INFLUENCE OF ALIEN GENOME COMBINATIONS IN PROTEIN SYNTHESIS IN CEREALS

by

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ABSTRACT

Starch gel electrophoresis of the water-soluble, salt-soluble, acid-soluble and alcohol-soluble proteins of Triticale 6A190, Triticum durum, Secale cereale, Triticum vulgare and Tritipyron 6A58 revealed both qualitative and quantitative differences. The experimental evidence obtained indicated that the biosynthetic integrity of the alien genomes in the synthetic species(Triticale) was not fully maintained. The apparent variable influence of the tetraploid wheat (Triticum durum) genomes on protein synthesis in the three hexaploid cereals (Triticale 6A190, Triticum vulgare and Tritipyron 6A58) was observed.

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INTRODUCTION

The first successful production of a synthetic cereal species, Triticale, was reported by Rimpau as early as 1888. Since then, intensive research has been carried out by cytologists and cytogeneticists with the aim of refining the development of these synthetic species both in the octaploid and the hexaploid levels. Biochemical effects presumably due to the presence of two alien genomes in these synthetic species had also been noted by some investigators in the past. Thus, Kowarski (1901) and Moritz (1933) both reported that serological similarities existed between the proteins found in the extracts of rye, wheat and the synthetic rye-wheat species (1,2). In recent years, some further research involving immunological techniques has been reported. Hall, in 1959, reported (3) results of immuno-electrophoresis studies of the octaploid Triticale and its parental species and implied that the integrity of the rye genomes was generally maintained in the species hybrid. More recently, Unrau and Vaisey (4), and Unrau and Jenkins (5) made a comparative survey of the milling, baking and some compositional characteristics between Triticale and their parental species and attributed some observed differences in these characteristics in the Triticale to the influence of the rye genome.

The investigations herein described and discussed were instigated in an attempt to establish whether an observable

change in the biosynthetic integrity of both the alien genomes present in the hexaploid synthetic species Triticale might occur. Since the hexaploid Triticale 6A190 is essentially derived through a combination of the genomes of Triticum durum and Secale cereale, both these parental species were included in the study. Furthermore, because Triticum vulgare (Kharkov) and Tritipyron 6A58 have the parental genomes, namely "AABB" of T. durum in common with Triticale 6A190, they were also included in the investigation.

REVIEW OF LITERATURE

As early as the late 18th century, attempts had been made to characterise the protein in wheat flour. A number of investigators such as Beccari (1745), Einhof (1805), Taddei (1820), de-Saussure, Berzelius, Boussingault, Liebig, Dumas and Ritthausen (1872) published reports on the isolation and characterisation of these wheat proteins. However, these early reports were confusing and somewhat contradictory due to a general lack of agreement in terminology and in the number of individual components believed to be present in the most thoroughly studied protein -- gluten (6,7). It can be stated that Osborne (1907) carried out the first comprehensive and systematic studies of wheat proteins. He characterised and identified the protein constituents of wheat flour on the basis of solubility in various solvents in conjunction with analytical analysis of the individual elements and amino acid composition of the protein fractions. In his investigations which were published in 1907, he stressed that gluten constitutes about 80 per cent of the total wheat flour protein and that gluten is an intimate mixture of two distinct individual proteins, glutenin and gliadin, and that both these were present in essentially equal amounts (7). With the introduction of ultracentrifuge and electrophoretic techniques in the 1930's, fairly convincing evidence became available indicating that gliadin

did not behave as a single molecular species (8,9,10). It must be pointed out that many of the electrophoretic studies carried out at that time, using the Tiselius electrophoresis apparatus, did not give symmetrical electrophoretic patterns (18). Even then, considerable work was conducted under these unfavourable conditions. Thus, in 1948, Laws and France reported that no significant differences could be detected in wheat gluten proteins derived from various sources (11). McCalla (1951) showed that electrophoresis of plant proteins in sodium salicylate solution was unsatisfactory (12). Kondo et al (1951) demonstrated by electrophoresis that gliadin and glutenin would only exist as a mono-component protein using an alkaline buffer (Kolthoff's buffer)(13,14). Lontie et al (1952) found that gluten consisted of three major and two minor components (15). Mills et al (1954) suggested that at least four protein components were present in gliadin (16). By using urea, Holme and Briggs (1959) succeeded in obtaining enantiographic patterns in moving boundary electrophoresis and found that three components were present in gliadin (17). In a search for a more suitable buffer system for wheat protein electrophoretic investigations, Jones et al (1959) discovered that aluminium lactate buffer was particularly effective (18). They showed that gluten contained at least four major and one minor component. One of the major components could be further resolved into two components when chloroacetate buffer

was used. Their findings were confirmed by Woychik et al (1960) who isolated these electrophoretic components by chromatographic fractionation and comparing their migration properties (19). Further investigations of Woychik et al (1961), using starch gel electrophoresis in the presence of urea, resolved gluten protein into nine components (20). At almost the same time, Elton in England also obtained similar results, by using the same technique (21) as that used by Woychik. Elton attributed the origin of the eight components that migrated into the gel to the gliadin fraction while the portion that remained at the origin corresponded to glutenin (22). Meanwhile, Zenter (1960) reported that seven components were detected in gluten by paper electrophoresis (23). Meredith et al (1960) re-examined gluten in the moving-boundary Tiselius apparatus and found that seven peaks were obtained, however, lack of symmetry in the patterns hindered interpretation of the results (24). Simmonds and Winzor (1961) separated the gluten proteins into eleven fractions by chromatography on carboxymethyl-cellulose columns (25). Graham (1963), using an improved apparatus and procedures for starch gel electrophoresis, showed that protein components having similar electrophoretic mobilities occurred in the extracts of wheat flour obtained when using a variety of solvents. However, there were marked differences in the proportions of these components in various

extracts (26). Pence and co-workers (1963) carried out electrophoresis of wheat proteins in poly-acrylamide gel. When electrophoresis was carried out in either phosphate or cacodylate buffer at pH 6, 15 to 17 protein components were apparently obtained for the acetic acid soluble fraction, whereas in aluminium lactate buffer, at pH 3.2, about 9 to 10 bands were observed. All the bands in the gel were accounted for in a fractionation involving the use of diethylamino-ethylcellulose ion-exchange resin (27). Lee and Wrigley (1963) investigated the gluten proteins of different wheat varieties and some tetraploid Triticum species by column chromatography on carboxymethyl-cellulose and by electrophoresis on polyacrylamide gel in basic buffer (28). Obvious differences in the electrophoretic patterns of the wheat varieties and the tetraploid species were observed. However, correlation of these patterns to baking quality could not be made because varieties having similar chromatographic and electrophoretic patterns were found to differ rather widely in baking quality. From results of moving boundary electrophoresis studies of some flour proteins, Kelly and Koenig (1963) suggested that wheats could be classified into groups according to their electrophoretic patterns (29). Wright et al (1964) demonstrated that gel-filtration could be applied to the study of cereal proteins together with starch gel electrophoresis (30). Most recently (1964) Elton and Ewart published re-

sults of electrophoretic analysis of cereal proteins by starch gel at higher resolution and suggested that the protein components that migrated into the gel were probably single proteins independent of each other (31).

In the field of species synthesis of cereals, much effort has been directed towards the production of the synthetic species appropriately referred to as "Triticale". These cereal species were synthesized by the combination of the genomes of Triticum durum (AABB) and Secale cereale (RR). O'Mara, in his review, had reported that successful crossing of Triticum with Secale had been performed as early as 1877 (32), but the first successful production of these new species was reported by Rimpau in 1888. Intensive studies carried out at that time and until the last decade were confined to the octaploid triticale (33). It was not until the last decade, when Sanchez-Monge (1959) suggested the hexaploids as possibly the optimum level of polyploidy, that efforts on the development and improvement of hexaploid Triticale were intensified (34). Investigations concerned with the potential practical application of these cereal crops are underway in the Department of Plant Science, University of Manitoba (35,5).

Biochemical studies of species relationships in cereals were first conducted by Kowarski (1). In 1901, he reported that there were antigenic similarities in the soluble substances of wheat and rye flours. Later, Moritz (1933)

carried out comparative immunological studies of the antigenic specificity of the flour of rye wheat (Triticale) and the two parental species (2). He concluded from his results that the antigen composition of triticale included the specific antigens of wheat and rye as well as the antigens they had in common. Noting that both Kowarski and Moritz had used unfractionated extracts for the serological reactions, thus permitting only a rough estimation of the similarities and dissimilarities between the antigen mixture, Hall (1959) re-examined the antigenic specificity of cereal proteins in triticale and the two parental species by immuno-electrophoresis (3). In this study, antigens identified by means of their electrophoretic mobilities were found to be shared by the allopolyploid as well as, either or both the parents. He thus concluded that the biochemical complexity of cereal proteins increased in proportion to the degree of polyploidy.

MATERIALS AND METHODS

1. FLOUR SAMPLES

Flour samples used in this investigation were derived from the grain of one hexaploid triticale (6A190), one hexaploid tritipyron (6A58), a bread wheat Triticum vulgare (Kharkov), a tetraploid durum (Stewart) and a diploid rye (Prolific). Triticale (6A190) is a cereal species synthesized by the combination of the two alien species Secale cereale and T. durum. Tritipyron (6A58) is a synthetic species derived in a similar manner from T. durum and Agropyron elongatum. T. vulgare (kharkov) contains the genomes of T. durum and Aegilops squarrosa. All the grain samples were harvested in the 1962-63 season. Grains of these cereals were milled in a Quadruplex Experimental Mill under identical conditions. The flour samples were stored in a cold room at $5^{o}C$.

2. EXTRACTION PROCEDURES

The extraction of proteins from the samples was carried out using a Lourdes Multi-mixer. Four different solvents, namely water, 0.2 M sodium chloride solution, 70 per cent ethanol and 0.05 M acetic acid were used.

Ten grams of each flour sample were macerated in the mixer at 20 rpm for 15 minutes with water. After centrifugation at 2000 rpm, the residue was given a second treat-

ment in the mixer and the suspension centrifuged. The supernatent from the second treatment was added to the first. The residue was submitted to a similar extraction with 0.2M sodium chloride solution buffered at pH 7. After the salt extraction, the residue was hand-kneaded in a stream of distilled water to remove all starch and water soluble materials. The gluten ball thus obtained was extracted twice with 70 per cent ethanol. In this extraction and also in the subsequent acetic acid extraction, the speed of the mixer was raised to 40 rpm. During the extraction, the outside of the container was cooled with cold water. After the second treatment with alcohol, the residue was washed with distilled water before being dispersed in 0.05 M acetic acid.

To inactivate proteolytic enzymes and the amylases (18, 36), the water, salt, alcohol and acetic acid extracts were heated at 98 - 100°C for three minutes and cooled quickly. The alcohol fractions were then reduced by approximately one-third their original volume in a flash evaporator (reduced pressure) followed by dispersion in 0.5 M acetic acid. Each of these four fractions of supernatent was further clarified by centrifugation at 13,500 rpm for 30 minutes after which the salt samples were dialyzed against distilled water for 12 hours. All the samples were then lyophilized and stored in a dessicator placed in a cold room at 5°C.

3. ESTIMATION OF THE CARBOHYDRATE CONTENT

The carbohydrate content of the water, salt and acid fractions was estimated by the phenol-sulfuric acid method (37). A portion (5 mg) of each sample was dissolved in an appropriate amount of solvent. An aliquot (1 ml) of each sample solution was transferred into optically-matched pyrex test-tubes. An aliquot (1 ml) of a 20 per cent phenol solution was added to each, followed by a rapid addition of concentrated sulfuric acid (5 ml) from a Machlett burette. The mixture was swirled and allowed to cool. The absorbance of the solution was measured in a colorimeter (Bausch and Lomb Spectronic 20) at 490 mμ. A reagent blank was determined in the same way. The total carbohydrate was expressed as glucose by reference to a standard curve of this hexose.

4. DETERMINATION OF THE NITROGEN CONTENT

The nitrogen content of the freeze-dried samples was determined by a slightly modified method of Unrau (38) and Hill-Cottingham (39). An aliquot (0.2 ml) of a solution containing 5 mg of freeze-dried protein per ml of solvent, was evaporated to dryness in a small pyrex test-tube at 80-90°C. An appropriate volume (0.4 ml) of 1:1.2 v/v sulfuric acid-water solution was added to each tube. The solution was refluxed by placing the test-tube into drilled holes in a brass block and heating on a hot plate. After 5 hours of refluxing, the tubes were removed, cooled and

2 drops of 30 per cent hydrogen peroxide added. The solution was refluxed again for 5 minutes, and then cooled. Another 2 drops of 30 per cent hydrogen peroxide were added to each tube and the solution refluxed for another 5 hours before being removed and cooled. The solution which was perfectly clear after the last reflux, was transferred quantitatively to a 25 ml volumetric flask to which 1 ml of gum ghatti solution (approximately 0.2 per cent concentration), 2.5 ml of Nessler reagent and 10 ml of 2N NaOH solution were added. The solution, made to volume with distilled water, was allowed to stand for 1 hour before measuring the absorbance in the colorimeter at 515 mμ. A reagent blank was determined in the same manner as described above. A standard curve was prepared by using ammonium sulfate in essentially the same way. Nitrogen content ranging from 0-150 μg per ml of sample solution could be determined in this manner.

