IMMUNOCHEMICAL AND ELECTROPHORETIC CHARACTERIZATION OF PROTEIN CHANGES IN THE WHEAT KERNEL OF HARD RED WINTER WHEATS DURING MATURATION

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CHAPTER I

INTRODUCTION

The proteins of wheat are the constituents mainly responsible for the position held by this product on the world market today. These proteins contribute the elastic, cohesive properties responsible for the porous structure of baked goods and they form the basic structure for a variety of baked products, especially bread.

Osborne (1) was the first to report a comprehensive investigation of these proteins classifying them by means of solubility into five fractions. Most of the earlier work was concerned with mature wheat and mainly the gluten fraction because of its apparent role in the baking process. However, much of the recent work has been on the albumin and globulin fractions which comprise the so-called "soluble fraction" that may also play a role in modifying baking properties.

Many conflicting results have been obtained from attempts to elucidate the chemical and physical properties of wheat proteins. The underlying factors for the differences in properties of the proteins among various species and varieties of wheat are at present unknown.

One approach to further insight into the make-up of wheat proteins is the study of their changes during maturation. The present study was undertaken to investigate this aspect of the problem using electrophoretic techniques with emphasis on immunochemical analysis.

CHAPTER II

LITERATURE REVIEW

Work on the proteins of wheat has been reviewed extensively over the years (2, 3, 4, 5, 6, 7). Some of the techniques used previously for the characterization of these proteins include ion-exchange chromatography (8), moving-boundary electrophoresis (9), gel filtration (10), starch gel electrophoresis (11-16) and immunoelectrophoresis (17). Most of these works were performed on the gluten and soluble fractions of mature wheat.

Woodman and Engledow (18), using the classical solubility procedure of Osborne (1) as a means of fractionation, were the first to report investigations of proteins of developing wheat endosperm. Later McCalla (19) performed similar work using this approach. Both authors concluded that the gliadin proteins were synthesized later than and independent of the other protein fractions. McCalla concluded also that the earliest formed gluten fraction (glutenin) became the most insoluble portion of the proteins in the mature kernels. It was suggested further that the non-protein nitrogen in the kernel at any one stage of development is the precursor of a definite fraction of the endosperm protein (gliadin), rather than of a portion of the whole gluten complex (gliadin-glutenin).

The use of radioactive tracers for studying protein changes in relation to endosperm maturity was initiated by Bilinski and McConnell

(20). These workers injected acetate-1-^{14}C and acetate-2-^{14}C into the stems of wheat plants at various stages of maturity. They not only concluded that the gliadins reached a maximum rate of biosynthesis at a later period than the other fractions but also suggested that these proteins were not derived through interconversion of the proteins present at an earlier stage of development. These results agree with those of earlier workers (18, 19), e.g., that the gliadin fraction is formed later than and independent of other proteins.

Finlayson and McConnell (21, 22) later employed performic acid to oxidize a gliadin fraction from wheat plants previously injected with acetate-2-^{14}C. Fractions were isolated from this performic acid-oxidized preparation by chromatogrphy on DEAE-cellulose. The fractions differed from each other in chemical properties, amino acid composition, and carbon-14 content. Two possible explanations for the results were offered: (1) 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; (2) 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 (23) injected ^{35}S-sulfate as a tracer in wheat 28, 23, 20, 13, and 8 days before harvesting. They found that at any stage of development the gliadin fraction increased in specific activity twenty per cent faster than the glutenin fraction, and higher still

than the albumins and globulins.

Morphological studies have also been applied to the study of maturing wheat proteins. Buttrose (24) used the electron microscope to study the ultrastructure of the developing wheat and found that during the first week after fertilization spherical protein deposits, loosely enclosed within sacs of the outer nuclear membrane, appeared. Jennings et al. (25) also noted these deposits using electron and fluorescence microscopy and gave them the name of "protein bodies". The protein bodies were isolated by density gradient centrifugation. As the wheat plant matured, notable enlargements of the protein bodies were observed beginning between the second and third weeks after flowering. However, mature endosperm also contain small bodies comparable to those seen in more immature endosperms. It was suggested that the enlargement of some protein bodies was due in part to formation of storage proteins soluble in acetic acid or sodium hydroxide (gliadins).

Jennings and Morton (26) studied four varieties of wheat and found that in each variety there was rapid synthesis of protein occuring mainly in the endosperm of developing grain. 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. Non-protein nitrogen constituted approximately 48% of the total nitrogen initially at day 8 after flowering and declined to about 15% at day 19 and to approximately 3% at maturity. They concluded from the patterns of the relative changes in total protein and in non-protein nitrogen of the maturing wheat that their results were indicative of a precursor pool-product relationship. This conclusion was based also on the fact that the non-protein nitrogen in the

immature samples was mostly in the form of free amino acids. The amino acid composition of the endosperm protein remained relatively constant from 19 days after flowering to maturity. The interpretation derived from these results suggested a rapid accumulation of storage (gliadin) proteins at day 19.

Protein fractions were extracted from developing wheats of three varieties by Graham et al. (27), using sodium pyrophosphate, acetic acid, and sodium hydroxide. In all three varieties, there was a rapid increase in endosperm nitrogen during development with the greatest increase due to changes in the acetic acid-soluble (gliadin) proteins, but increases did occur in the other fractions. They chromatographed the sodium pyrophosphate-soluble proteins on DEAE-cellulose and the acetic acid-solubles on CMC. These results indicated that only quantitative changes occur in protein composition during maturation and were contrary to earlier reports (18, 19, 20, 26) that gliadin proteins were synthesized later than or independent of the other protein fractions.

To obtain information of possible relationships between the various protein components during the rapid synthesis and accumulation of storage proteins, Graham and Morton (28) measured rates of incorporation of ^{35}S-sulfate into the endosperm. The endosperm protein was extracted with sodium pyrophosphate and acetic acid after exposure to the isotope for a period of between 3 and 48 hours. The results obtained indicated to these workers that synthesis of acetic acid-soluble protein occured more rapidly than incorporation of ^{35}S into this protein. Also it has been found (24) that salt-soluble proteins have a greater proportion of sulfur-containing amino acid residues than do gluten proteins. This may account for the results obtained. These results can

be correlated with those of Bilinksi and McConnell (19). Starch-gel electrophoresis of the extracted proteins showed the slow-moving components in the acetic acid extract. Radioautographs of the starch gels showed a correspondence of label with dyed protein bands.

The study of the amino acid composition of proteins in maturing wheat offers another approach that might distinguish among the constituents of wheat at various stages of maturity since Waggle et al. (29) and Woychik (12) showed that the insoluble proteins (gliadins and glutenins) exhibit amino acid compositions different from those of the solubles (albumins and globulins).

Jennings and Morton (30) showed that the amino acid composition of whole endosperm changes considerably during development due to changes in the composition of both the non-protein nitrogen fraction (pool of free amino acids) and of the protein. The endosperm was extracted with pyrophosphate buffer, acetic acid and dilute sodium hydroxide. The composition of the fraction extracted by acetic acid remained relatively constant from fourteen days after flowering through maturity. The change in amino acid composition, e.g., glutamic, proline, lysine, occurs chiefly in the fraction extracted by pyrophosphate buffer and by sodium hydroxide. This change in composition was explained as resulting from extraction of more of the gluten-type proteins with these solvents as the endosperm matured. Ewart (31) found that gluten proteins are high in glutamic acid, proline, and lysine, and that their concentrations increase with maturity of the endosperm.

Pomeranz et al. (32) studied the amino acid composition of two Hard Red Winter wheats at 23, 17 and 0 days before maturity. They found that during maturation of the endosperm, the concentration of lysine,

aspartic acid, glycine, alanine and valine decreased while that of glutamic acid and proline increased.

Coulson and Sim (33) studied the changes in wheat endosperm proteins during the life cycle of the plant. Starch-gel electrophoresis was employed for characterization and showed that fractions of low electrophoretic mobility (gliadins) were progressively degraded during germination and progressively synthesized towards the end of the ripening period. In contrast, fractions of higher mobility (albumins, globulins) were less affected during germination and were utilized at a later stage. Similarly, these components were synthesized first during ripening and remained at a relatively constant level during the build-up of apparently high molecular weight material.

Fiellet (34) also studied the development of wheat proteins during maturation by use of starch-gel electrophoresis. He found that the electrophoretic mobility of the albumin-globulin fraction and gliadins remained unchanged during maturation. Heterogeneity was observed in the solubles from the first days of development and remained relatively unchanged until the thirty-fifth day after flowering, when one or two additional constituents of greater mobility appeared. Except for a few constituents of very low mobility, no definite zones could be seen in gliadins until day 35. Beyond this day, however, this fraction acquired a definitive structure and fifteen constituents appeared and remained unchanged in number and staining intensity. Glutenin, on the other hand, did not migrate into the gel at any stage of maturity.

Feillet drew two general conclusions from his study concerning the evolution of protein material in wheat. First, there is a period of intense synthesis relative to a massive deposit of nitrogen in the

kernel and corresponding with the increase in its dry weight. Second, that a period exists when the grain begins to dehydrate and stores the total of its nitrogen in the form of protein. It is at this time that the gliadin acquires its definite structure. He also concluded that gliadin is the last fraction formed.

Using gel filtration on G-200 columns, Fish (35) studied changes in the lactic acid-extractable proteins from two Hard Red Winter wheat varieties at various stages of maturity. The over-all patterns from gel filtration were qualitatively the same for both varieties and all intervals of development. The differences found were quantitative only. These results were confirmed by starch-gel electrophoresis comparing the peaks obtained from gel filtration. The electrophoretic pattern of a given molecular weight group of proteins illustrated that practically no qualitative variation of the proteins within a group were found. Fish's conclusions, in general, agree with those of Graham (27). Fish also correlated the fluctuations in the levels of free amino acids with the synthesis of higher molecular weight proteins. No general trends were observed when the fluctuations of the amino acids present in large amounts in the glutenins were compared with those of the gliadins. The absolute amount of each amino acid decreased with maturation of the kernel. He observed that the residues of arginine, aspartic acid, phenylalanine, tyrosine, and isoleucine showed substantial increases in relative amounts, while alanine, serine, proline, and glutamic acid exhibited marked decreases in relative amounts. These results partially confirm the observations of Jennings and Morton (30), who found increases in the free amino acids, aspartic acid and arginine, and decreases in lysine and proline.

