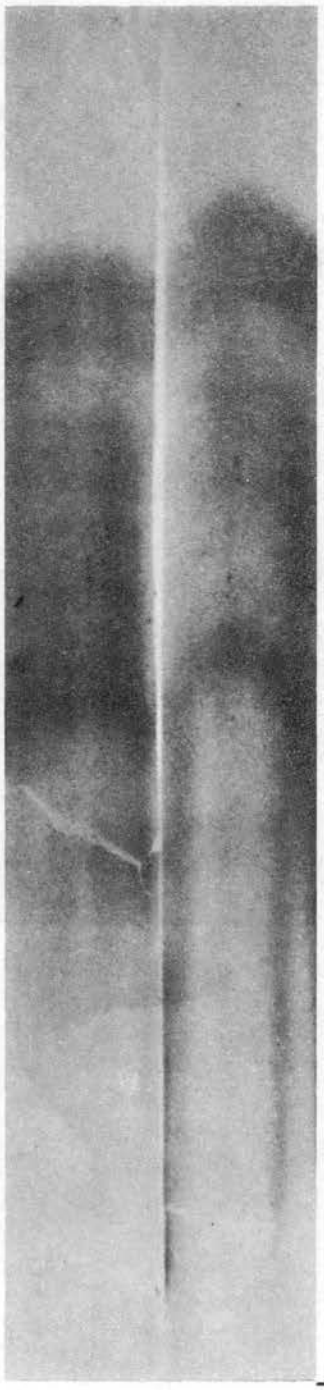

Origin
1
2

PLATE II

Starch Gel Patterns of Comanche Gliadin (Alcoholic extract)