5. STARCH GEL ELECTROPHORESIS

a. Apparatus

The starch gel tray was made of lucite, with dimensions 30 x 22 x 0.6 centimeter. The tray had three removable sidewalls and a lid. Moulds (0.4 x 0.1 x 3 cm) for casting sample slots in the gel, were fastened at appropriate distances on the tray by use of the adhesive "Dope" (Eastman Kodak Co. USA).

Electrophoresis was carried out inside a migration chamber especially built for this purpose. In this chamber were two buffer containers and a support for the gel-plate which was placed in between the two buffer tanks. The buffer chambers were constructed of lucite. Each chamber was divided into two compartments by partition so that two different buffers could be used simultaneously. In each compartment, an internal partition with a narrow opening at the bottom, further divided each compartment, thus serving as a salt bridge. In the outer chamber was placed a platinum wire electrode which extended across the two compartments. The platinum electrode was fastened tightly to a brass-rod, fitted at one end of the buffer chamber. A brass socket which was soldered with insulation to the floor of the chamber, connected the brass rod and the external power supply. Electrical connections between the gel resting on the support and the buffer solution in each tank were made with filter paper strips.

Direct current was supplied from a stabilized power unit (Kensington Scientific Corporation) capable of delivering 150 - 400 volts and 150 milliamperes.

b. Preparation of buffer

A stock solution of 0.17 M aluminum lactate (18) was prepared by activating 5.47 grams aluminum foil with a small amount of saturated mercuric chloride solution and then dissolving the metal in 38.5 ml of lactic acid. The

reaction was allowed to proceed overnight followed by filtration of the solution through celite. The solution was made to a volume of 1 liter. In the preparation of the aluminum lactate - lactic acid buffer, pH 3.1, a volume (100 ml) of the stock solution was added to a solution containing 2 M urea in a 2-liter flask. After adjusting the pH to 3.1, the solution was made to volume with distilled water. The concentration of the solution was 0.0085 M and the calculated ionic strength was 0.05 assuming that aluminum exists as the trivalent ion Al^{+++}.

c. Preparation of gel

A quantity (65 gms) of partially hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada) was mixed thoroughly with 500 ml of aluminum lactate buffer in a 2 liter round bottom flask. The starch suspension was heated on a water bath 80°C and the flask mechanically rotated until a viscous mobile liquid was formed. The liquid was poured immediately on to the gel-tray and then covered with a sheet of polyethylene taking precautions to exclude air bubbles. A lid was screwed tight to the tray in order to extrude excess gel.

d. Electrophoresis

After allowing the gel to set, the lid was removed from the tray and the gel transferred to a glass-plate which was placed on the support stand inside the migration chamber. An appropriate amount of the sample solution, dis-

persed in aluminum lactate buffer, was applied to the sample slots. Electrophoresis was carried out with the gel in a horizontal position at 12 v/cm for 15 to 24 hours depending on the type of protein samples under investigation. After electrophoresis, the gels were sliced to a thickness of 2 mm by a thin wire held firmly between two supports. A mixture (3:1 v/v) of 0.1 per cent Amido black 10B in 5 per cent acetic acid solution and 0.007 per cent Nigrosine in 0.2 per cent trichloroacetic acid solution were used to stain the gels overnight. The stained gels were washed repeatedly with a mixed solvent of methanol-water-acetic acid (50:50:10 v/v) until greatest contrasts between bands in the gel were observed. Usually 5 washings were found to be sufficient. The developed gels which were shrunk somewhat after destaining, were immersed in 2 per cent trichloroacetic acid for 3 hours to restore them to the original size. The stained gels were scanned in a recording densitometer (Photovolt Corporation). Finally, the gels were photographed and wrapped in "saran-wrap" for further storage and reference.

6. PREPARATION OF POLYACRYLAMIDE GEL

Gels containing 5 per cent (w/v) Cyanogum 41 (American Cyanamid Company, N.Y.) were prepared according to the method described by Raymond and Wang (40). It was noted that gelations could be achieved easily in neutral and basic buffers.

However, attempts to prepare the gels in acid buffers such as aluminum lactate-lactic acid buffer were not successful, hence, further attempts were not made.

RESULTS AND DISCUSSION

1. FLOUR SAMPLES

The texture and appearance of the flour samples may vary from one variety to another. Triticale (6A190) gave a pale-white flour with a texture quite similar to those obtained from Prolific rye. Stewart durum, Kharkov and Tritipyron (6A58) gave flours with a light yellow appearance. Unrau and Vaisey (4) reported that many of the hexaploid triticale species contained less yellow pigment than the durum varieties. These observations give an indication of an expression of a more dominant character of the rye genomes in the synthetic cereal species; that is, where the rye genomes and durum genomes existed together in one cellular environment.

2. EXTRACTION

In some initial experiments, flour samples were extracted with water-saturated n-butanol to remove lipids prior to extraction of the proteins by the method described in the Material and Methods section. When a sample of triticale flour, which had been defatted by the water-saturated n-butanol treatment, was compared with a control sample of the untreated flour (no butanol extraction), the electrophoretic patterns showed no significant differences (see Fig.1). In this figure, patterns in the odd numbered

FIGURE 1. Starch-gel electrophoresis of proteins from non-defatted and defatted flours of Triticale (6A190).
Channel 1-water fraction of non-defatted flour;
Channel 2-defatted flour;
Channel 3-salt fraction of non-defatted flour;
Channel 4-defatted flour;
Channel 5-acid fraction of non-defatted flour;
Channel 6-defatted flour;
Channel 7-alcohol fraction of non-defatted flour;
Channel 8-defatted flour.

Al-lactate buffer 0.0085M+2M urea, pH 3.1

Potential gradient 12 v/cm, time-20 hrs.

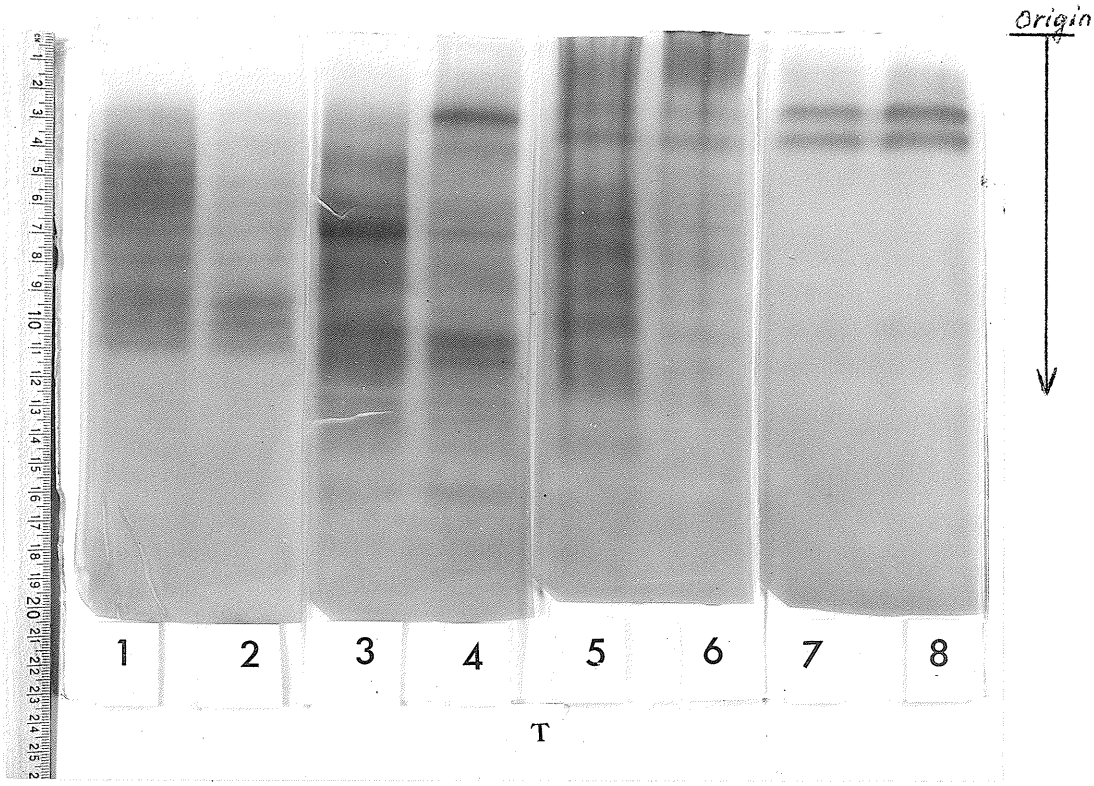


Fig. 1

gels were of the non-defatted flour while patterns in the even numbered gels were from the defatted flour. Considering the gels in the figure from left to right, they are, the water-soluble proteins (gel No.1 and 2), salt-soluble proteins (gel No.3 and 4), acid-soluble proteins (gel No.5 and 6) and the alcohol-soluble proteins (gel No. 7 and 8). With one exception in the case of the pattern of the water-soluble proteins, all the other electrophoretic patterns showed practically no detectable differences between the butanol extracted and non-extracted samples. In the water fraction, it was noted that a component with a migration distance of 4.6 cm was present in the electrophoretic pattern of the non-defatted flour samples but not in the defatted flour sample. The absence of this component in the defatted flour sample might be due to the removal of lipids or a lipoprotein from the sample; alternatively, the lipo-protein might be present but in minute quantities and which did not contrast sufficiently against the background stain. In view of the general similarity of the other electrophoretic patterns of the various fractions, the possibility that this particular component was present but was obscured because of background staining seemed quite probable. Jones et al (18) and Elton et al (22) also reported no change in pattern in the gluten fraction of the defatted and non-defatted flours. Because of their observations and the above mentioned results, untreated flours

were used in the subsequent investigations of the cereal proteins.

Conditions of extraction of the flour proteins were maintained constant throughout so that any differences in the electrophoretic patterns of these samples could be attributed to molecular size and electrical differences which might be a reflection of specific genome combinations rather than to variations in the isolation techniques.

The inactivation of the proteolytic enzymes and amylase activities in the extracts was effected by heating the extracts at 98 - 100°C for 3 minutes and then cooling the solutions quickly. It was noted that if the process of heating was maintained longer than 3 minutes, coagulation of the soluble proteins would occur, especially the water-soluble and the salt-soluble proteins. Elton et al (22) compared the electrophoretic pattern of the gluten fractions treated as described above with those containing 50 ppm of mercuric chloride which acted as an enzyme inhibitor and found no significant change in the electrophoretic patterns. This indicated that the time of heating of the protein preparations was somewhat critical in that, to obtain distinct electrophoretic patterns, enzymatic activity must be eliminated with minimum physical change (coagulation) of the particular protein preparations.

Prior to freeze-drying, the alcohol fractions were

evaporated under reduced pressure to remove most of the solvent. Generally one-third of the original volume of solvent was removed in this way and replaced by an equal volume of 0.5 M acetic acid. This also aided in freezing the solutions easily prior to lyophilization. The salt samples were dialyzed against distilled water to remove most of the salts before being freeze-dried.

The freeze-dried materials had a white, fluffy and soft texture. These freshly prepared materials were easily soluble or dispersed in the aluminum lactate buffer at pH 3.1, but they tended to become more difficultly dispersed when stored in room temperature and prevailing humidity. This phenomenon was particularly characteristic of the acid-extracted protein samples. Such solubility changes were not significant when the materials were stored in a dessicator which was placed in a cold room at $5^{o}C$.

3. ESTIMATION OF THE CARBOHYDRATE CONTENT

The carbohydrate content of the freeze-dried samples was determined by the phenol-sulfuric acid method (37). The results are shown in Table I. The water fractions of all the different cereal species contained about 60 per cent carbohydrate while the salt-fractions had about 15 to 20 per cent. The acid and alcohol fractions contained

less than 5 per cent carbohydrates with the exception of the acid fraction from rye which had 11.7 per cent. It is interesting to note the fairly even distribution of the amount of carbohydrate within the samples in each fraction.

TABLE I. Carbohydrate Content of Freeze-Dried Samples.