Jennings (36) used gel elctrophoresis to characterize the proteins extracted from wheat flour at various maturities. He found evidence to support the conclusions of various workers (18-20, 23) that the synthesis of the gliadin fraction commences later than and proceeds independent of that of other endosperm proteins. He found also that a recalculation of the data of Graham and Morton (28) shows the same relationship. In addition, his results are consistent with a precursor-product relationship between proteins extracted by pyrophosphate buffer and dilute alkali. Qualitative similarities in starch gel zone patterns shown by immature and mature endosperm extracted with these solvents provided further evidence for this relationship. Some of the results obtained by Graham and Morton (28) also appear to support this conclusion.

Jennings further concluded that there are two distinct groups of storage proteins present in the wheat grain. The components of one group are characterized by their solubility in dilute acetic acid, low mobilities in gels (pH 3), high content of glutamine and proline, and low content of basic amino acids (30). Proteins in the second group are characterized by their high mobilities in gels (pH 3), a lower content of glutamine and proline, a higher content of basic amino acids, their solubility in pyrophosphate buffer, and their aggregation into insoluble complexes, probably after their interaction with proteins or other compounds with net negative charges produced in stoichiometric amounts.

Research on protein development in the wheat kernel has been carried out using a variety of techniques. From the use of radioactive tracers, solubility techniques and starch-gel electrophoresis, there

has been suggested a late and rapid synthesis of the gliadin proteins. Starch-gel electrophoresis has shown that the protein bodies isolated from endosperm during maturation are gluten-type proteins of slow electrophoretic mobility. Ion-exchange chromatography, gel filtration and starch-gel electrophoresis of isolated protein fractions indicate a quantitative rather than a qualitative build-up of proteins in the wheat kernel.

Work in the field of immunochemistry of wheat proteins has been quite limited, but it is becoming an important method in comparative studies of these proteins. Although no published immunological work has appeared dealing with wheat protein during maturation, it is pertinent to review immunological research related to the proteins of wheat in general.

As early as 1901, Kowarski (37) investigated the immunological properties of soluble substances in wheat and rye seeds and demonstrated similarities between these two species. Later Moritz (38) made a comparative study of the antigenic specificity of the seeds of ryewheat hybrids and the two parental species by means of anaphylactic reactions. Results demonstrated antigen composition of ryewheat includes the specific antigens of wheat and rye as well as the antigens they have in common.

In the early fifties, Grabar and Williams (39, 40) described a technique called immunoelectrophoretic analysis (IEA) to define proteins by two completely distinct criteria. This method allows one to characterize a protein in a mixture by two different criteria: electrophoretic mobility and immunochemical specificity. The first depends on the number and charge of ionizable groups on the molecule, while the

latter is based on the steric configuration of certain groups on the molecule. The reaction being specific, each antigenic protein gives an independent arc. Thus, ideally IEA allows the enumeration and definition of every component of a mixture or the comparison of components in different mixtures.

In 1955 Scheidegger (41) developed a method of performing the entire operation of IEA on a microscope slide. Since this technique requires only a few hundredths of a milliliter of serum and even less antigen, it is quite useful when these reactants are scarce.

Later, Hall (42) demonstrated a very useful technique of immunoabsorption. A small amount of an antigen is added to an antiserum causing precipitation of the specific antigen-antibody complex. Removal of the precipitate leaves the serum minus antibodies to the added antigen. Hall used this technique along with IEA in analyzing allopolyploid ryewheat and its parental species. It was concluded that wheat extract contains proteins which are lacking (or present in only small quantities) in the rye extract. The converse was true also. In addition, biochemical complexity increased in proportion to the degree of polyploidy.

Grabar and Daussant (43) used simple electrophoresis and IEA in the study of water-and salt-soluble proteins from wheat and barley. IEA revealed 8 to 10 protein constituents in wheat and 17 to 22 in barley. Of these several were of similar mobilities, but of distinct antigenic specificity. They also showed that the pattern of arcs was very similar whether a given wheat was treated with its own antiserum or one immune to another variety. This is very interesting evidence that similar proteins are present in different varieties, because the

antibodies to one wheat react with the antigens of another.

Elton and Ewart (44) used a rabbit serum against Manitoba #2 gluten to compare protein extracts of cereal flours from several varieties of Triticum vulgare and Triticum durum. All varieties appeared to share at least four immunologically similar gluten proteins. Double diffusion reactions were emphasized but IEA experiments at pH 5 in acetate buffer were conducted. However, with IEA three lines near the origin were obtained, and they concluded that the serum is not compatible with the low pH at which good separation of gluten proteins is achieved. These investigators concluded that changes in baking quality are due to quantitative changes in protein composition or some other as yet undetermined factor rather than qualitative differences of major components.

Benhamou et al. (45) also studied the gluten fraction of wheat using immunochemical methods. Ten gluten proteins were extracted with 3M urea pH 7 or in 0.05M acetic acid and electrophoresis was carried out at pH 8.2 in 0.05M veronal. They found that the proteins from both extracts were qualitatively the same and also that the major component migrated little at pH 8.2. A comparative study of gliadin and glutenin showed that these two protein complexes were immunochemically identical though they behaved differently in electrophoresis.

Grabar et al. (46) utilized simple electrophoresis and IEA to analize the salt-soluble proteins in wheat, barley, and malt extracts. Ten, twenty and twelve independent constituents were found, respectively, and their relative mobilities were calculated. These workers compared whole wheat with flour and found that they contained the same components and that their mobilities were practically the same. The only apparent difference was that the extract of the grain contained a

lipoprotein not found in the flour. They concluded that some proteins of wheat are found in the barley. Their conclusion was based on the observation that when wheat extract is reacted with antibarley serum, some of the component arcs appear as when reacted with the antiwheat serum, with the mobilities being similar if not the same. Gliadin and glutenin fractions obtained by chromatography on CMC were also analyzed by simple electrophoresis. It was found that the gliadin fractions were immunochemically identical and that glutenin migrates in agar gel containing 3M urea and gives an immunochemical identity reaction with gliadin as observed by Benhamou et al. (45).

IEA in the study of wheat protein was continued with an investigation of wheat flour albumins by Hamauzu et al. (47). Albumin preparations from four varieties of wheat were used and seven to eleven components was essentially the same in all four varieties of wheat used although considerable variations were shown in the durum wheat.

Nimmo and O'Sullivan (17) compared a Hard Red Winter wheat and Durum wheat by IEA using acid extracts, sodium barbital extracts and urea extracts of both wheats. They found that, among the gliadins (urea soluble) and salt-soluble proteins, no components unique to one type of wheat were present. They concluded that the proteins of the two wheat types were qualititatively matched. Their results appeared to support previous conclusions that baking quality differences among wheat must be realted to factors other than individual protein differences (27, 35, 36, 43, 47).

CHAPTER III

MATERIALS AND METHODS

Wheat Samples

Two varieties of Hard Red Winter (HRW) wheat, Triumph and Kaw, grown at the Oklahoma State Agriculture Experiment Station in 1968, were used in this investigation. Samples of Kaw were collected 4, 6, 8, 11, 13, 15, 18, 21, 24, and 27 (mature) days after heading. Triumph samples were collected 4, 6, 9, 11, 13, 16, 20, 23, 26, 29, 32, and 36 (mature) days after heading.

All samples of each variety were collected by removing the heads in the fields and placing them in the freezer at -20°C until the grain was collected by hand threshing. Each sample was weighed, frozen, dried from the frozen state, and reweighed. The dried grain was then ground (in a Waring blender cooled by dry ice) to pass through a 60mm sieve and stored at -20°C until use. Microkjeldahl protein assays were run on each sample using the technique of Mettler, et al. (48)

Protein Extraction

The extraction procedure for the soluble proteins was a modified method of Grabar and Daussant (43). Five grams of each sample were extracted overnight with 20 ml of 0.5M NaCl-0.025M sodium phosphate buffer pH 6.8 at 4.0°C with constant mixing. These were centrifuged at 25,000xg on a Servall Ultracentrifuge for twenty minutes, the

residues re-extracted three times with the salt buffer for one and one-half hours, and centrifuged as before. The supernatants from all four extractions were then combined and saved.

The gluten protein was extracted from the buffer-extracted residue using 2M urea following the procedure described above for the buffer solubles. The supernatants of all urea extracts were combined. Both types of extracts were filtered through one-half inch thickness of glass wool to remove any solid residues.

The extracts were dialyzed versus distilled water for six days with three changes of water daily. They were then lyophilized, weighed, and transferred into vials. The modified biuret method of Jennings (49) was used in determining the protein concentration. The dried extracts thus prepared were used for immunizations and for starch-gel electrophoresis.

Protein Extracts For IEA

As indicated by starch-gel electrophoresis, most of the changes that occured were in the immature samples. Therefore a representative set of samples was selected for IEA. Kaw samples collected 4, 6, 8, 11, 13, 15, 21, 24, and 27 days after heading, and Triumph samples collected 6, 9, 11, 13, 16, 20, 29, 32, and 36 days after heading were used. A 1:3 ratio of ground wheat to the buffer was employed to increase the protein concentration. A thirty-minute extraction time at $4^{\circ}C$ was used and the extracts were centrifuged at 27,000xg for twenty minutes. The supernatant solutions were used without concentrating. Protein determinations by the Lowry method (50) were run on the supernatants.

Immunization

Twelve mature rabbits were used. These were divided into four groups consisting of three rabbits each for immunization with buffer soluble proteins of Triumph and Kaw and urea-soluble proteins of Triumph and Kaw. Prior to inocculation the serum of each rabbit was tested for non-specific reactions with wheat antigens.

Preparation of Antiserum

Each rabbit was injected with 1.2 ml of the appropriate antigen-emulsion which was prepared by mixing the antigen solution with Freunds complete adjuvant in a ratio of 1:1. Total protein concentrations were as follows: Triumph and Kaw buffer-soluble preparation 7.5 mg, Triumph and Kaw urea-soluble preparation 4.7 mg. The antigen was given by multiple subcutaneous injections over six areas of the back. After one month a second injection of 1.2 ml of antigen-emulsion was given subcutaneously.