Fractionated on Sephadex G-200

1. Peak one
2. Peak two
3. Peak three
4. Peak four

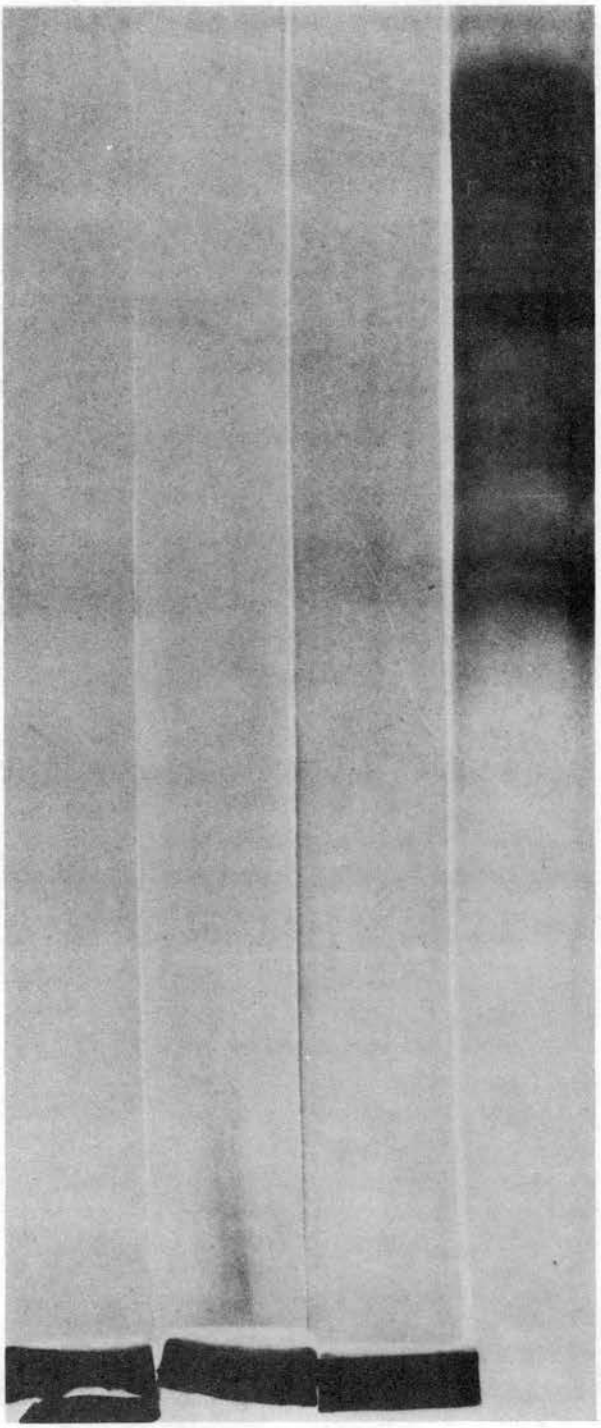

Origin
1
2
3
4

PLATE III

Starch Gel Patterns of Comanche Gluten Fractionated on Sephadex G-200

1. Peak one
2. Peak two
3. Front portion of peak three
4. Middle portion of peak three
5. Rear portion of peak three

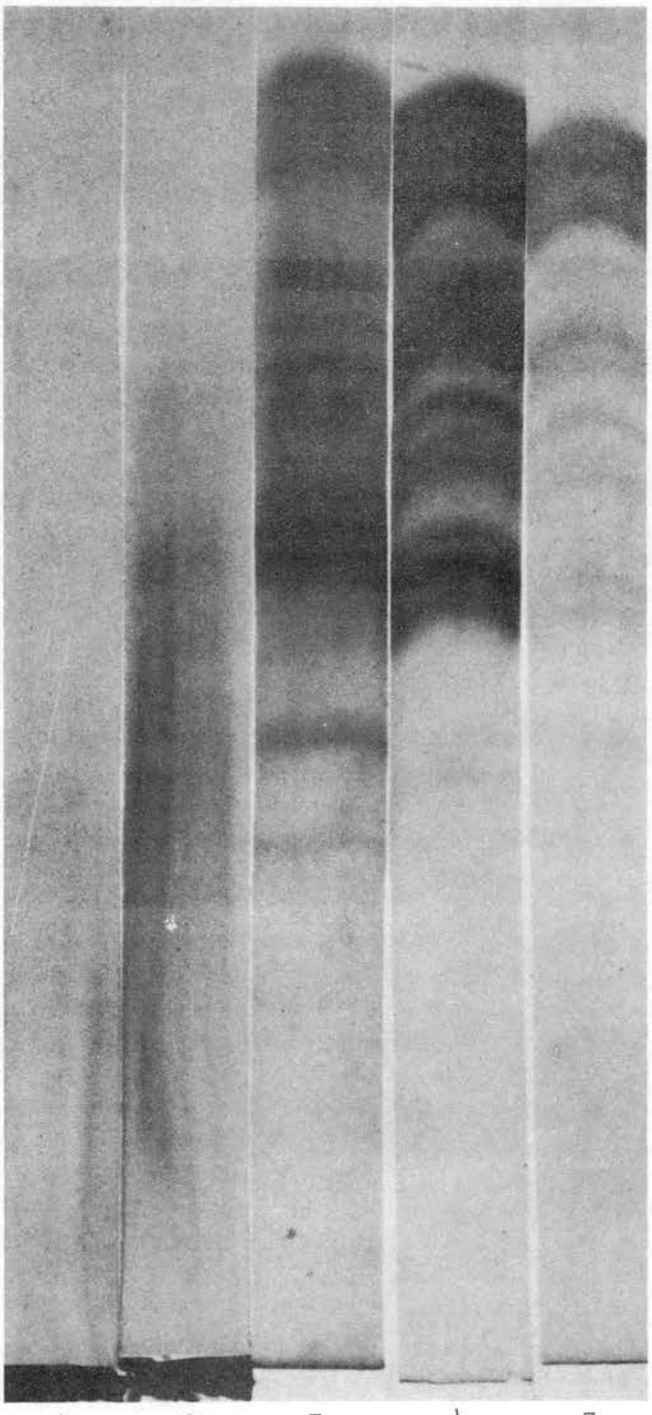

Origin
1
2
3
4
5

PLATE IV

Comparison of the Starch Gel Pattern of Comanche Gliadin Prepared by pH Fractionation with Those of Comanche Gliadin Prepared by Fractionation of Gluten on Sephadex G-200

1. Front portion of peak three from Sephadex fractionation
2. Middle portion of peak three from Sephadex fractionation
3. Rear portion of peak three from Sephadex fractionation
4. Gliadin from pH fractionation