	Water Soluble Fraction	Salt Soluble Fraction	Acid Soluble Fraction	Alcohol Soluble Fraction
Rye (Prolific)	65.7%	17.3%	11.7%	2.0%
Durum (Stewart)	59.2	-	1.7	1.1
Triticale (6A190)	59.3	22.9	2.2	1.4
Wheat (Kharkov)	58.8	13.9	2.2	0.4
Tritipyron (6A58)	59.2	22.1	2.3	1.0

This probably indirectly reflects the consistency of the conditions of extraction and the treatment of the various fractions. The small amount of carbohydrate detected in the acid and alcohol fractions (less than 2 per cent) was probably due to physical entrapment or protein-polysaccharide interactions of these materials in the gluten ball. The relatively high content (11.7%) of carbohydrate in the acid soluble fraction of rye was not unexpected because the parent materials which had been previously extracted with water and salt solution, did not form a gluten ball that could be hand-kneaded under a stream of distilled water. Instead, these

rye residues were washed and the supernatent repeatedly decanted until no starch granules appeared to be left. The inability of this treatment to remove all the carbohydrate material was therefore reflected in the observed higher carbohydrate content of the acid soluble protein fraction of rye particularly when compared to the other samples which readily formed gluten balls that could be hand-kneaded in the conventional manner.

4. DETERMINATION OF THE PROTEIN CONTENT

The protein content of the freeze-dried samples was estimated by first determining the nitrogen content using the modified method described in the Materials and Methods section. The calculated nitrogen content was multiplied by the usual factor of 5.7 to give the protein content of the various fractions. The results are presented in Table II.

TABLE II. Protein Content of Freeze-Dried Samples

	Water Soluble Fraction	Salt Soluble Fraction	Acid Soluble Fraction	Alcohol Soluble Fraction
Rye (Prolific)	31.2 %	78.2 %	80.2 %	94.2 %
Durum (Stewart)	36.4	—	92.6	94.4
Triticale (6A190)	34.9	76.1	90.0	92.8
Wheat (Kharkov)	37.1	82.6	93.2	95.2
Tritipyron (6A58)	36.6	74.5	94.2	96.2

The fraction extracted by water for the five species of cereals ranged from 31 to 37 per cent protein while those extracted by salt solution were 75 to 83 per cent. The acid and alcohol fractions contained more than 90 per cent protein except the acid soluble fraction from rye. These two fractions, with the above mentioned exception of the acid soluble fraction of rye, could therefore be considered to contain relatively small amounts of impurities. The reason for the apparent lesser proportion of acid soluble proteins in rye was pointed out in the previous section.

A comparison of Table I and II show that approximately 95 per cent of the material present in the freeze-dried samples could be accounted for. Since the accuracy of the phenol-sulfuric acid determination of carbohydrates lies in the range of ± 5 per cent, the above results can therefore be considered significant.

An interesting fact revealed by the results in Table I and II is the considerable amount of carbohydrates present in the water and salt fractions. These carbohydrates are possibly associated with the proteins in the form of glycoproteins. As indicated earlier, the occurrance of rather strong protein-polysaccharide interactions is an alternative possibility. Both fractions produced a relatively large amount of humin during acid hydrolysis which is a further reflection of the rather significant amount of associated polysaccharide.

When the total recovery was mathematically normalised to 100 per cent and the corresponding percentages of carbohydrate and protein were calculated on this basis the figures shown in Table III were obtained for Prolific rye, Stewart durum and Triticale (6A190). It was noticeable that the protein content of Triticale (6A190) was consistently intermediate between that of rye and durum. This observation is interesting since Triticale (6A190) is a synthetic species containing the genomes of both diploid rye (Secale cereale) and the tetraploid wheat (Triticum durum).

5. STARCH GEL ELECTROPHORESIS

Since the introduction, by Smithies (41), of starch gel as a medium for zone electrophoresis to study serum proteins, its use as an analytical tool for the investigation of cereal proteins had been envisaged. Woychik (20) first applied this technique to study the protein components of gluten obtained from a hard red winter wheat, Ponca, and demonstrated the greater resolving power of this technique. In this laboratory, this technique had not been attempted, hence, a standard method of preparing the gels, the buffer, and generally suitable conditions for electrophoresis had to be developed before any detailed study of the cereal proteins could be carried out. To this effect, a series of

TABLE III. Normalized Value of Protein and Carbohydrate Content of the Freeze-Dried Samples.

	Water-Sol. Fraction			Salt-Sol. Fraction			Acid-Sol. Fraction			Alc. Sol. Fraction		
	% Protein	% Carbohydrate	Total	% Protein	% Carbohydrate	Total	% Protein	% Carbohydrate	Total	% Protein	% Carbohydrate	Total
Rye(Prolific)	32.2	67.8	100	82.0	18	100	87.3	12.7	100	97.8	2.2	100
Durum (Stewart)	38.0	62.0	100				98.1	1.9	100	98.8	1.2	100
Triticale (6A190)	37.1	62.9	100	76.9	23.1	100	97.6	2.4	100	98.4	1.6	100

exploratory experiments were conducted in order to select the most appropriate buffer system to be used for dispersion of the protein samples, and to standardize the technique of starch gel preparation and determine the optimum conditions for electrophoresis.

a. Buffer

In the past, the greatest difficulty encountered in the study of cereal proteins had been the inability to find a suitable buffer system whereby the gluten proteins could be homogeneously dispersed. This problem had been overcome in recent years by using aluminum lactate - lactic acid buffer (18). Hence, selection of a suitable buffer narrowed down to this particular one.

Preparation of this buffer was essentially the same as that employed by Jones (18), except with the addition of 2 M urea.

The concentration of the buffer used throughout the work was 0.0085 M, pH 3.1 and containing 2 M urea. This concentration was found to give a well defined electrophoretic pattern of the cereal proteins in the gels. The effect of varying the concentration of the buffer on the protein pattern had been studied. If the concentration was doubled (0.017 M), a blurred, ill-defined electrophoretic pattern with extensive trailing was obtained. Such an alteration of the pattern might be due to the effect of

heat which was produced in the gel because of the increased ionic strength. The calculated ionic strength of the 0.017 M buffer solution was 0.1 while that of 0.0085 M was 0.05, assuming that aluminum existed as a trivalent ion, Al^{+++}.

The presence of urea in the buffer helped to reduce intermolecular attractions in proteins and, therefore, aided in the improvement of the resolving power of the starch gel.

b. Preparation of starch gel

A standardized ingredient and standard method of preparing starch gels was provided by the Connaught Research Medical Laboratory (Canada) which manufactures the partially hydrolysed starch. However, this method was found to be inadequate when a large quantity of the gel was to be prepared. A more refined and incidentally more simplified procedure was developed for making larger quantities of starch gel. Instead of heating the starch suspension over a free flame and removing the air bubbles from the boiling viscous liquid by suction, the starch was suspended in buffer in a large round-bottom flask and the contents were stirred manually by rotating the container on a water bath maintained at 80°C. It was found that by heating a 500 ml quantity of starch suspension in this manner, a mobile liquid would be formed in less than 5 minutes which could be easily poured onto the gel tray. After the gel was set, no

air bubbles were found to be present in the gel provided sufficient care had been exercised not to mix the starch suspension too vigorously during preparation. If a few air bubbles were present, they could easily be dispelled by puncturing them with a capillary tube. It was found that the physical properties of the gel were the same as those when the standard method was used and consequently, the simplified procedure of preparing the starch gel was used throughout.

A number of different concentrations of starch in the gel were tested to determine whether this would have any effect on the protein pattern. As anticipated, it was found that no change was evident in the patterns when the proteins were subjected to electrophoresis in gels containing 10,12,15 and 18 per cent of starch. The only difference observed in the gel itself was of a physical nature rather than chemical, in that a gel with a high concentration of starch (18 per cent) was rigid while one containing a low concentration of starch (10 per cent) was soft. It was difficult to cut a rigid gel into thin sections having an even thickness. After several trials, a gel of 13 per cent concentration was found most useful and chosen for all succeeding experiments.

c. Electrophoresis

Electrophoresis was carried out in a cold room main-

tained at 5°C. Buffer used was 0.0085 M, adjusted to pH 3.1 with lactic acid and containing 2 M urea. The buffer solution was changed after each experiment. Electrical connection between the gel and the buffer in the buffer chambers was made with wetted (buffer) filter paper strips. A potential gradient of 12 volts per centimeter was maintained throughout the duration of electrophoresis which usually lasted from 15 to 24 hours (standard condition). The optimum time of electrophoresis was determined by subjecting the various protein fractions (water, salt, acid and alcohol soluble fractions) to different time intervals of electrophoresis. It was found that 15 to 20 hours was sufficient for migration of the faster components of the salt soluble proteins to near the edge of the gel. For the resolution of the other protein fractions, 20 to 24 hours were required.

The freeze-dried protein samples were dispersed in aluminum lactate buffer before being placed in the sample slots in the gel. A suitable concentration of each different protein fraction to be used was determined by running a series of concentrations of the protein sample side by side in one gel. As would be expected, the intensities of the bands in the stained gel diminished as the dilution increased. It was found that a solution generally containing 100 mg protein per ml buffer gave a distinct electrophoretic pattern for the salt soluble, water soluble and the alcohol soluble pro-

teins, while 75 mg protein per ml buffer was the upper limit that could be used for the acid soluble fractions due to difficulty of dissolving this protein. A volume of 100 µl of the sample solution was usually applied to each slot.

At the end of an electrophoretic run, the gel-plate was removed from the migration chamber and the gel sectioned into 3 layers, each with an even thickness of 2 mm.

All the 3 layers (the top, centre and bottom) were stained to determine whether any variation in the migrated distance of each component occured within the gel body. The developed gels showed no such noticeable differences, hence, in subsequent staining procedures, only the second layer of each gel was stained while the two remaining sections were covered with saran wrap and stored in a cold room at 5°C. Initially, the entire section of the gel strip was stained but it was found in due course that no protein components migrated from the origin into the gel towards the anode. This observation was in agreement with those reported by Woychik (20) and Elton (22) who explained that under the conditions in which acidic buffers (pH 3.1) were involved, the proteins would definitely be positively charged and consequently they would migrate into the gel towards the cathode. In view of this, only the portion of the gel extending from the point of sample insertion and extending towards the cathode was stained.

three different staining methods were tried. In one procedure the gels were immersed in 0.1 per cent Amido Black 10B dissolved in 5 per cent acetic acid for 15 minutes and destained by washing repeatedly in 5 per cent acetic acid solution. The developed gels were transparent and soft. It was noticed that some of the stained bands, especially those of the water soluble and salt soluble proteins, tended to fade away when the gels were left to stand overnight in the wash solution. This phenomenon was observed even when the moistened gels were dried with paper-towel strips and then stored. Attempts were made to transform the developed gels into permanent records by the glycerol-benzyl alcohol "drying" method of Jones (42), however the results were not satisfactory in that, before subjecting the developed gels to the glycerol-benzyl alcohol treatment, they were dehydrated in 95 per cent ethanol which not only removed the water but also a great proportion of the dye from the stained protein components. This resulted in the loss of many faintly stained bands in the gels and consequently this method of making permanent records of the gels was therefore not pursued further.

A second method of staining the gels involved the use of nigrosine. The gels were soaked overnight in 0.007 per cent nigrosine dissolved in 0.2 per cent trichloroacetic acid and then washed repeatedly in 0.2 per

cent trichloroacetic acid until a maximum contrast of the protein patterns was achieved. The developed gels were also transparent and soft. Although this staining method reduced fading of the stained bands to a minimum, it suffered the drawback in that most of the protein components were only faintly stained in comparison to the background.

Eventually, a combination of the above two methods was found to give superior results. The gels were immersed in a mixture of 0.1 per cent Amido Black 10B solution and 0.007 per cent Nigrosine solution overnight. The stained gels were washed in a mixture of methanol-water-acetic acid solution (5:5:1 v/v). Washing with this solvent made the gels translucent and rigid and some shrinkage of the gels occurred with this treatment. Usually five washings were found to be sufficient to attain maximum contrasts in the electrophoretic patterns. The gels were then immersed in 2 per cent trichloroacetic acid solution for 3 hours which restored the gels to their original size. This staining procedure had the advantage of preventing the stained components from fading and also provided greater contrasts between the stained bands and the background color.

The procedure of removing the background stains by washing was found to be a time consuming process. Considerable success had been reported by Ferris and co-workers (43) in reducing the destaining time to half an hour by subjecting the stained gels to electrolysis in a 10 per

cent solution of acetic acid. This method was simplified further by using a cylindrical tank of 5 liter volume as the electrolytic cell, and two stainless steel sheets folded into two cylinders of different diameter as electrodes. When these electrodes were immersed in the acetic acid solution (10 per cent) contained in the cell, the outer electrode was connected to the anode and the inner electrode to the cathode of a 12-volt storage battery. The gels were sandwiched between wet paper toweling and positioned next to the inner surface of the anode. Current was passed through the system for 30 minutes and the apparatus then disconnected. The gels were removed, washed with water and stored in saran wrap. This procedure gave satisfactory results. During the electrolytic process of destaining, it was found necessary to agitate the sandwitched gel occassionally in order to remove gas bubbles (hydrogen) trapped between the paper toweling and the gel.