The double diffusion test of Ouchterlony (51) was used to determine when antibody levels of concentration sufficient to perform further tests had been reached. These tests showed very low antibody titers after two subcutaneous injections; therefore another route of injection and antigen preparation was used. Footpad injection was decided upon due to its reportedly greater sensitivity and the low quantity of antigen solution needed. The protein solution was prepared as follows. For the buffer solubles, approximately 250 mg of each variety of ground whole wheat (25 mg of each maturity) were extracted with 0.75 ml of the salt-buffer for one-half hour, and the suspension

centrifuged at 27,000xg for twenty minutes. The supernatants (0.42 ml) were diluted to 0.70 ml with deionized distilled water to give a protein concentration of 75 mg per 0.70 ml. This solution was mixed with an equal volume of adjuvant to form the emulsion for injection. An aliquot of 0.10 ml of the antigen-emulsion was injected into each footpad of the rabbits. After a one-week interval, another footpad injection was made, followed by an interperitaneal injection seven days later (0.50 ml, total protein of 15 mg). For the urea solubles the lyophylized fraction described earlier was used for the injection.

Collection of Serum

For all preliminary bleedings 3-5 ml of blood were taken from the marginal vein of the ear. For the final bleeding, heart punctures as described by Campbell (52) were performed one week after the last injection. By this method approximately 50 ml of blood could be taken without sacrificing the rabbit. The blood was allowed to stand in the cold overnight in centrifuge tubes. The serum was pipetted from atop the blood clot after centrifuging and then frozen in one and two milliliter aliquots without preservative.

An antiserum to buffer-soluble wheat proteins prepared in a similar manner was obtained from the Pasteur Institute in Paris, France. Because it appeared to contain antibodies to more of the wheat proteins, and in higher amounts than the antiserum prepared locally, this serum, #202, was employed for most of the comparisons described here. In some cases the two antisera were compared.

IEA Experiments

The immunoelectrophoretic technique is a combination of electrophoresis (in translucent gels) with double-diffusion. First the constituents of the antigen are separated by electrophoresis and then are allowed to react with antibodies in an antiserum which is placed in a trough parallel to the electrophoretic track. When the separated antigens and the antibodies meet in adequate proportions, they form stabilized precipitates in the shape of arcs which are characterized by their position, curvature and length.

In the present investigation, the micro-method of Scheidegger (41) was used throughout. Molten gels of 1.25% ionagar #2 (Colab laboratories) were prepared in 0.025M veronal-HCl buffer pH 8.2 and poured on a template (Gelman Instruments) holding six microscope slides in rows of three (230mm x 25mm). Resulting gels were 1.5mm thick. The antigen wells were 1.0mm in diameter and held 2 µl of solution. The voltage across the gels during electrophoresis was 75-80 volts, a value which corresponds to a potential fall of about 3.5 volts/cm in the separation zone. The agar-covered slides and the buffer solution (0.05M veronal-HCl pH 8.2) in the immunoelectrophoretic chamber were connected by cellulose acetate strips 25mm x 65mm. All IEA experiments were run approximately two and one-half hours at room temperature.

The antiserum troughs (65mm x 1mm) which hold 175 µl of serum were cut in the gel after electrophoresis. The immune serum was added by means of drawn-out Pasteur pipettes. The reaction time for the precipitations was twenty-four hours during which time the plates were

kept in the closed electrophoretic chamber at room temperature. Excess antigens and serum proteins were removed by washing the plates for three days in 0.9% NaCl-sodium barbital solution (sterilized) with two or three changes of wash solution daily. The gels were then covered with wet filter paper strips and dried for three hours at 37°C. They were then stained in saturated amido black dye in 5% acetic acid for fifteen minutes and washed in 5% acetic acid to removed excess dye.

Starch-Gel Electrophoresis

Starch gels of 15% in 3M urea-aluminum lactate buffer pH 3.3 were prepared and starch-gel electrophoresis conducted at 4°C on an apparatus as previously reported by Abbott and Johnson (10). All gels had dimensions of 1/8" x 7" x 9". The time of electrophoresis varied depending upon the samples being analyzed: the buffer-soluble proteins were run for seven to eight hours, while the urea solubles were run for 15 to 18 hours.

Absorption Techniques

Using the procedure of Hall (42), the absorption experiments were conducted in the following manner: to 0.045 ml of serum in centrifuge tubes, 0.05 ml of buffer-soluble protein solution was added. The tubes were placed in an incubator at 37°C for one hour, and shaken at about fifteen-minute intervals. Then the tubes were held in the cold at 4°C overnight. The tubes were centrifuged at 2,000 rpm for about ten minutes (longer times and higher speeds were needed in some instances) to remove the precipitated antigen-antibody complex. The supernatant was placed in another centrifuge tube and, using only one-half the

amount of antigen, the absorption procedure was repeated until no further precipitate occured. A control or blank on the serum was conducted at the same time using buffer in place of antigen solution.

Enzyme Assays

Peroxidase and esterase enzyme assays were run according to histochemical techniques described by Ureil (53) on washed and dried gels after antigen-antibody reactions were performed. Amylase assay on washed but not dried gels were conducted as described by Daussant (54).

Materials

All reagents were of analytical grade, and prepared in deionized distilled water.

CHAPTER IV

RESULTS AND DISCUSSION

Protein Assays

Protein nitrogen in the lyophilized whole wheat samples at various maturities was determined by the microkjeldahl technique. The results obtained were similar to those of earlier workers (30, 34), e.g., high nitrogen content in immature samples which decreased as the plant matured and then increased again. Modified biurets were run in an attempt to show that a high percent of the nitrogen in these immature samples was not protein nitrogen but nitrogen from other sources, i.e. free amino acids. This assay was also performed to demonstrate that the protein content of wheat kernels increases as the plant matures. However, there were some substances present in the immature samples, probably free sugars, which interfered with color development. For this reason these results are not presented; however, it has been shown by earlier workers that the protein content of the wheat kernel does indeed increase as the plant matures. Other methods of determining the protein content of these whole wheat samples were not applied due to the insufficient quantity of immature samples.

Starch-Gel Electrophoresis

Preliminary Experiments:

Preliminary experiments compared the protein constituents obtained

from the extractions with the salt-phosphate buffer and with urea. Starch gel patterns of a mature Triumph sample for the four successive extractions with the NaCl-PO_4 buffer and the four 2M urea extracts of the residue obtained from the above procedure are shown in Figure 1. The first buffer extract contained all of the protein components present in each of the succeeding extracts plus some additional bands. Biuret assays showed that the first buffer extract contained 46.6% of the protein material obtained by all four of the buffer extractions, the other extracts contained 15.7%, 15.5%, 11.1% of the total protein extracted. The exact concentration of protein on the gel was not known accurately, since the samples were dialyzed and concentrated prior to electrophoresis.

Urea extracts the gluten-type proteins by breaking the hydrophobic and hydrogen bonds of the proteins. As can be seen in Figure 1, each successive urea extract contained the full complement of protein components relative to the first. By biuret assay of these extracts it was determined that the first urea extract contained about 35.3% of the protein extracted by the urea solution while the three following extracts contained 23.3%, 20.83%, and 20.51%, respectively.

Since these experiments demonstrated that a representative set of protein components could be extracted from the whole wheat by four buffer and four urea extractions, this procedure was adopted for the extraction for these proteins from the maturing kernels of Triumph and Kaw.

Comparison of Maturities:

The starch gel patterns for Triumph buffer extracts at various stages of development are shown in Figure 2. At 4 days after heading,

Figure 1. Starch-Gel Electrophoresis of Four Extracts with NaCl-PO_4 Buffer and Four Extracts with Urea from a Mature Triumph Sample.

1. Buffer extract one
2. Buffer extract two
3. Buffer extract three
4. Buffer extract four
5. Urea extract one
6. Urea extract two
7. Urea extract three
8. Urea extract four