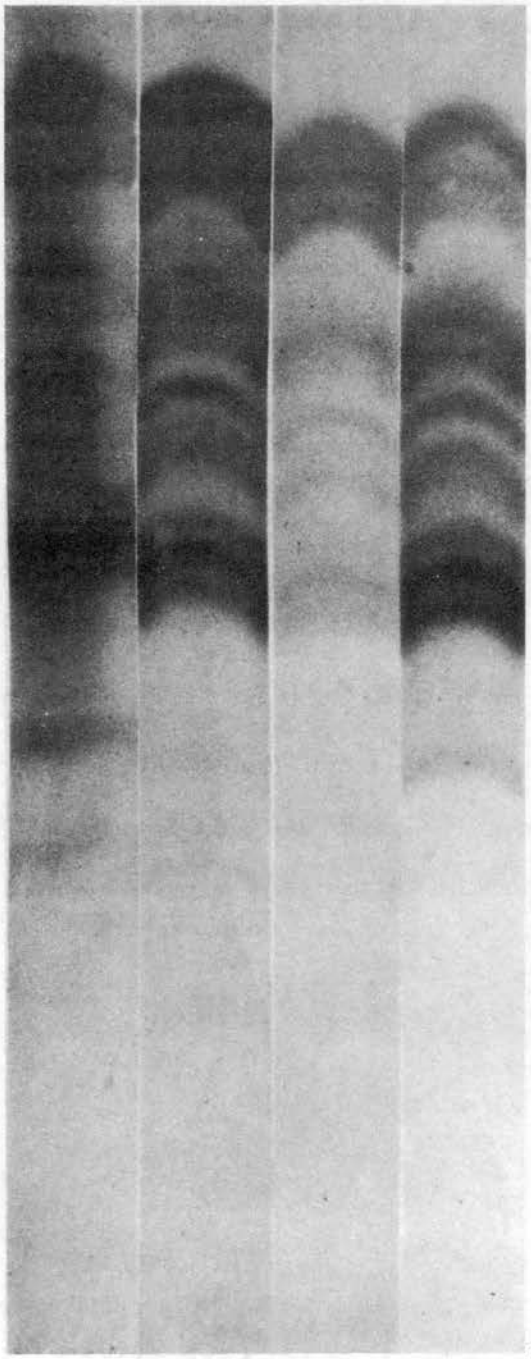

Origin
1
2
3
4

PLATE V

Starch Gel Patterns of Comanche Gliadin Further Separated on Sephadex G-100

1. Peak one (Electrophoresis - 36 hours)
2. Peak two (Electrophoresis - 36 hours)
3. Peak three (Electrophoresis - 36 hours)
4. Peak three (Electrophoresis - 12 hours)

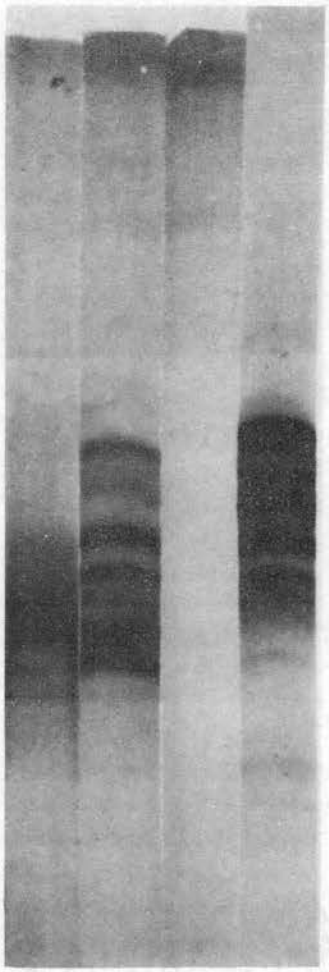

Origin
1
2
3
4

PLATE VI

Starch Gel Patterns of Ponca Gliadins Prepared by

Various Fractionation Procedures

1. Gliadin prepared by pH fractionation of gluten from 0.01 N acetic acid
2. Gliadin prepared by pH fractionation of gluten from 0.017 M aluminum lactate
3. Gliadin prepared by fractionation of gluten on Sephadex G-200 using 0.01 N acetic acid as eluent
4. Gliadin prepared by fractionation of gluten on Sephadex G-200 using aluminum lactate buffer, pH 3.1, 0.05 μ as eluent