The developed gels were photographed and then scanned in a recording densitometer modified to accomodate gel strips. The recorder was set on logarithmic response to give readings in terms of absorbance.

6. PREPARATION OF POLYACRYLAMIDE GEL

Preparation of poly-acrylamide gel

It has been reported by many investigators that polyacrylamide gel possessed certain advantages over starch gel. Its greater rigidity, which facilitated easier manipu-

lations, and its greater transparency, which provided better transmission in densitometry studies, were cited as some examples. Preparation of polyacrylamide gels with desirable physical properties in neutral and basic mediums by the method of Raymond and Wang (40) could be easily accomplished. Lee (28) has reported results from investigation of cereal proteins in which he used polyacrylamide gel electrophoresis exclusively. However, the results did not show that polyacrylamide gel had a greater or even comparable resolving power as that of starch gel. The difference might probable be due to the different pH used in polyacrylamide gel (basic) and starch gel (acid). Preparation of polyacrylamide gel in an acidic medium such as in aluminum lactate buffer of pH 3.1 had not met with success. In the writer's attempts, aluminum lactate buffers of different pH (3.1 to 4.5) and containing different concentrations of urea ranging from 0.to 3 molar were tried. None of these favored gel formation even after standing fro 48 hours. It had been suggested (44) that atmospheric oxygen exerts a delaying action on gel formation under the conditions used. Since a device or apparatus to envelope the gel solution in a nitrogen atmosphere was not available, this suggestion was not tested experimentally and further experimentation with polyacrylamide was discontinued. Later, Reisfeld and co-workers (45), reported the use of disc electrophoresis to study peptides and proteins and described a method

of preparing polyacrylamide gel (small pore) at pH 4.3. Their method was tried and was found workable but only when small glass tubing containers were used (as specified) in which the gel solution could be photopolymerised. On the other hand, an attempt to prepare the gel (large pore) at pH 6.8 by the specified method was not successful. Since this technique was obviously still in the developmental and refinement stages, no further experiments were carried out. A recent comprehensive discussion of the theory and application of disc electrophoresis has been prepared by Davis and Ornstein (46).

7. ELECTROPHORETIC PATTERNS OF CEREAL PROTEINS

a. Designation

Figures 1 to 7 show the electrophoretic patterns of the different cereal proteins while figures 8 to 12 show the densitometer spectra and calculated mobilities of the various bands in the afore-mentioned gel electrophoresis patterns.

Abbreviations used in these figures include:

R - Rye (Prolific)

D - Durum (Stewart)

T - Triticale (6A190)

K - Wheat (Kharkov)

Py - Tritipyron (6A58)

R/D - A mixture of the salt soluble proteins of rye (Prolific) and Durum (Stewart).

FIGURES 2 to 12 INCLUSIVE

FIGURE 2. Starch-gel electrophoresis of the water, salt and acid and alcohol soluble protein fractions of rye (Prolific) and Durum (Stewart).

1W - water fraction, Rye
5W - water fraction, Durum
S2 - salt fraction, Rye
S6 - salt fraction, Durum
3A - acid fraction, Rye
7A - acid fraction, Durum
A4 - alcohol fraction, Rye
A8 - alcohol fraction, Durum

A1-lactate buffer, 0.0085 M containing 2 M urea pH 3.1.
Potential gradient 12 v/cm. Time - 20 hours.

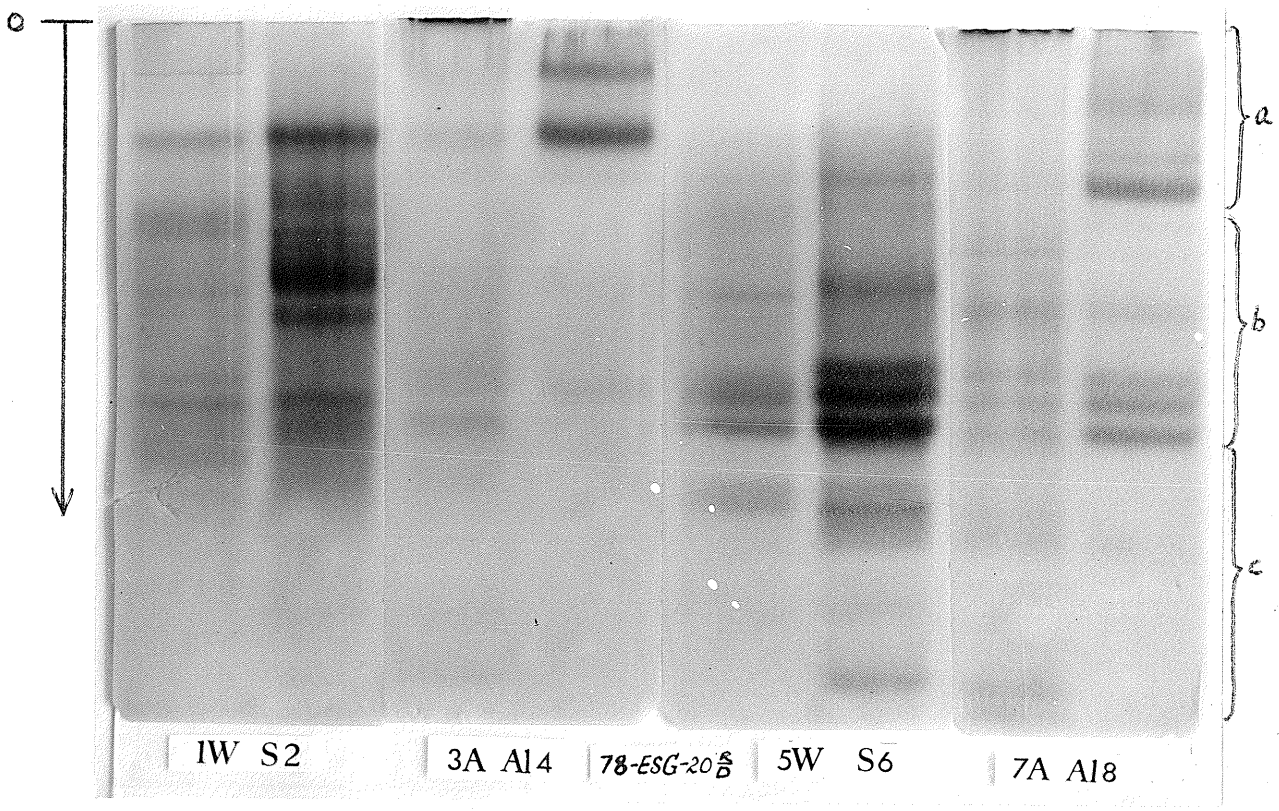


Fig. 2

FIGURE 3. Starch-gel electrophoresis of the water, salt, acid and alcohol soluble protein fractions of Triticale (6A190) and Tritipyron (6A58).

1W - water fraction, Triticale
5W - water fraction, Tritipyron
S2 - salt fraction, Triticale
S6 - salt fraction, Tritipyron
3A - acid fraction, Triticale
7A - acid fraction, Tritipyron
A4 - alcohol fraction, Triticale
A8 - alcohol fraction, Tritipyron

A1-lactate buffer, 0.0085 M containing 2 M urea pH 3.1

Potential gradient 12 v/cm. Time - 20 hours.

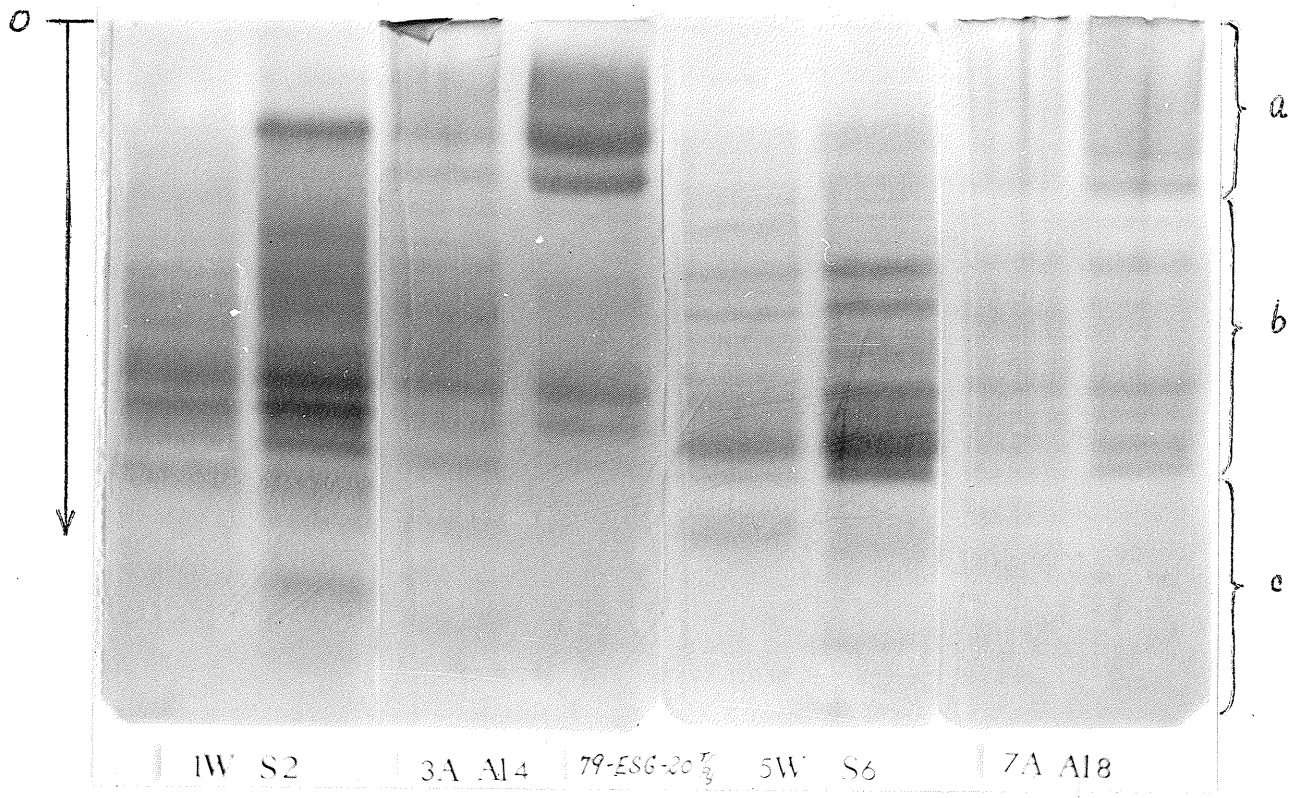


Fig. 3

FIGURE 4. Starch gel electrophoresis of the salt soluble protein fractions of Rye (Prolific), Durum (Stewart) and a mixture of both.

5R - salt fraction, Rye.
R/D 6 - mixture of salt fractions of Rye and Durum (1:1)
7D - salt fraction, Durum.

Al-lactate buffer, 0.0085 M containing 2M urea, pH 3.1.

Potential gradient 12 v/cm. Time - 20 hours.

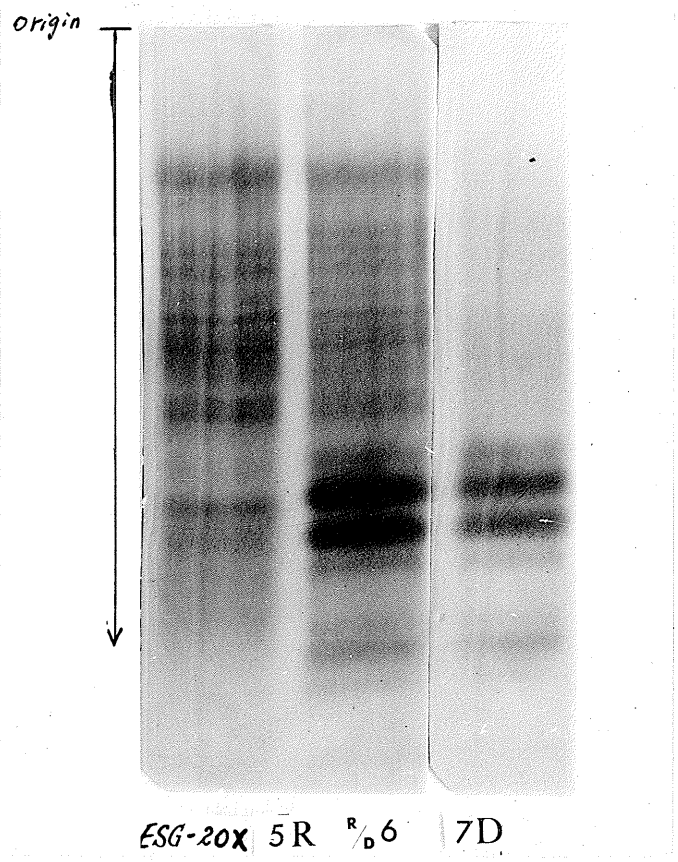


Fig. 4

FIGURE 5. Starch-gel electrophoresis of the water soluble protein fraction of different cereal species.