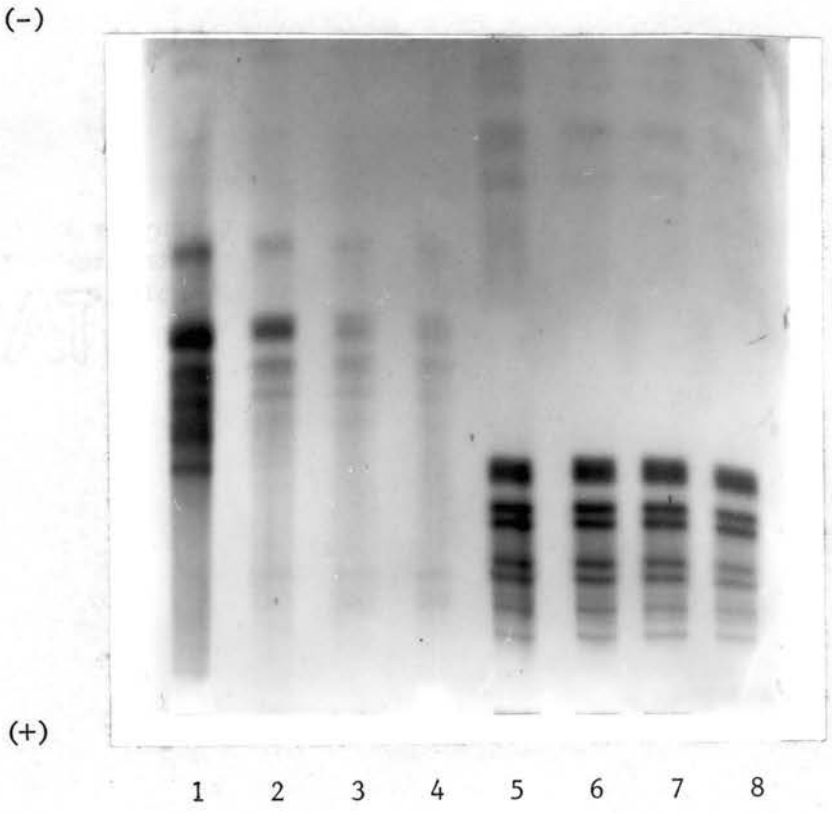
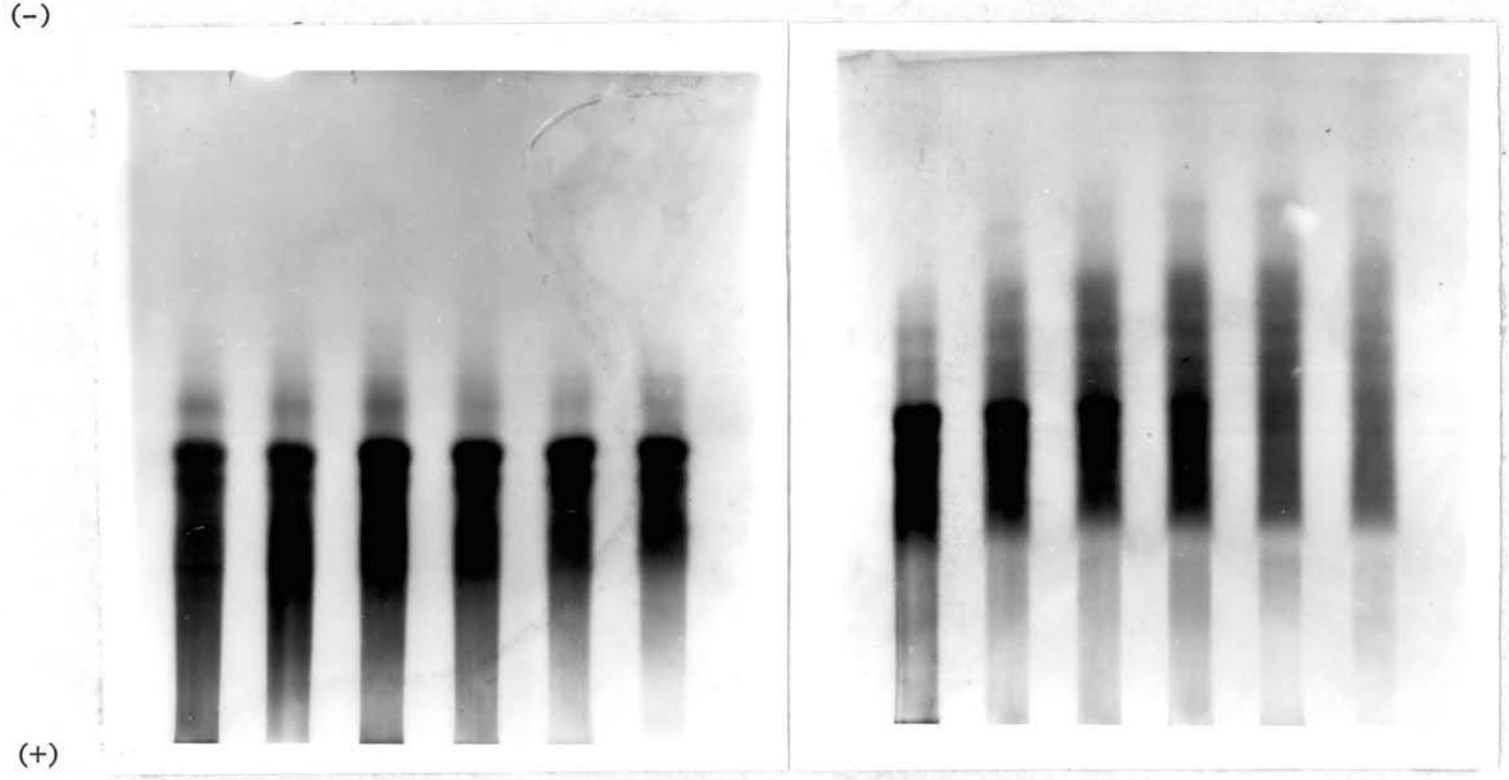

(-)
(+)
1
2
3
4
5
6
7
8

Figure 2. Starch-Gel Electrophoresis of Lyophilized Triumph Fractions Extracted with NaCl-PO_4 Buffer.

Approximately 220-340 µg. material on sample wicks.


(-)
(+)
36
32
29
26
23
20
16
13
11
9
6
4
DAYS AFTER HEADING

little discrete band formation is observed, but there is staining (due to protein) seen the length of the gel. Days 6 to 13 appear to show more band formation with a build-up of protein material at intermediate electrophoretic mobility. From day 16 after heading through maturity the only difference observed is quantitative. The high mobility weak components seen at days 4 to 13 have disappeared by day 16. The Kaw buffer-soluble starch-gel patterns are shown in Figure 3 and all are qualitatively the same as the Triumph samples. It should be noted that the Kaw variety headed later and matured earlier than the Triumph.

The starch-gel patterns for the urea-soluble proteins of both Triumph and Kaw are also qualitatively very similar. Figure 4 represents the Triumph urea-solubles and Figure 5 the Kaw urea-solubles. At day 4 in both varieties, there is no staining in the lower region of the gel but a constant build-up of material in this area is seen as the plant matures. At day 6 for Kaw and day 11 for Triumph, there may be noted the formation of components of high mobility though they are weak in appearance. Along with the formation of these fast moving components is noticed the appearance of low mobility material. By day 15 for Kaw and day 23 for Triumph, no further qualitative changes in protein composition are observed, each extract containing the full complement of components present in the ripe kernel. The synthesis of all fast moving material appears to be completed prior to that of the low mobility material.

From the above results it can be concluded that there is a qualitative as well as quantitative formation of proteins in the developing wheat kernel. In the buffer-soluble samples there is a quantitative build-up of material of intermediate mobility with a loss of the high

Figure 3. Starch-Gel Electrophoresis of Lyophilized Kaw Fractions Extracted with NaCl-PO_4 Buffer.

Approximately 230-290 µg. of material on sample wicks.

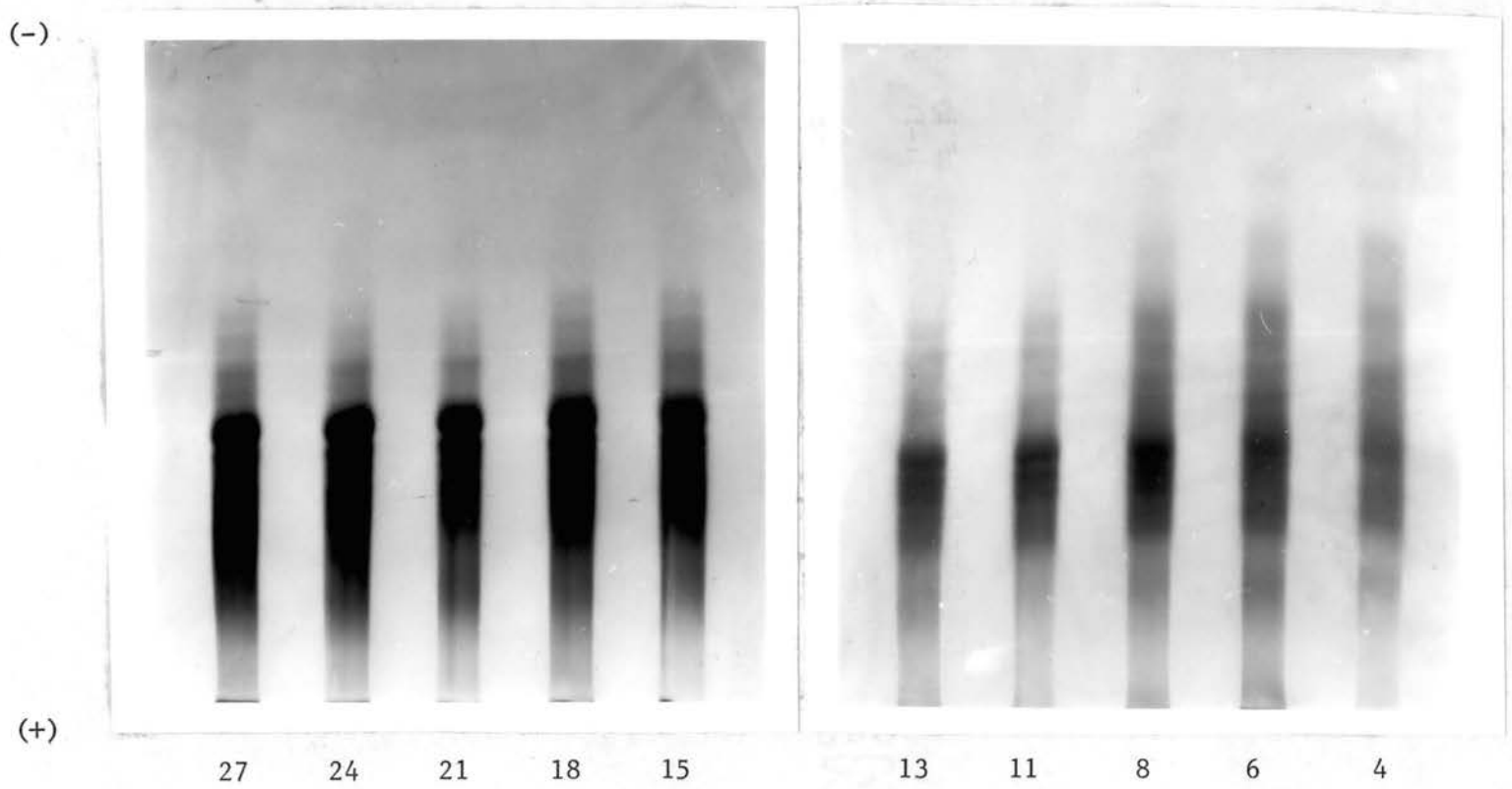

(−)
(+)
27
24
21
18
15
13
11
8
6
4
DAYS AFTER HEADING

Figure 4. Starch-Gel Electrophoresis of Lyophilized Triumph Fractions Extracted with Urea.

Approximately 260-370 µg. material on sample wicks.