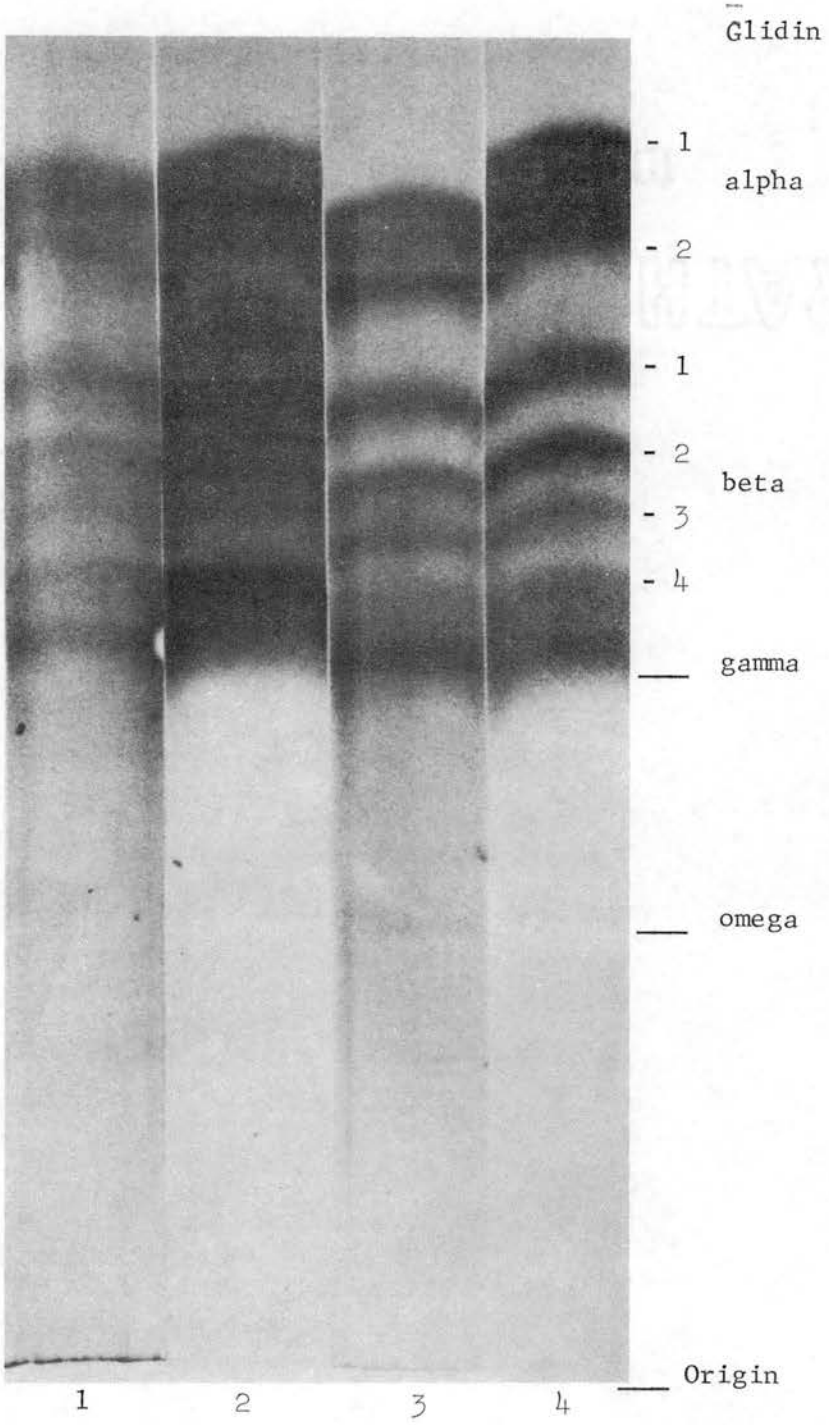

Glidin
- 1
alpha
- 2
- 1
- 2
beta
- 3
- 4
gamma
omega
Origin
1
2
3
4

PLATE VII

Comparison of the Starch Gel Pattern of Ponca and Comanche Gliadins Prepared by Sephadex Fractionation