R - Rye (Prolific)
D- Durum (Stewart)
T - Triticale (6A190)
K - Wheat (Kharkov)
Py- Tritipyron (6A58)

Al-lactate buffer 0.0085 M containing 2M urea. pH 3.1.

Potential gradient 12 v/cm. Time - 24 hours.

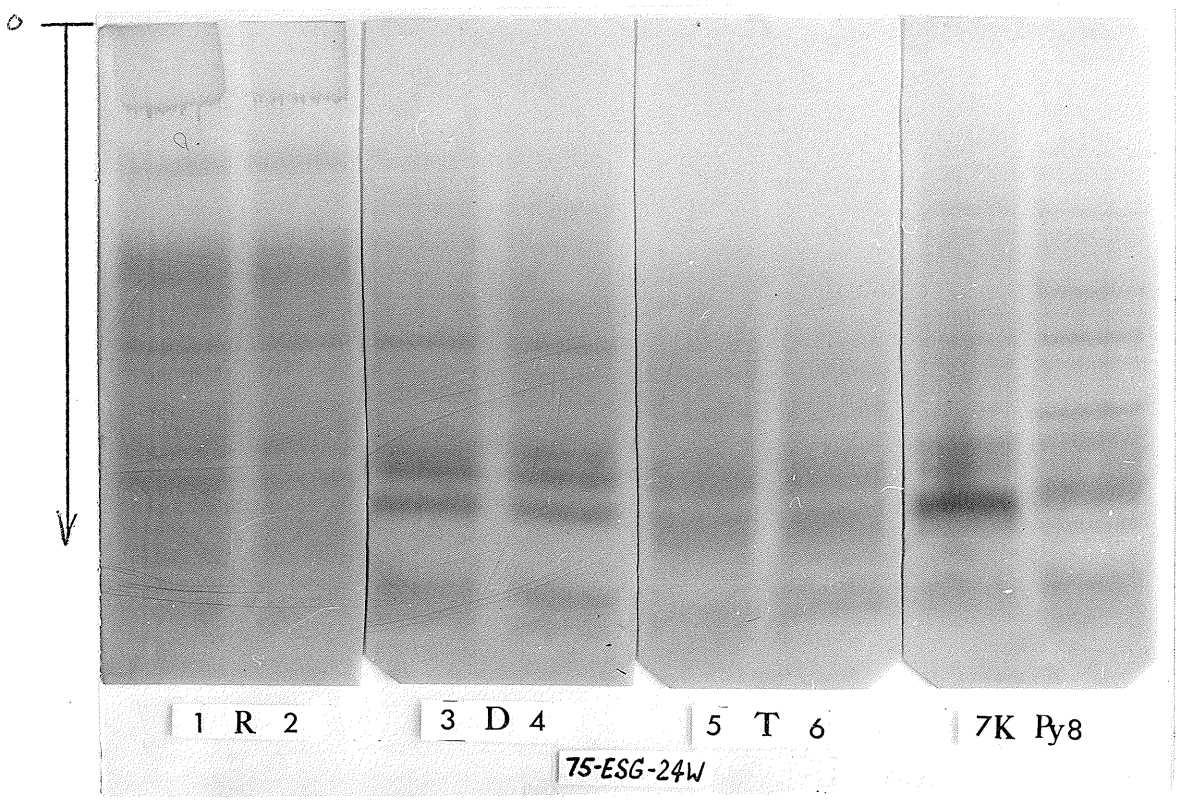


Fig. 5

FIGURE 6. Starch-gel electrophoresis of the salt soluble protein fraction of different cereal species.

R - Rye (Prolific)
D - Durum (Stewart)
T - Triticale (6A190)
K - Wheat (Kharkov)
Py- Tritipyron (6A58)

Al-lactate buffer, 0.0085M containing 2M urea, pH 3.1.

Potential gradient 12 v/cm. Time - 15 hours.

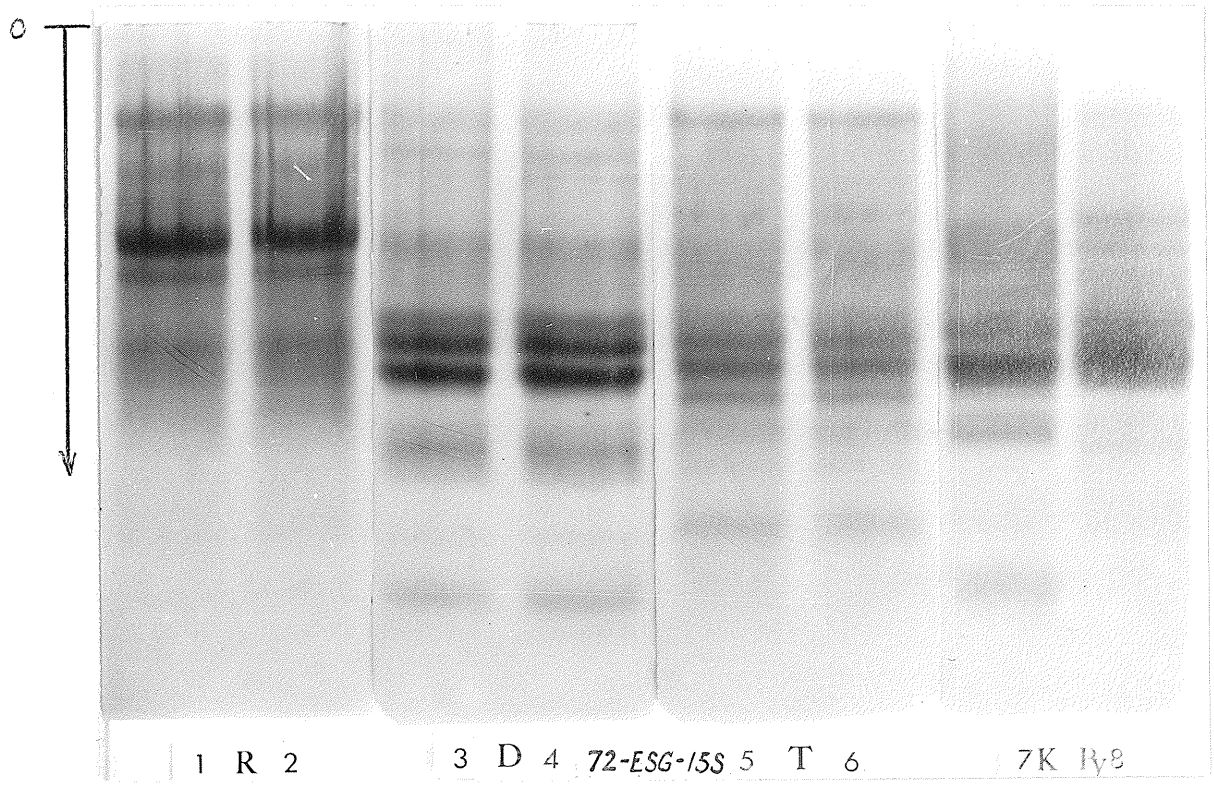


Fig. 6.

FIGURE 7. Starch-gel electrophoresis of the acid soluble protein fractions of different cereal species.

R - Rye (Prolific)
D - Durum (Stewart)
T - Triticale (6A190)
K - Wheat (Kharkov)
Py- Tritipyron (6A58)

Al-lactate buffer, 0.0085 M containing 2M urea, pH 3.1.

Potential gradient 12 v/cm. Time - 20 hours.

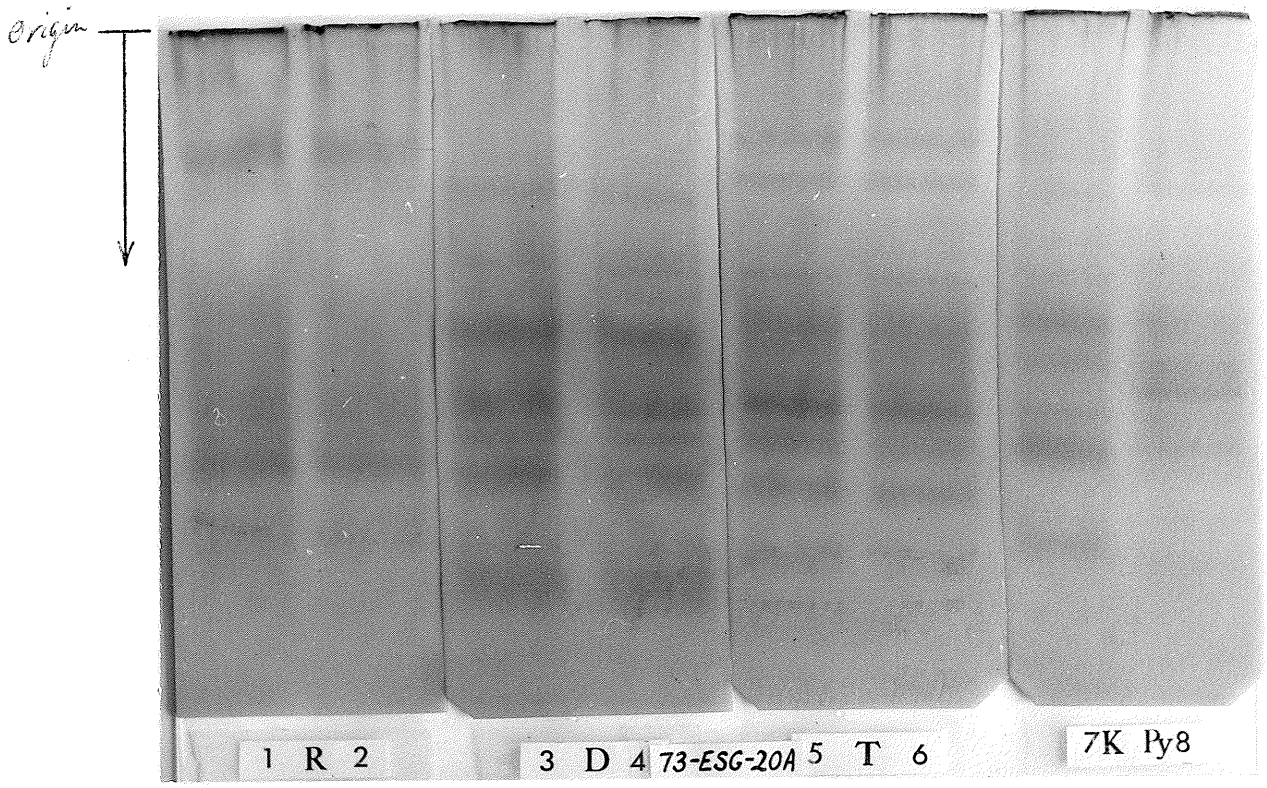
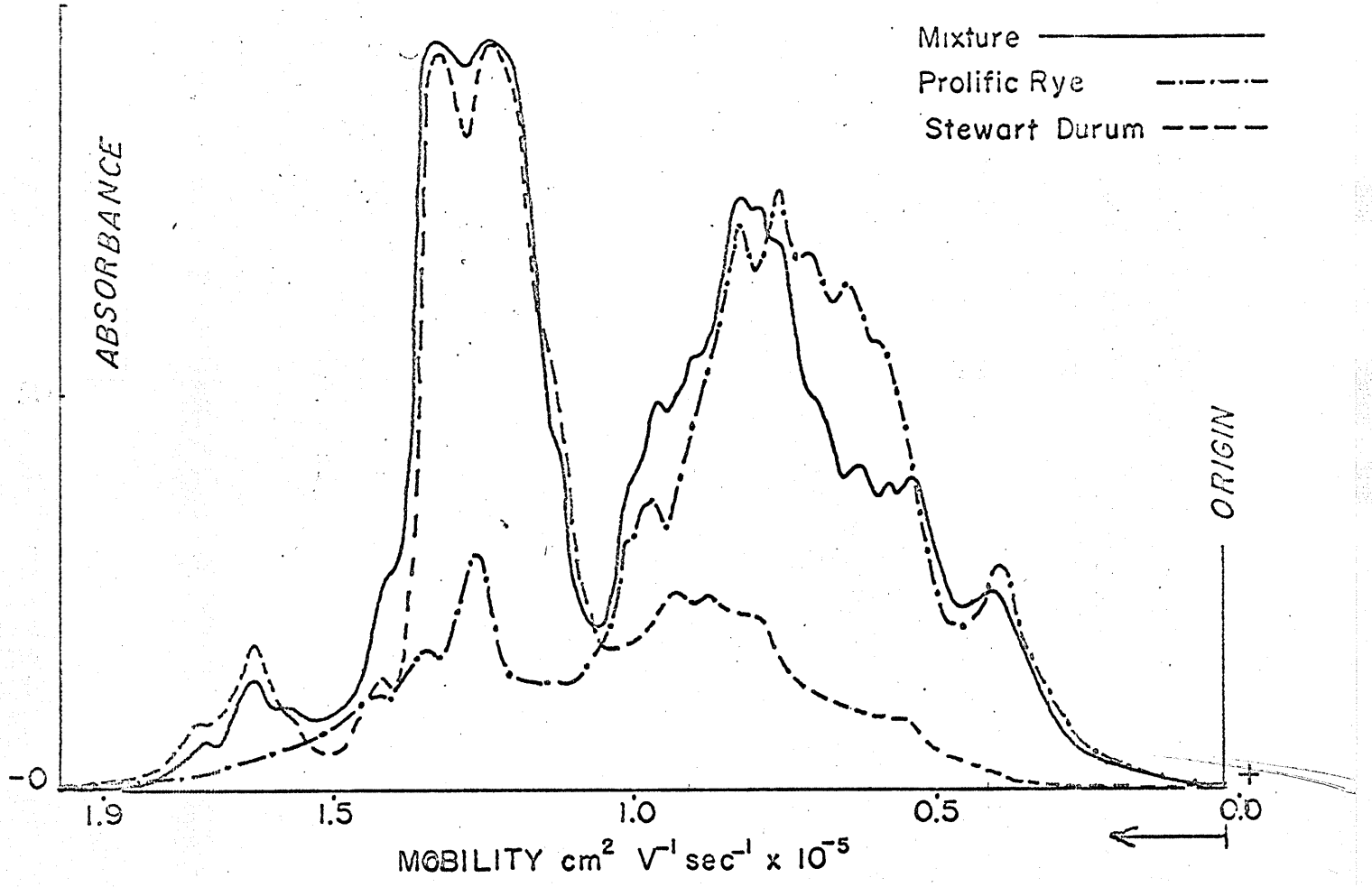


Fig. 7

Figure 8

Electropherograms and calculated mobilities of the salt-soluble proteins of Prolific Rye, Stewart Durum, and a mixture of both.