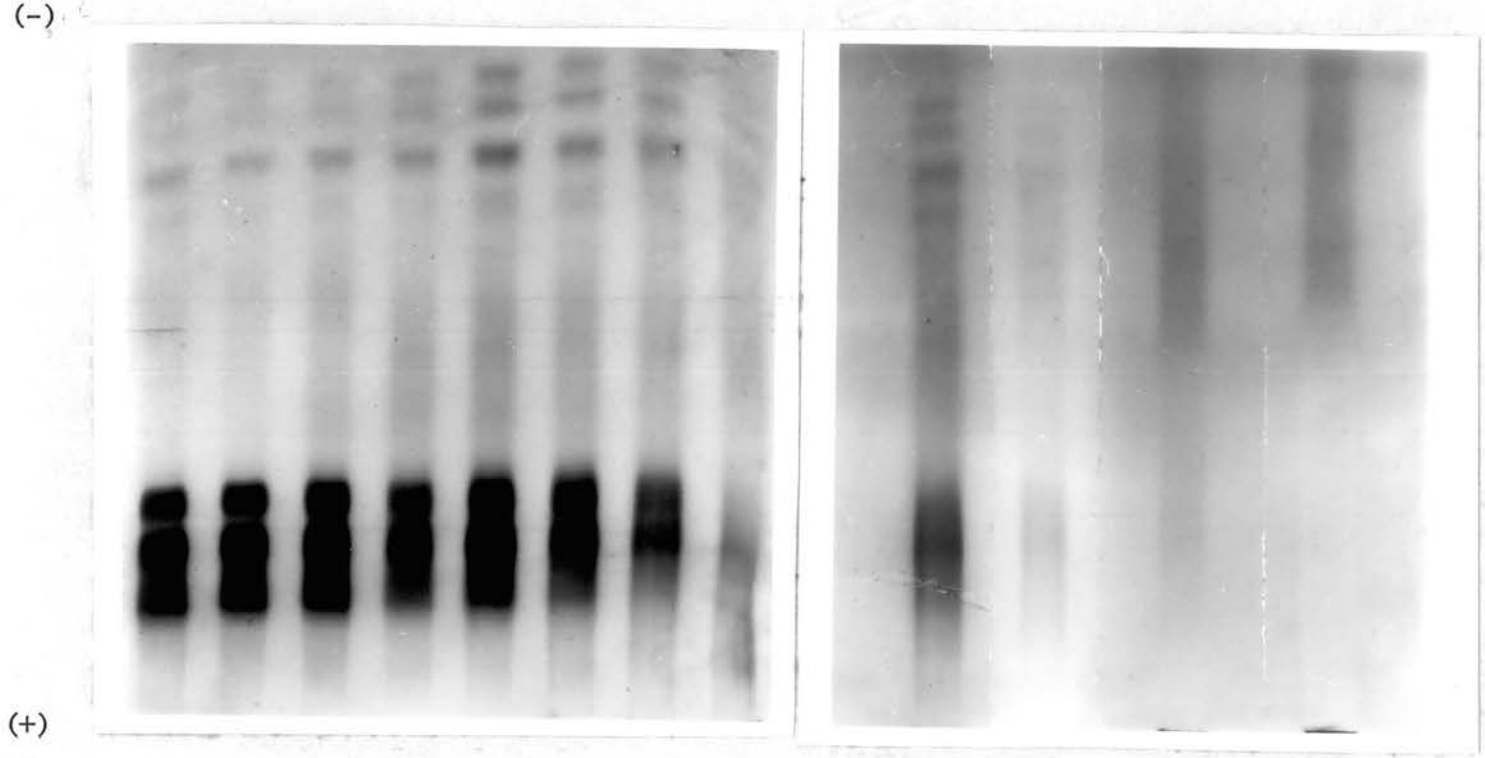
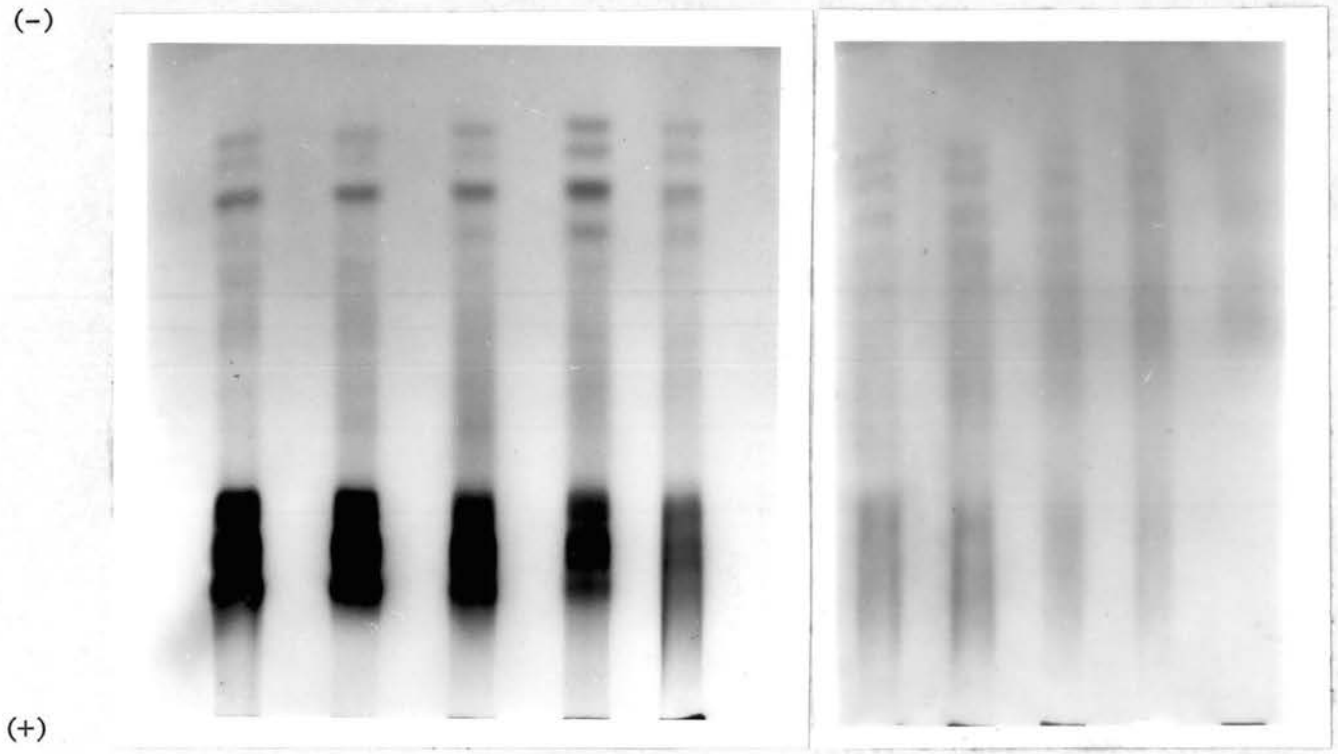

(-)
(+)
36
32
29
26
23
20
16
13
11
9
4
DAYS AFTER HEADING

Figure 5. Starch-Gel Electrophoresis of Lyophilized Kaw Fractions Extracted with Urea.

Approximately 250-300 µg. material on sample wicks.


(−)
(+)
27
24
21
18
15
13
11
8
6
4
DAYS AFTER HEADING

mobility components present in the immature samples as the plant matures. These observations agree with the findings of some of the earlier workers (23, 36), but are in contrast with those of others (27, 34, 35) who found only quantitative changes.

In the urea-soluble samples there is a simultaneous increase in components of high and low electrophoretic mobility. The results indicate that qualitatively the formation of gluten proteins is complete about 15 days and 20 days before the grain is ripe for Kaw and Triumph respectively. The synthesis of urea-soluble protein components of ripening wheat kernels seems to substantiate further the view of earlier workers (28, 30, 33) that the components of low electrophoretic mobility may represent the storage proteins of the grain. Protein assays of the Triumph maturity series show that as the plant matured, there was a rapid increase in the amount of urea-extractable protein from 6 to 16 days after heading. The rate of the protein accumulation decreased after this date (Figure 6). This fact correlated with the starch-gel patterns would further indicate the synthesis of gluten proteins as the plant matures.

Immuno-Electrophoretic Analysis (IEA)

Buffer-Soluble Proteins:

IEA experiments were conducted in an attempt to further distinguish between proteins of the mature and immature wheat kernels. Comparative analysis of the Triumph and Kaw buffer-soluble extracts at various maturities are presented in Figures 7, 8, 9, and 10. AntiKaw-buffer-solubles serum #6 prepared in this laboratory was used for experiments shown in Figures 10 and 11 while antiwheat-buffer-solubles serum #202

Figure 6. Lowry Assay on Triumph Urea-Soluble Extracts at Various Stages of Maturity.