1. Front portion of Comanche gliadin peak
2. Middle portion of Comanche gliadin peak
3. Rear portion of Comanche gliadin peak
4. Ponca gliadin

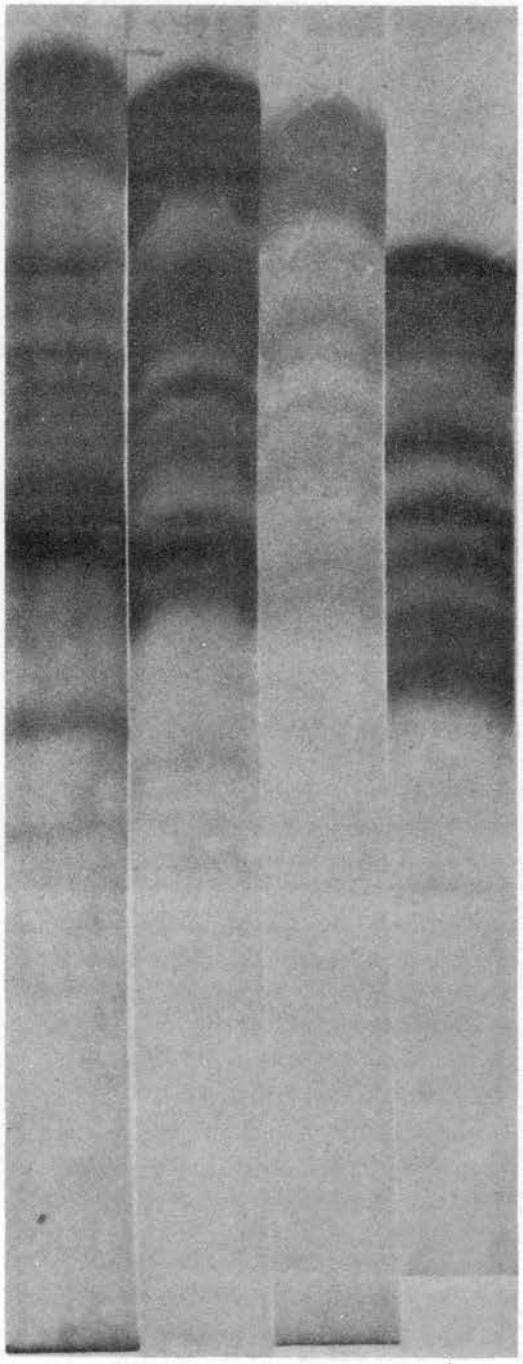

Origin
1
2
3
4

PLATE VIII

Comparison of Starch Gel Patterns of Water Extracts with Those of Glutens (12 hours)

1. Superking water extract
2. Superking gluten
3. Comanche water extract
4. Comanche gluten
5. Ponca water extract
6. Ponca gluten