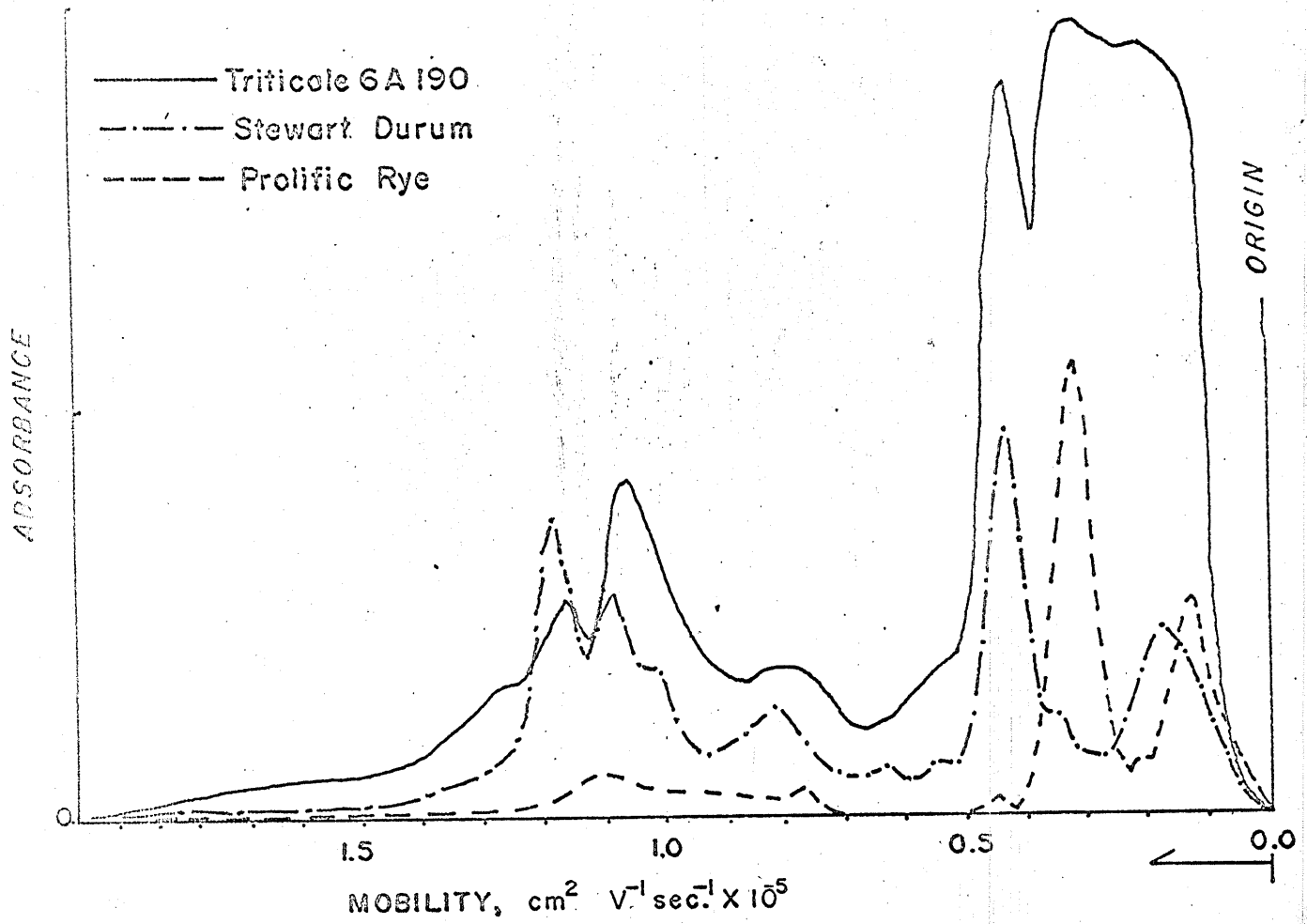


R			1.43	1.37	1.27			1.02	.97			.83		.76	.71	.64	.59		.38
R/D	1.74	1.66	1.60	1.42	1.34		1.24	1.13	1.01	.96		.89	.82	.80	.76	.71	.63	.58	.53	.39
D	1.74	1.66	1.60	1.42	1.34		1.24	1.13			.94	.89		.80					.53	

R ..... Rye (Prolific)
R/D ..... Mixture (1:1)
D ..... Durum (Stewart)

Figure 9

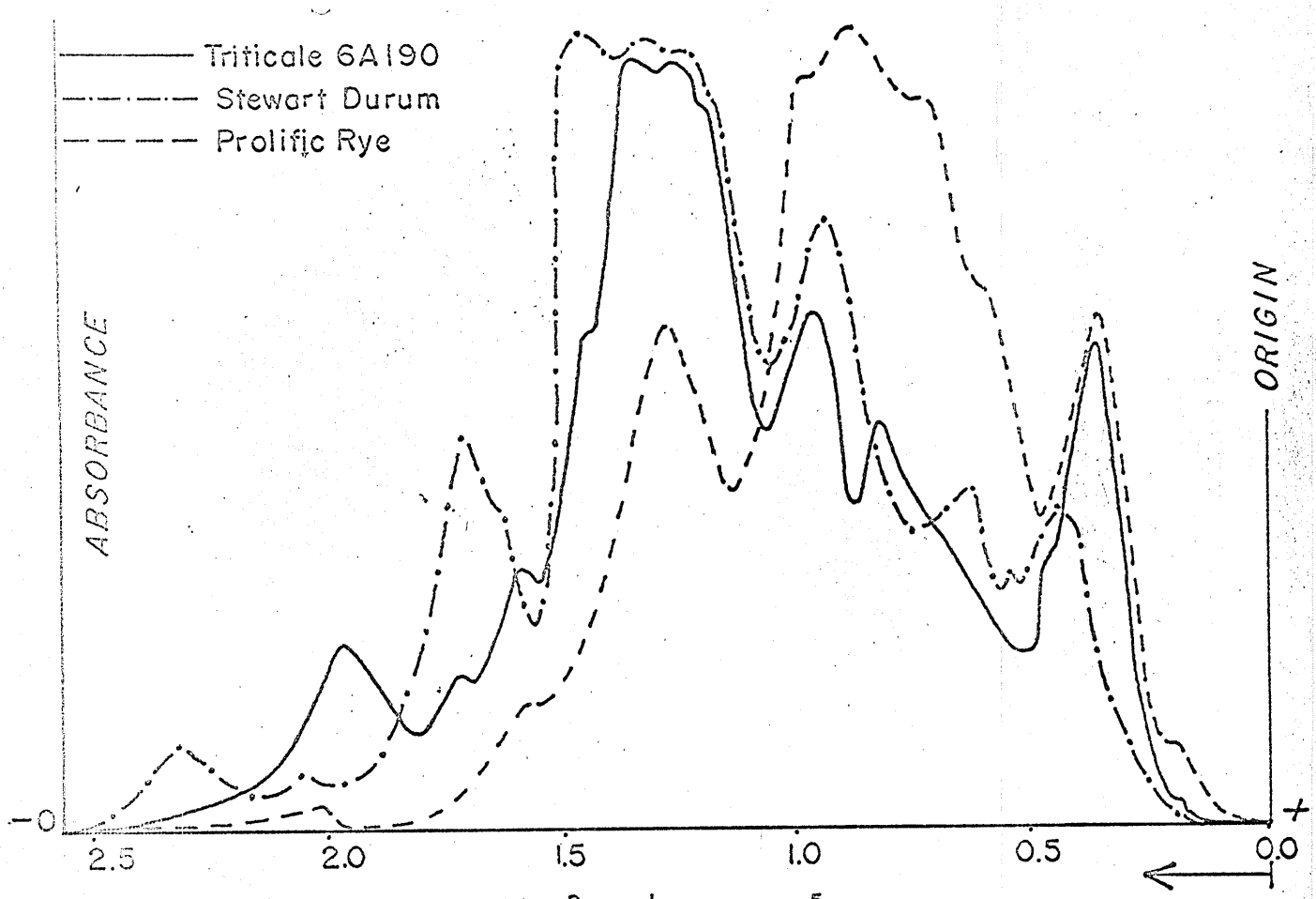
Electropherograms and calculated mobilities of the alcohol-soluble proteins.



Prolific Rye			1.10			.75			.44 .31 .21 .13	
Triticale (6A190)	1.25	1.17		1.06		.79			.42 .33 .21	
Stewart Durum	1.26	1.19	1.10	1.03		.81	.64	.56	.43 .36 .29 .17	
Tritipyron (6A58)			1.16	1.10	1.02	.86	.79	.65 .57	.43 .37	

Figure 10

Electropherograms and calculated mobilities of the salt-soluble proteins.