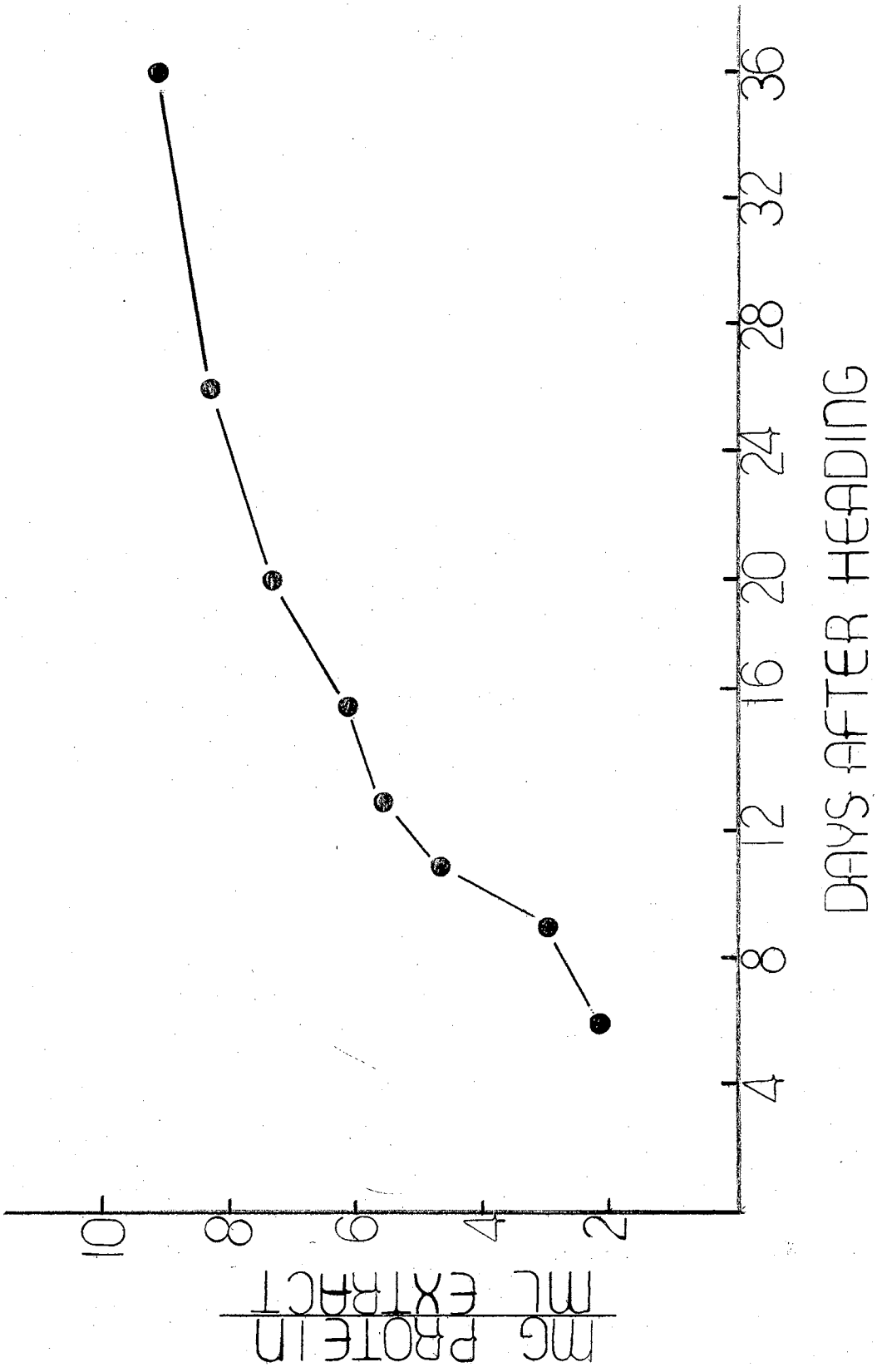
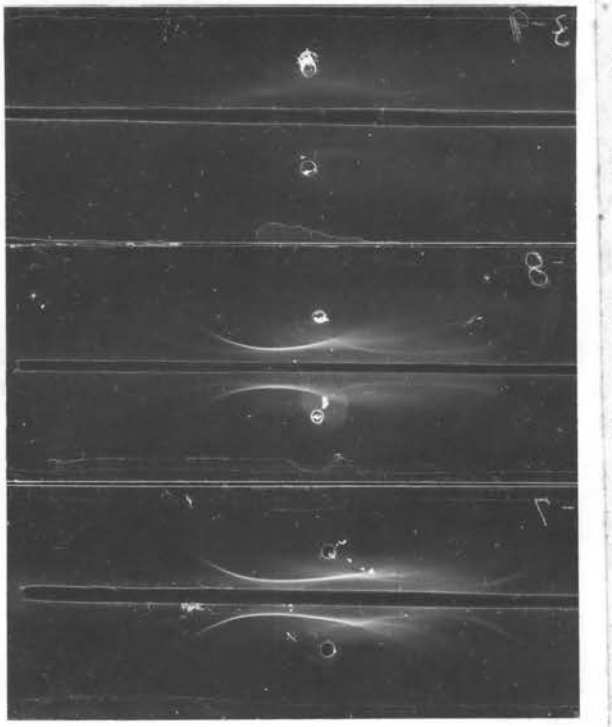

MG PROTEIN
ML EXTRACT
10
8
6
4
2
4
8
12
16
20
24
28
32
36
DAYS AFTER HEADING

Figure 7. IEA of Triumph and Kaw Buffer-Solubles Extracts as Developed with AntiKaw #6 Serum.

K = Kaw
T = Triumph
days = days after heading

(-) (+)

SLIDE #



1 K, 4 days

T, 6 days

2 K, 6 days

T, 9 days

3 K, 8 days

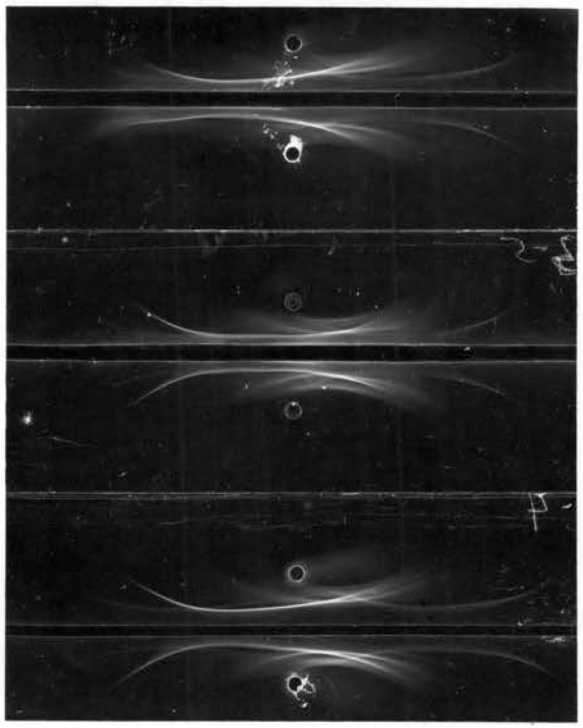
T, 11 days

Figure 8. IEA of Triumph and Kaw Buffer-Solubles Extracts as Developed with AntiKaw #6 Serum.

K = Kaw
T = Triumph
days = days after heading

(-) (+)

SLIDE #



1 K, 11 days

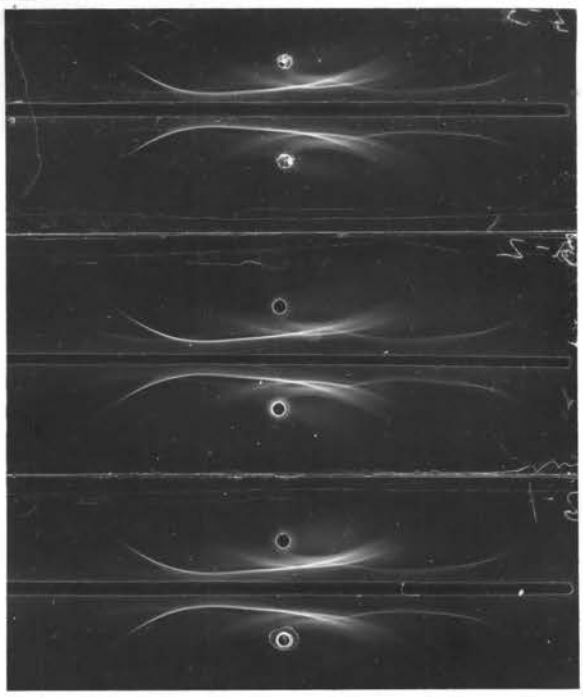
T, 13 days

K, 13 days

2 T, 16 days

K, 15 days

3 T, 20 days



K, 21 days

4 T, 26 days

K, 24 days

5 T, 32 days

K, 27 days

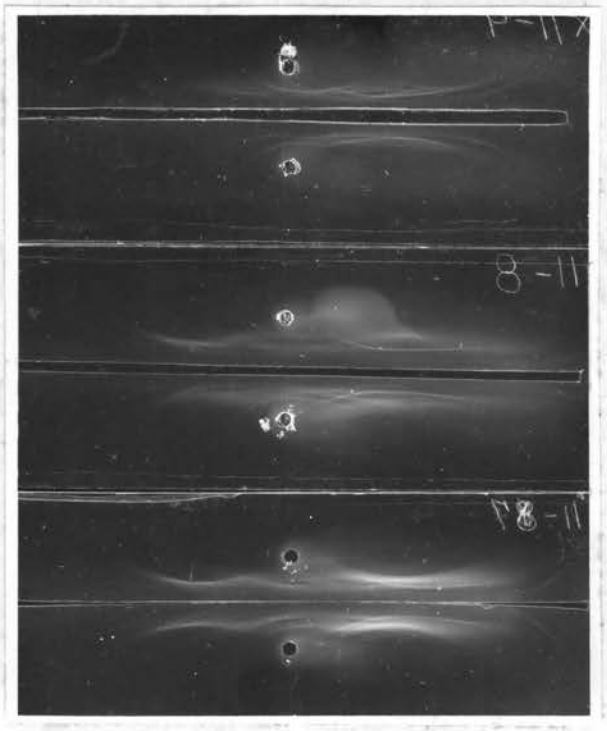
6 T, 36 days

Figure 9. IEA of Triumph and Kaw Buffer-Solubles Extracts as Developed with Antiwheat Serum #202.

K = Kaw
T = Triumph
days = days after heading

(-) (+)

SLIDE #



1

K, 4 days

T, 6 days

2

K, 6 days

T, 9 days

3

K, 8 days

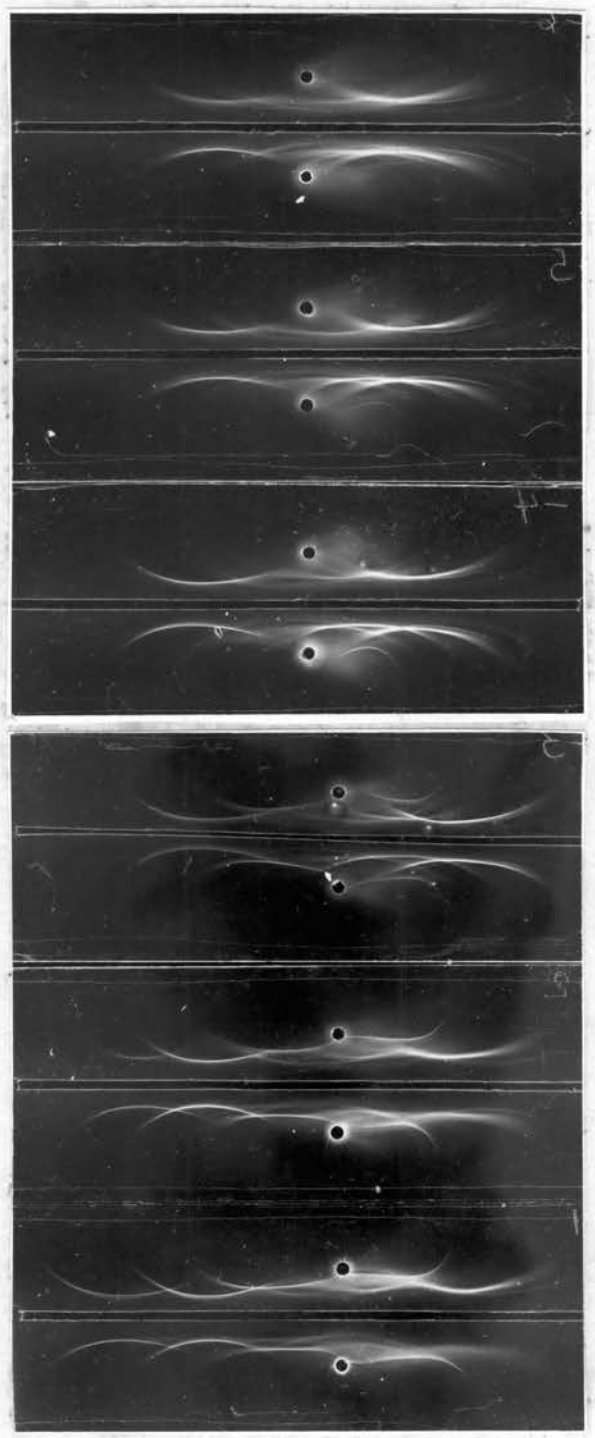
T, 11 days

Figure 10. IEA of Triumph and Kaw Buffer-Solubles Extracts as Developed with Antiwheat Serum #202.