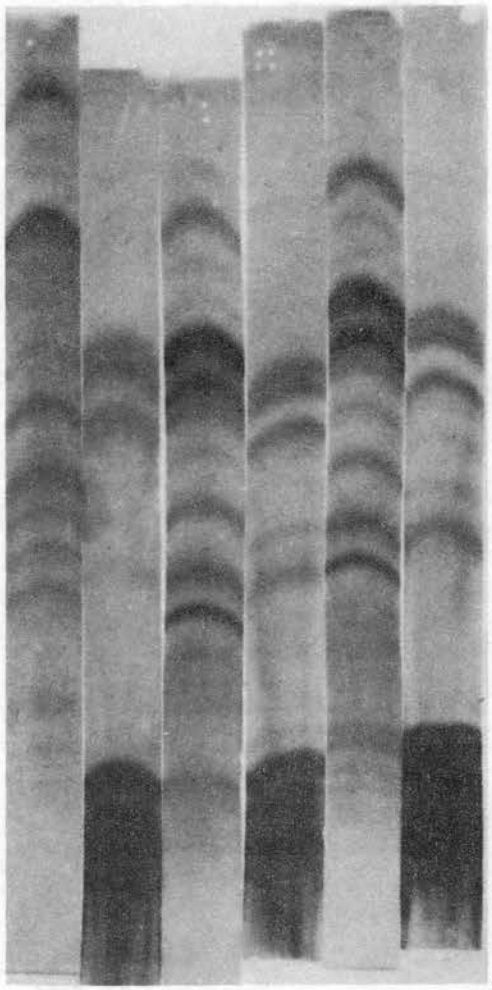
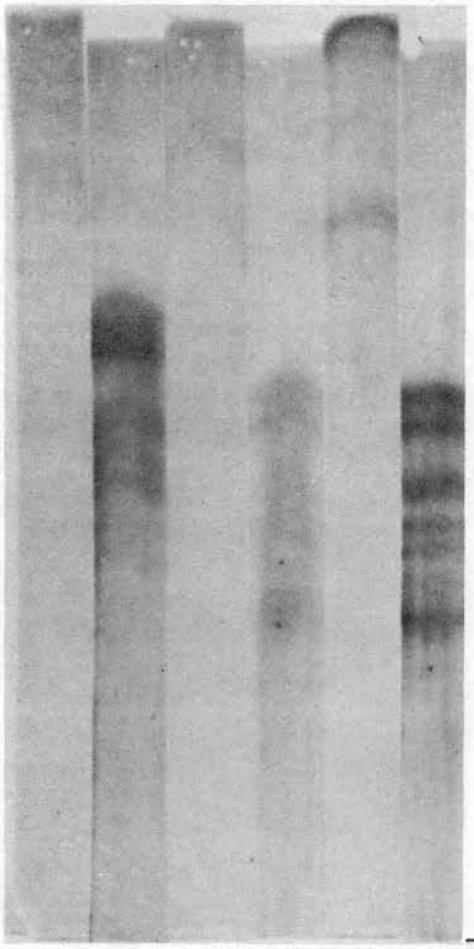

Origin
1
2
3
4
5
6

PLATE IX

Comparison of Starch Gel Patterns of Water Extracts with Those of Glutens (36 hours)

1. Superking water extract
2. Superking gluten
3. Comanche water extract
4. Comanche gluten
5. Ponca water extract
6. Ponca gluten


Origin
1
2
3
4
5
6

SUMMARY

Three procedures for the separation of wheat gluten protein into its major fraction, glutenin and gliadin, were investigated. These procedures included alcoholic extraction of flour or gluten, pH fractionation of gluten, and fractionation of gluten by gel filtration on Sephadex G-200. The last method effected the most clean cut separation of gliadin and glutenin as evaluated by starch-gel electrophoresis of the products. Attempts to separate the proteins within the gliadin fraction by gel filtration on Sephadex G-100 were unsuccessful, but the use of this material permitted the separation of the gliadin proteins from glutenin and water soluble proteins which were present as contaminants in the gliadin preparation. Gel filtration on Sephadex G-200 and Sephadex G-100 appears to be a suitable means for separating wheat flour proteins into three fractions: glutenin, gliadin and water soluble proteins.

The number of components present in Comanche and Ponca flour gliadin prepared by fractionation on Sephadex G-200 was evaluated by starch gel electrophoresis. Seventeen protein components were found in Comanche gliadin while only ten were observed in Ponca gliadin. Although improved techniques might reveal additional components in Ponca gliadin, it appears that there may be a varietal difference between the two wheats.

The elution characteristics of Ponca gliadin proteins on Sephadex G-200 suggest that all of these proteins have similar molecular weights. This finding is in contrast to an earlier report in which the weight

average molecular weight of impure alpha gliadins was estimated to be about four to five times greater than that of the other gliadin proteins.

Starch-gel electrophoretic patterns of water soluble proteins from three flours were compared with those for the corresponding glutens in order to determine whether or not some of the bands observed in gluten and gliadin were water soluble contaminants. It was concluded that, although gluten does contain some of the water soluble proteins, these components migrate far ahead of the gluten proteins and do not contribute to the starch gel pattern for gliadin or gluten under the conditions routinely employed.

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33. Descriptive Brochure - Sephadex for Gel Filtration.

VITA

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