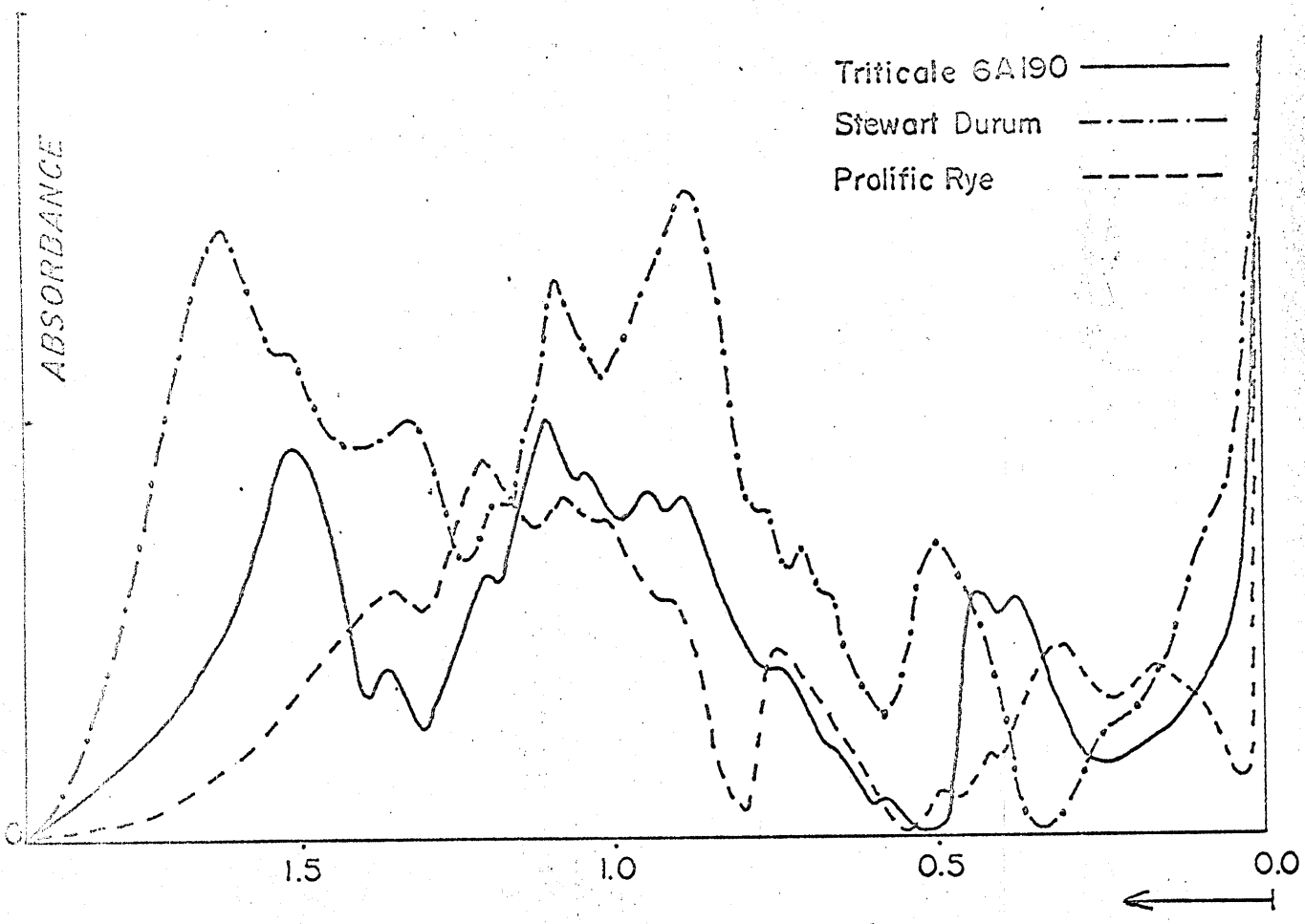


R	2.02				1.57			1.26			.99		.86	.69	.59			.36	.19						
D	2.32	2.05		1.82	1.70	1.64		1.44	1.32	1.23			.92			.62	.56	.44						
T		1.96.		1.71	1.59			1.44	1.35	1.24	1.17		.96		.82				.45	.36	.19			
K	2.27		1.96			1.62	1.54	1.44	1.34	1.22		1.10		.91	.82									.08
Py		1.96		1.67	1.59			1.39	1.31		1.16		.99	.90	.77			.54	.48	.39				

R ..... Rye(Prolific)
D ..... Durum(Stewart)
T ..... Triticale(6A190)
K ..... Wheat(Kharkov)
Py ..... Tritipyron(6A58)

Figure 11

Electropherograms and calculated mobilities of the acid-soluble proteins.

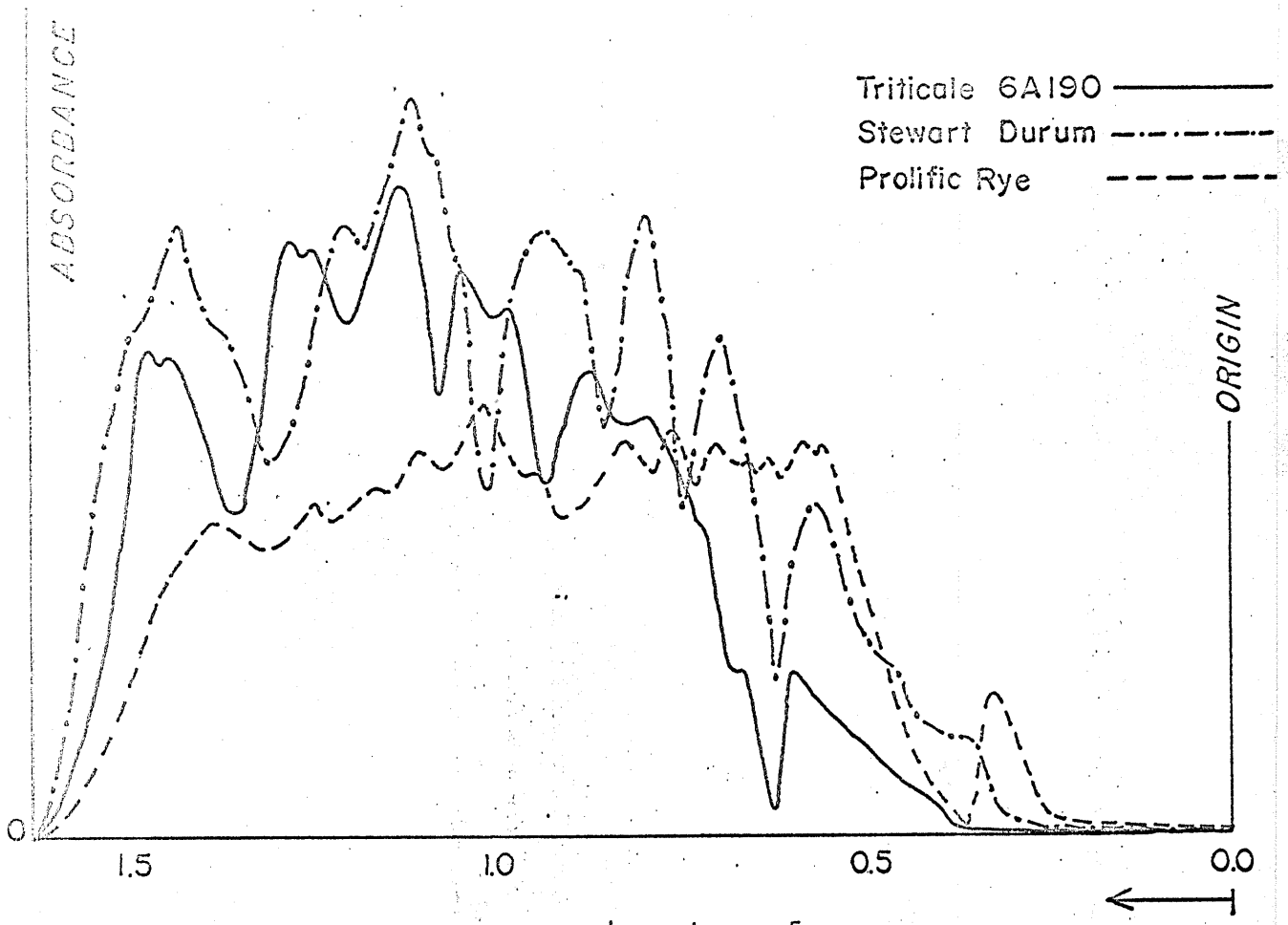


R			1.37		1.22	1.09	1.02	.91		.76			.51		.41	.32	.17	.00
D ...	1.63	1.53		1.33	1.20	1.10		.88		.78	.71	.68	.51					.00
T		1.54	1.38		1.22	1.14	1.07	.96	.91		.76		.68	.60		.45	.39	.00
K		1.54			1.26	1.15	1.08	1.01	.90		.76		.64		.52		.40	.00
Py	1.58	1.46		1.31		1.22	1.15	1.05	.99	.91	.86	.80	.78		.69		.49	.00

R ..... Rye(Prolific)
D ..... Durum(Stewart)
T ..... Triticale(6A190)
K ..... Wheat(Kharkov)
Py ..... Tritipyron(6A58)

Figure 12

Electropherograms and calculated mobilities of the water-soluble proteins.



R ..... 1.38 1.25 1.10 1.02 .95 .83 .77 .71 .64 .59 .56 .32
D ... 1.51 1.44 1.21 1.12 1.08 .93 .89 .80 .70 .66 .57 .46 .35
T ... 1.48 1.44 1.28 1.26 1.13 1.05 .99 .87 .80 .72 .67 .59
K ..... 1.42 1.38 1.28 1.26 1.20 1.09 1.05 .95 .83 .78 .70 .62 .52 .45 .39
Py .... 1.42 1.36 1.21 1.12 1.06 1.00 .94 .81 .76 .70 .60 .52 .45

R ..... Rye (Prolific)
D ..... Durum (Stewart)
T ..... Triticale (6A190)
K ..... Wheat (Kharkov)
Py ..... Tritipyron (6A58)

W - water soluble protein fraction

S - salt soluble protein fraction

A - acid soluble protein fraction

Al - alcohol soluble protein fraction

Numbers shown on the plates in Figures 1 to 7 correspond to the respective channel on the gel-plates.

b. Reproducibility of patterns

The electrophoresis of the various fractions of cereal proteins demonstrated excellent agreement of the duplicate patterns (Figs.5,6,7). Reproducibility of the patterns was also obtained when two separate electrophoretic runs were performed under identical conditions. This was evident by comparing the patterns shown in Figures 5, 6 and 7 with the patterns of the corresponding fraction in Figures 2 and 3. For example, the over-all pattern of the water soluble fraction of Prolific rye as shown in Fig.5 was found to be the same as that shown in Fig.2 (1W). The difference in the distance migrated by these protein components in the two gels was due to the difference in the total time of the individual electrophoretic runs.

c. Calculation of mobilities

After a densitometric scanning of the entire gel-strip, the positions of the various protein components were notated by visual observations and further checked with the mapped out electropherograms. The distance migrated by each com-

ponent was measured from the point of insertion (origin) in the gel to the center of each band (component). The identity or a characteristic of each component in an electrophoretic pattern and also in the electropherogram is given by its mobility which was calculated as follows:

$$\mu = \frac{dx}{dtdE}$$

where μ = mobility of a protein component

dx = distance migrated (cm)

dt = time of electrophoresis (sec)

dE = applied potential per centimeter (V/cm)

The mobility of a protein component therefore contains the dimensions or parameters of $cm^2V^{-1}sec^{-1}$.

To avoid lengthy repetition, all mobilities which will be quoted later will be given in numerical form, with the magnitude ($\times 10^{-5}$) and the dimensional units ($cm^2V^{-1}sec^{-1}$) omitted.

d. Characterisation of the migrating components

In an attempt to determine whether the protein components that migrated into the gel were independent of each other and were not co-acervates or polymeric or hydrogen-bonded complexes of larger molecules, a mixture (1:1) of the salt fractions of rye and durum was run side by side together with the original two fractions in one and the same starch gel plate. The electrophoretic patterns are

shown in Fig.4. The electropherograms and calculated mobilities of these patterns are presented in Fig.8. Each peak or hump in the electropherogram corresponded to a protein component whose mobility was indicated in the legend below the electropherograms. It was observed that all the components present in the electrophoretic patterns of the two original salt fractions could be accounted for in the electrophoretic pattern of the mixture with possibly two minor exceptions. Referring to the mobilities in Fig. 8, a rye component with a mobility of 1.27 and a durum component with a mobility of 0.94 were both absent in the mixture. However, close examination of the electropherogram of the mixture and those of the two pure fractions indicated that in the regions where these two components (1.27, 0.94) should be present, high non-specific absorption was evident. In the mobility region of 1.27, absorbance was approximately 95 per cent for the mixture and only 30 per cent for the rye component (1.27). In the 0.94 mobility region, the absorbance was approximately 54 per cent for the mixture and 24 per cent for the durum component (0.94). Therefore it seemed very probable that both these two components could have been present in the mixture but were not revealed visually or in the electropherogram because they were obscured by the much higher absorption intensities of the neighboring components.

Elton and Ewart (31) established the independent nature of the protein components that migrated into the gel by eluting four components from a developed gel and then running each of them separately under identical conditions. Each of these components gave the same distance of migration as before in the mixture. From these results, it seemed likely that the protein components that migrated into the gel represented single proteins. Furthermore, because the mobilities of the protein components were the same when run either in pure fractions or in the mixture, this established the criterion whereby protein components of similar mobility in the electrophoretic patterns developed under identical conditions were presumed to be structurally similar if not identical. To further emphasize this, it may be pointed out that Elton and Ewart (22) have suggested that the mobility of a protein component would change under different conditions of electrophoresis.

e. Comparison of the electrophoretic patterns of various protein fractions within a species

Figures 2 and 3 show the electrophoretic patterns of the various fractions extracted from Prolific rye, Stewart durum, Triticale (6A190) and Tritipyron (6A58). In Fig.2, channels 1W, 2S, 3A and 4A1 represent respectively, the electrophoretic patterns of the water soluble, salt soluble, acid soluble and alcohol soluble proteins of Prolific rye.

Channels 5W,6S,7A and 8A1 are the electrophoretic patterns of Stewart durum. In Fig.3, channels 1W,2S,3A and 4A1 are the electrophoretic patterns of Triticale (6A190) while channels 5W,6S,7A and 8A1 are those of Tritipyron (6A58). For ease of comparison and discussion, the electrophoretic pattern in each channel has been divided into three regions, namely region (a) which included those protein components with low mobility, region (b) for those protein components with intermediate mobilities and region (c) for protein components with faster mobilities (see Figs.2 and 3). A cursory examination of the electrophoretic patterns in Figs 2 and 3 showed that both qualitative and quantitative differences in the four protein fractions within a cereal species existed. For example, although the leading band in region (a) of the alcohol fraction of rye was also present in the other three fractions, they appeared in markedly different intensities, hence in different concentrations. The same was also true for the three leading bands in region (b) of the alcohol fraction of durum. Most of the components in region (c) and some in region (b) were not present in all the four fractions of a species. Coulson and Sim (47) attributed the presence of similar proteins in different fractions to incomplete extraction. However, Graham (26) found that such similarity still persisted in different fractions even after exhaustively extracting the sample with each solvent. Recently, Elton and Ewart (31)reported that some of the

alcohol soluble components in wheats were electrophoretically similar to those in the water extracts although they differed markedly in quantities. It might be appropriate to point out that although marked similarities of certain protein components were observed in different fractions extracted from the same cereal species, there were also reproducible differences in the patterns of the various fractions as was demonstrated in Figs. 2 and 3. A coherent explanation of these observed facts might await a more comprehensive study with pure proteins.