K = Kaw
T = Triumph
days = days after heading

(-) (+)

SLIDE #



1 K, 11 days

T, 13 days

2 K, 13 days

T, 16 days

3 K, 15 days

T, 20 days

4 K, 21 days

T, 26 days

5 K, 24 days

T, 32 days

6 K, 27 days

T, 36 days

obtained from Pasteur Institute was used for experiments pictured in Figures 9 and 10. As was expected, there was essentially no difference between varieties. It is evident, however, that serum #202 evoked more precipitin arcs than the antiKaw serum. Further discussion will be limited to work with this serum except where otherwise noted.

It is apparent that a number of changes in protein occured as the wheat kernels developed. The general trend noted in Figures 9 and 10 is the gradual build-up of material that migrated toward both the anode and the cathode. In the least mature samples of Triumph and Kaw (slide 1), Figure 9) very little material that migrated toward the cathode is seen, but as the plant developed at least four proteins migrating toward the cathode were formed. Most of the proteins that migrate toward the anode in the immature samples appear to be present at all stages of maturity with increases in their concentration as maturity was approached. A new protein with low electrophoretic mobility (toward the anode) and low diffusion rate began to appear at day 13 in the Triumph (slide 1, Figure 10) and day 13 in the Kaw (slide 2) although it is very faint in staining intensity. This arc increased in intensity and extent as maturity of the plant increased. The most cathodic arc seen in slide 5 (Triumph 32 and Kaw 24 days after heading) first appeared at 20 days and 21 days after heading for Triumph and Kaw, respectively, although it was too faint to appear in the photographs. Another change noted is the gradual change in the rather diffuse area of precipitation just adjacent to the sample well on the cathode side. At about 21 days for Kaw and 20 days for Triumph a definite precipitin arc appeared and increased in intensity from that time through maturity.

It is clear from these IEA experiments that at least four new

proteins make their appearance during the period from heading to maturity. Proteins already present in the earliest samples appear to increase in concentration throughout maturation.

To more clearly demonstrate changes between the different stages of maturity, absorption experiments were performed using buffer extracts of the Triumph variety at intervals where definite changes seemed to occur. IEA of several samples using antisera #202 absorbed with extract of the Triumph sample taken six days after heading is seen in Figure 11. When the six-day extract was reacted with the absorbed serum, no precipitin arcs appeared. This result indicates antibodies to antigens in this extract were absorbed from the antiserum (slide 1). The reaction of the six-day extract with the blank serum and with the untreated serum (slide 2) showed identical patterns except for the greater intensity of the precipitin arcs with the untreated serum. This must be expected since the blank serum was slightly diluted due to the addition of the buffer.

The extract of Triumph nine days after heading shows the presence of two proteins not present in the six-day Triumph extract, as indicated when this extract is reacted with the absorbed serum (slide 1). At this developmental stage the first protein band that migrated toward the cathode appeared, and it increased in concentration at day 11 and 13 (slide 2). The difference between the patterns of these extracts (11 and 13 day) as compared with the nine-day extract reacted with the six-day absorbed serum is the greater intensity and extension of the arcs produced by the 11-and 13-day extracts when reacted with the absorbed serum. There is a splitting of the arc migrating toward the anode in the 11-and 13-day extracts. This splitting is not indicative

Figure 11a. Antiserum #202 Absorbed with Triumph Buffer-Soluble Extract 6 Days after Heading.

Days = Days after heading.
Abs. = Antiwheat #202 absorbed with extract 6 days after heading.
Blank = Antiwheat #202 absorbed with NaCl-PO_4 buffer.
Unabs. = Untreated antiwheat serum #202.

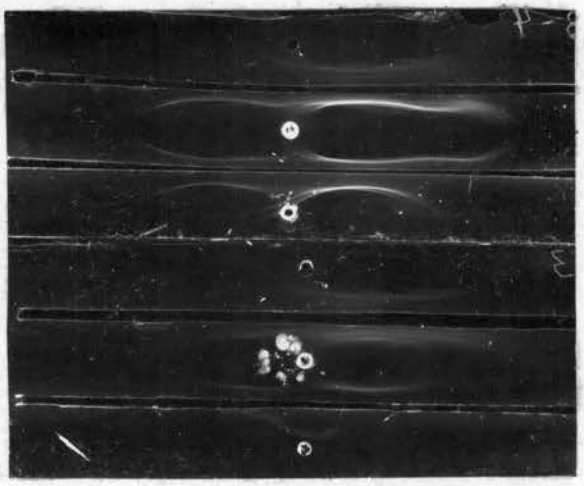
Figure 11b. IEA of Antiwheat Serum #202 Absorbed with Triumph Buffer-Soluble Extracts 13 and 26 Days after Heading.

Days = Days after heading.
Abs. 13 = Antiwheat #202 serum absorbed with Triumph extract, 13 days after heading.
Abs. 26 = Antiwheat #202 serum absorbed with Triumph extract, 26 days after heading.
Blank = Antiwheat #202 serum absorbed with NaCl-PO_4 buffer.

(-) (+)

A

SLIDE #



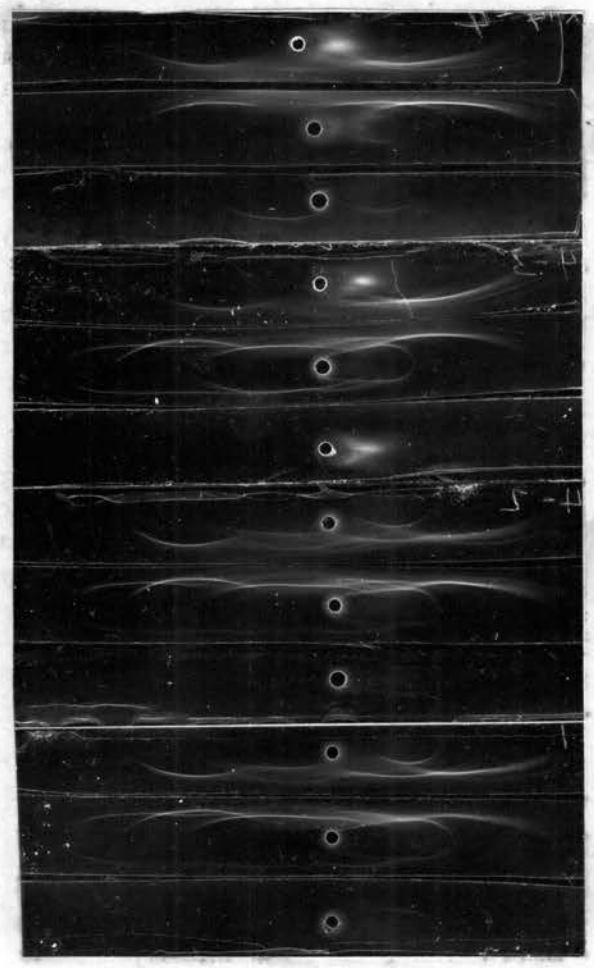
2

6 days
Unabs
13 days
Abs
11 days
6 days
Blank
9 days
Abs
6 days

1

B

SLIDE #



1

13 days
Unabs
16 days
Abs 13
26 days

2

13 days
Blank
32 days
Abs 13
13 days

3

26 days
Unabs
32 days
Abs 26
26

4

26 days
Blank
36 days
Abs 26
26 days

of the formation of a new protein but demonstrates that this antigen's concentration in the extract is in excess of its antibody concentration in the serum (quantitative increase of this protein with development).

When compared with each other the 11 and 13 day precipitin arc patterns are relatively the same except for the further extension of the arc migrating toward the anode in the extract 13 days after heading. This could be due to two proteins having the same antigenic groups but differing in their electrophoretic mobility. The lengthening and flattening of the cathode arc in these extracts suggest that this arc is increasing in concentration as the plant matures, since it is longer and flatter in the 13-day extract than in either the 11- or 9-day extracts.

Antisera #202 was also absorbed with buffer extracts of Triumph samples taken 13 and 26 days after heading. The resulting IEA are presented in Figure 11a. It is noted from this Figure that all the antibodies to antigens in the 13-day extract were absorbed out of the antiserum (slide 2). The extract of 16 days after heading appears to be rather similar to that of the 13-day extract since no precipitin arcs appeared except for a faint initial appearance of an arc on the anode side of the central reservoir (slide 1). This faint arc lengthened somewhat by 26 days after heading (slide 1). At this stage of maturity (26 days), another protein appeared, one which migrated toward the cathode (slide 1) and which first appeared 20 days after heading (Figure 10, slide 3). At 32 days the faint arc, which first appeared in the 16-day extract reacted with the 13-day absorbed serum, has lengthened to the point that it begins at the central reservoir. This indicates an increase in quantity. It should be noted that this arc

first appeared faintly in the 13-day extract reacted with the antiwheat #202 serum (Figure 10, slide 1). Its electrophoretic mobility and diffusion coefficient is low; this suggests that it may be a high molecular weight protein. It is at this time (32 days after heading) that the most cathodic arc was present in significant enough amounts to be detected by photography (Figure 10, slide 5). When reacted with the 13-day absorbed serum, its presence is very evident in the 32-day extract (Figure 11b, slide 2).

Reaction of the 26-day extract with antiserum #202 absorbed with this extract shows that all of the antibodies to the antigens were absorbed from the serum except for a small amount of one corresponding to the arc extending from the anode side of the origin (Figure 11b, slide 4). The same relative pattern as that obtained when the 32-day extract was reacted with antiserum absorbed with the 13-day extract is seen when the 32-day extract was reacted with antisera absorbed with the 26-day extract (slide 3). The only difference noted is that the staining of the pattern is less intense at this period of development than at the earlier stage (slide 2). This indicates that these components are present in the 26-day extract as well as the 32-day extract. More complete absorption should have eliminated these arcs completely. The reaction of the 36-day extract with the antiserum absorbed with the 26-day extract revealed patterns both qualitatively and quantitatively the same as the one obtained by reaction of the 32-day extract with this serum (slide 4). These results offer substantiating evidence of a quantitative build-up and qualitative formation of proteins as the plant develops. From these experiments the formation of at least four different proteins have been ascertained from day six through maturity.

Urea-Soluble Proteins:

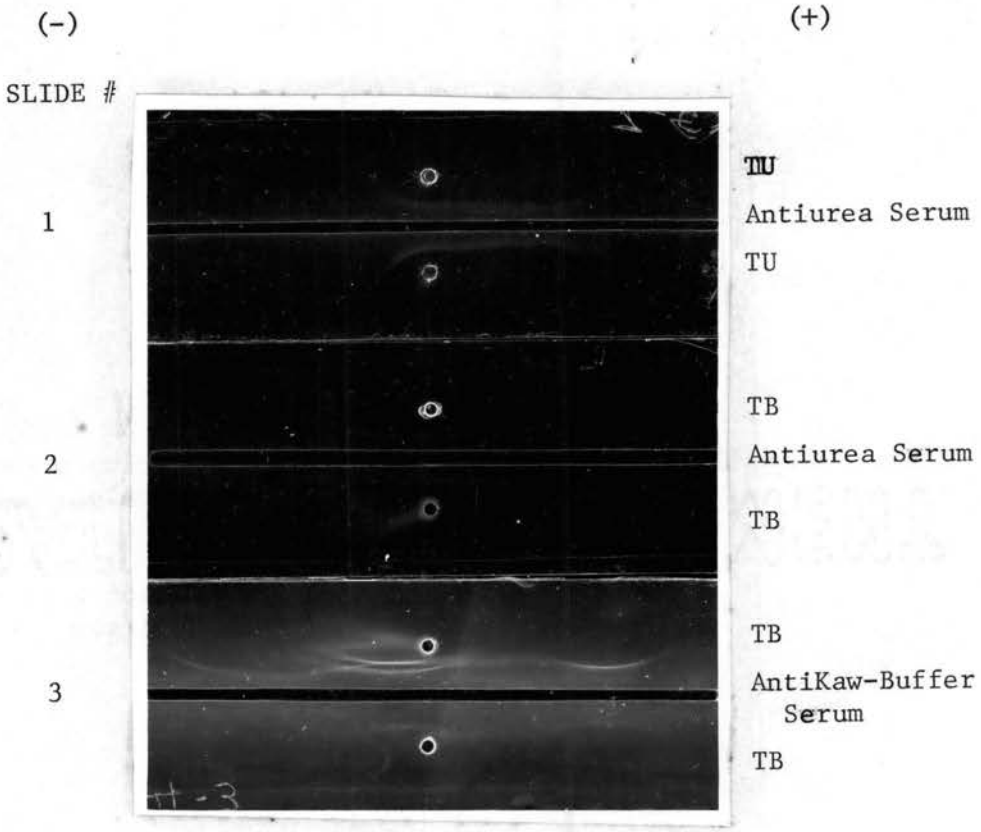
A limited number of IEA experiments were performed involving the urea-soluble proteins. The extracts of these proteins seemed to precipitate in the sample reservoir when experiments were run at pH 8.2. Also at this pH the urea-soluble proteins show little electrophoretic mobility or diffusion. Therefore, to remedy this problem IEA experiments were performed in the presence of 3M urea at a low pH and the gel was briefly washed with pH 8.2 buffer prior to addition of the antiserum (antibody-antigen reactions are hampered by low pH and urea). Even with this treatment, no arcs developed, although it had been reported by Nimmo and O'Sullivan (17) that this method is successful.

An antiurea-solubles serum prepared in this laboratory was reacted with a Triumph urea-solubles extract (mature, Figure 12, slide 1) and buffer-soluble extract (mature, Figure 12, slide 2) in veronal pH 8.2. Both extracts showed a protracted line of precipitation which was more pronounced in the urea-soluble extract. The only apparent difference was that the urea-soluble extract migrated further toward the cathode than did the buffer-soluble extract. The reason for this could be due to the relative position of the slides during the electrophoresis part of the experiment. To determine whether the precipitin arc patterns obtained were analogous to each other, absorption experiments would have to be run.

When earlier maturity extracts were reacted with this serum, the precipitin arcs obtained were too faint to be photographed, but they did have the same relative patterns exhibited by the mature sample. It should be mentioned that the appearnace of precipitin arcs was not

Figure 12. Comparison of Buffer- and Urea-Soluble Proteins with Antiurea and Antibuffer-solubles Serum.

TU = Triumph urea-soluble extract (mature).
TB = Triumph buffer-soluble extract (mature).


(-)
(+)
SLIDE #
1
2
3
TU
Antiurea Serum
TU
TB
Antiurea Serum
TB
TB
AntiKaw-Buffer Serum
TB

observed with the antiurea serum until 13 days after heading (Triumph), but these arcs gradually increased in intensity as the wheat kernel matured.

When a mature urea-soluble extract was reacted with antiKaw buffer-solubles serum #4, three arcs that migrated toward the cathod appeared (Figure 12, slide 3). From this observation one could conclude that the anti-buffer-solubles serum contains some antibodies to the antigens present in the urea-soluble extract. This could be caused by either of two reasons. First, there could have been some buffer-soluble material (albumins and globulins) present in the residue as a contaminant prior to urea-extraction. This material would be extracted with urea together with the gliadin proteins. The buffer extract was used to prepare the anti-buffer-solubles serum in this experiment; therefore when the contaminated urea-extract was reacted with the antiserum, the resultant arcs appeared. On the other hand, the buffer may have extracted quantities of gliadin protein in sufficent concentration to cause formation of antibodies. Therefore when this serum was reacted with the urea-solubles, the arcs appeared.

There are several ways of verifying the above assumptions. One could absorb the anti-buffer-solubles serum with a pure albumin-globulin preparation, and react the antiserum with the urea-extract. If the first suggestion was true, no precipitin arcs corresponding to some of the albumins or globulins would appear. Also a pure gliadin sample could be used to absorb the antibuffer-soluble serum, and when this absorbed serum is reacted with the urea-soluble extract, no arcs should appear corresponding to gliadin protein. The use of both of these techniques should determine what caused the precipitin arcs when

the urea-soluble extract was reacted with the antiKaw-buffer-soluble serum.

Enzyme Assays:

Enzyme assays were performed on the buffer-soluble samples developed with the antisera #202 and antisera as set forth in the MATERIALS AND METHODS. Esterase was the only enzyme that gave a strongly positive reaction with the antiserum (Figure 13). The β-amylase gave a very faint reaction, but the peroxidase showed no sign of activity. These results could be due to a low antibody content in the antisera to amylase and peroxidase or inactivation of the enzymes in the samples. The latter would not seem to be the case for the amylase, since reaction of amylase with the starch gel was very evident in some earlier experiments. A schematic diagram is used for Figure 13 because the amylase band was too faint to photograph. This was not the case for the esterase enzyme but it is presented schematically also.

CONCLUSIONS

Separations of buffer-soluble fractions by IEA produced more protein bands than are evident in the starch gels. The opposite is true for the urea-soluble protein fraction. It can be concluded from both of these types of analyses that as the wheat plant matures there is an increase in the amount and number of proteins extracted.

Fish concluded that, qualitatively, the same proteins are extracted from the endosperm of the wheat kernel at all stages of maturity (35). The results obtained in this thesis are contrary to Fish's finding, probably because plant sampling was performed at earlier stages of wheat kernel development in the present study.

Figure 13. Schematic Diagram of Esterase and Amylase Enzyme Activity on Precipitin Arcs.

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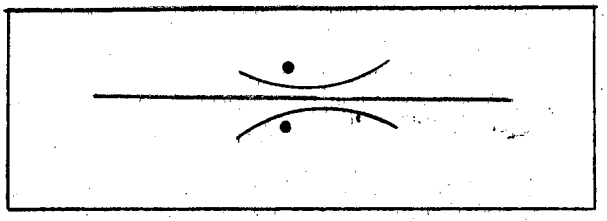
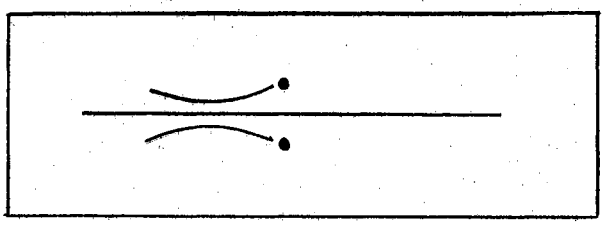


−

ESTERASE

+



−

AMYLASE

From the starch-gel patterns of the urea-soluble proteins, these quantitative and qualitative changes are quite easily observed. At four days after heading no band formation was present, but by 20 days after heading the full complement of components present in the mature protein samples had formed. The same was true for the buffer-soluble samples except more sharpness in the bands was observed in the four-day sample.

It has been determined from this investigation that IEA is a useful tool for observing the different protein constituents of maturing wheat. Immuno-electrophoretic results from work on the buffer-soluble proteins were similar to those obtained by starch-gel electrophresis, e.g., from 20 days after heading to maturity no further qualitative changes were noted. At least four different buffer-soluble protein components were formed between four and 20 days after heading as determined by IEA.

CHAPTER V

SUMMARY

The results of the present investigation indicate that with increasing maturity of the wheat plant, there were both quantitative and qualitative increases of proteins until about 20 days after heading, after which only quantitative changes occured. This phenomenon was noted for both the buffer-soluble and urea-soluble proteins as indicated by starch-gel electrophoresis.

From immuno-electrophoresis the same type results were observed for the buffer-soluble proteins. At least four immuno-chemically distinct proteins were formed in both varieties Triumph and Kaw from four to 20 days after heading, with increases in concentration as maturity was approached.

The two varieties were found to be qualitatively the same in protein constituents as determined by starch-gel electrophoresis and immuno-electrophoretic analysis.

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