It is of interest to point out that in the electrophoretic pattern of the water extract of rye (Fig. 2, 1W), a thin fringe-like appearance was observed near the point of insertion in region (a). Such a fringe-like appearance was also observed by Elton and Ewart (22) and Pence and co-workers (27). These investigators attributed this fringe development to β-amylase activity by suggesting that the enzyme enlarged the pores of the gel thus allowing large particles to penetrate into the enlarged gel network. These particles then gathered at the apices of these pores thus accounting for the appearance of visually discernable particles. A view of this fringe development is shown in Fig. 5, top left-hand corner of the plate.

A typical characteristic in the acid soluble fraction of each cereal species was the relatively large quantity of

protein material remaining at the point of insertion (see Figs. 2 and 3). Woychik (20) eluted these proteins from the origin and subjected them to moving boundary electrophoresis. The result showed that these proteins exist as a single homogeneous component which was later i-dentified by the same investigator to be glutenin.

f. Comparison of electrophoretic patterns of homologous fractions between the different species

i) Alcohol soluble protein fractions

Figure 9 depicts the electrophoretic patterns and calculated mobilities of the alcohol soluble proteins. Only the electropherograms of Prolific rye (Secale cereale), Stewart durum (Triticum durum) and Triticale 6A190 (T.durum x S. cereale) were shown as these three were the focus of interest in this investigation. Since Triticale (6A190) is a hexaploid cereal derived by a combination of the genomes of diploid rye (S. cereale) and tetraploid wheat (T.durum), a comparison of the mobilities which, in a sense, correspond to the migrated protein components in the electrophoretic patterns might present the possibility of establishing whether the individual biosynthetic integrity of the alien genomes was maintained when both were in intimate contact in the intact cell. On the basis that protein components with similar mobilities were structurally similar under identical conditions of electrophoresis as discussed in a

previous section (item d of section 7), all the protein components (represented by mobilities)shown in Fig.9 for Triticale (6A190) could be considered as similar to those having similar mobilities in either or both the parent species (S. cereale, T.durum). In other words, this implies that all the protein components present in the alcohol fraction of Triticale (6A190) were derived from either or both the parent species. For ease of comparison and discussion, the mobilities of all the components of the proteins of the cereal species were classified into the following groups according to whether a particular protein component was present or absent in the electrophoretic pattern of the synthetic cereal species.

Group a — Protein components similar to or common to all the four species --- 0.44 of rye.

Group b — Protein components similar or common to rye, durum and Triticale --- 0.21 of rye.

Group c — Protein components similar or common to durum and Triticale --- 0.33, 0.42, 0.79, 1.06, 1.17 and 1.25 of Triticale.

Group d — Protein components present in rye and durum but not in Triticale --- 0.31, 1.10 of rye.

Group e — Protein components present in rye but absent in Triticale --- 0.13, 0.75 of rye.

Protein components present in durum but absent

in Triticale --- 0.56, 0.64 of durum.

Group f — Protein components present in Tritipyron but absent in durum --- 0.86 of Tritipyron.

The presence of similar protein components of Triticale (6A190) in either or both parent species suggested a possible direct influence or contribution of the parent genomes in the synthesis of these similar or comparable protein components in the species hybrid (cf. group a, b and c). This would also mean that these alien genomes possibly maintained partial or complete biosynthetic integrity in the cell.

Since Tritipyron (6A58) is a hybrid derived in effect by a combination of the genomes of T. durum and Agropyron elongatum, those protein components that were found in Tritipyron but not in T. durum (ref. group f) might be attributed to contributions from the "EE" genomes of A. elongatum or, alternatively, they might be new proteins synthesized through a combination of the codes controlling protein synthesis of the alien genomes present in the cell. In view of the fact that Triticale (6A190) and Tritipyron (6A58) have one of the parental genomes in common, namely, T. durum a comparison of the pattern contributed by the "AABB" genomes of this tetraploid wheat to those of the synthetic hexaploid cereal species indicated different degrees of influence.

ii) Salt soluble protein fractions

Fig. 6 shows the electrophoretic patterns of the

salt soluble protein fractions of the five different cereals while Fig.10 presents the electropherograms and the calculated mobilities for the patterns. By classifying all the mobilities according to the method mentioned above, the following groups are obtained.

Group a — Protein components similar to all the five cereal species --- 1.57 of rye.

Group b — Protein components similar to rye, durum and Triticale --- 1.26, 1.57 of rye.

Group c — Protein components similar to rye and Triticale --- 0.19, 0.36, 0.99 of rye.

Proteins components similar to durum and Triticale --- 0.44, 1.32, 1.44, 1.70 of durum.

Group d — Protein components present in rye and durum but not in Triticale --- 0.59, 0.86, 2.02 of rye.

Group e — Protein components present in rye but absent in Triticale --- 0.69 of rye.

Protein components present in durum but absent in Triticale --- 0.56, 1.82, 2.32 of durum.

Group f — Protein components present in Tritipyron but absent in durum --- 0.39, 0.77, 0.99, 1.16 and 1.96 of Tritipyron.

Protein components present in Kharkov but absent in durum --- 0.08, 0.82, 1.10, 1.54, 1.96 and 2.27 of Kharkov.

Group g Protein components present in Triticale but absent in rye and durum --- 0.82, 1.17 and 1.96 of Triticale.

Protein patterns as classified in group (a) (b) and (c) suggested direct contribution of the parent genomes responsible for the synthesis of these groups of protein components in the species hybrid.

Group (d) and (e) suggested some possible interaction or inhibition between the related alien genomes in the hybrid species with the result that protein components which were similar to either or both the parent species were apparently not synthesized in the hybrid.

Proteins in group (f) were probably contributed by the other parent species which, in the case of Tritipyron (6A58), was A.elongatum, and in the case of Triticum vulgare (Kharkov) was Aegilops squarrosa. It is of course also possible that these "unaccounted" protein components were new proteins synthesized through a combination or interaction of the alien genomes in the species hybrid.

A comparison of the protein patterns of T.durum with the protein patterns of the three hexaploid cereals (Triticale 6A190, Kharkov and Tritipyron 6A58) further demonstrated the fact mentioned earlier that the influence of the "AABB" genomes of T. durum on the pattern of protein synthesis of these three hexaploid cereals was not constant.

Protein components of Triticale (6A190) classified in

group (g) were probably new proteins because they were not present in either of the parent species (T. durum and S.cereale). A careful comparison of these new Triticale proteins with the neighbouring proteins in T.durum and S.cereale (see Fig.10,) revealed a rather striking characteristic which was also observed in the other protein extracts. Referring to the Triticale proteins of 0.82 and 1.96 mobility range, it was found that in the mobility range of these two values, there appeared in the parent species (T.durum, S.cereale) protein components which had no complementary proteins in the hybrid (Triticale 6A190). Such observations again suggested possible interactions between the two alien genomes in the synthetic species.

The pattern of synthesis of the protein components with mobility 1.17 in Triticale (6A190) might differ from that mentioned above because a careful examination of the protein components of the two parent species in this mobility range (1.17) showed that they had complementary bands in the synthetic species. There was also the possibility that the three new proteins (0.82, 1.17 and 1.96) detected in the hybrid (Triticale 6A190) were experimental artifacts. However, the fact that all these three proteins were also present in the extracts of such different cereals species such as Kharkov and Tritipyron (6A58) and that such patterns of protein formation were also observed in the acid and water soluble protein fractions, the observation should

therefore be considered significant.

iii) Acid soluble protein fractions

Fig.7 shows the electrophoretic patterns of the acid soluble proteins and Fig.11 gives the electropherograms and calculated mobilities of these acid soluble proteins.

Classifying all these protein components as before, they are:

Group a — Protein components similar to all the five cereal species --- 0.0, 0.76, 0.91 and 1.09 of rye.

Group b — Protein component similar to rye, durum and Triticale --- 1.22 of rye.

Group c — Protein components similar to durum and Triticale --- 0.68 and 1.53 of durum. Protein components similar to rye and Triticale --- 0.41 and 1.37 of rye.

Group d — Protein components present in rye and durum but not in Triticale --- 0.51 of rye.

Group e — Protein components present in durum but absent in Triticale --- 0.71, 1.33 and 1.63 of durum. Protein components present in rye but absent in Triticale --- 0.17, 0.32 and 1.02 of rye.

Group f — Protein components present in Tritipyron but absent in durum --- 0.80, 0.86, 0.99, 1.15 and 1.46 of Tritipyron.

Protein components of T.vulgare (Kharkov) but absent in durum --- 0.40, 1.01, 1.15, 1.26 of T.vulgare.

Group g Protein components present in Triticale but absent in rye and durum --- 0.45, 0.60 and 1.14 of Triticale.

Patterns of synthesis of the protein components in group (a), (b) and (c) were probably due to direct contribution of the parent genomes present in the synthetic species. Again, the absence of the protein components of group (d) and (e) in the species hybrid might possibly be due to some form of interaction or inhibitory response between the alien genomes.

The formation of group (f) proteins in Tritipyron and in T.vulgare possibly came about through a contribution from the other parent species, or alternatively, they were formed as a result of certain interactions between the alien genomes in the species hybrid.

The protein components in group (g) were probably new components. They (0.45, 0.60) possibly arose through interaction of genes of the parent species. Evidence to this effect was the observed presence of non-complementary protein components of quite similar mobilities in the parent species (0.51 of rye, see Fig.11, bottom).

The protein component of 1.14 mobility in Triticale

formed the same pattern as did the protein of 1.17 mobility mentioned for the salt soluble protein fractions.

iv. Water soluble protein fractions

Fig.5 shows the electrophoretic patterns of the water soluble protein fractions of the five cereal species and the electropherograms and calculated mobilities are shown in Fig.12. According to the classification used before, the protein components represented by the respective mobilities were classified as follows:

Group a — Protein components similar to all the five cereal species --- 0.71, 0.83 of rye.

Group b — Protein components similar to rye, durum and Triticale --- 0.64 of rye.

Group c — Protein components similar to durum and triticale --- 0.89, 1.12, 1.44 and 1.51 of durum.
Protein components similar to rye and Triticale --- 0.59, 1.02 and 1.25 of rye.

Group d — Protein components present in rye and durum but not in Triticale --- 0.32, 0.56, 0.95 and 1.10 of rye.

Group e — Protein components present in durum but not in Triticale --- 0.46 and 1.21 of durum.
Protein components present in rye but absent in Triticale --- 0.83 and 1.38 of rye.

Group f Protein components present in Tritipyron but absent in durum --- 0.52, 0.60, 0.81, 1.00, 1.06 and 1.36 of Tritipyron.
Protein components present in T. vulgare but absent in durum --- 0.39, 0.52, 0.62, 0.83, 1.05, 1.26, 1.28 and 1.38 of T. vulgare.

Group g Protein components present in Triticale but absent in rye and durum --- 1.05 and 1.28 of Triticale.

The formation of proteins in group (a), (b) and (c) were probably due to direct contribution of the parent genomes present in the hybrid species. The absence of the protein components of group (d) and (e) in the species hybrid again point to possible interaction or inhibition of the alien genomes in the cell.

The presence of group (f) proteins in Tritipyron and in T. vulgare may be due to direct contribution of the other parent species, or, alternatively, they were formed as a result of certain interactions between thealien genomes in the species hybrid.

The protein components in group (g) were probable new components. They were possibly formed as a result of interaction of genes of the parent species. Evidence to this effect was the observed presence of non-complementary protein components of quite similar mobilities from those in the parent species (1.00 of rye, see fig. 12).

Alternatively, it definitely appeared advantageous to consider all the mobilities of the protein components ofthe various fractions of a single cereal species together with a similar composite of the other species and thus form one general, over-all pattern of mobilities representative of all the electrophoresis experiments. A comparison (see Table IV) of these over-all mobility patterns between the different cereal species might prove more revealing in considering the biosynthetic integrity of the alien genomes when in intimate cellular association.

It is interesting to note that the general pattern of relationships of the mobilities of the protein components between rye, durum and Triticale, and that between durum and the three hexaploid cereal species were found to be virtually the same as those observed when homologous protein extracts of these cereals were considered separately (cf. previous section). Classifying these over-all mobilities as before, the following groups were obtained.

Group a — Protein components similar to all the five cereal species --- 0.42 and 1.24 of rye.

Group b — Protein components similar to rye, durum and Triticale --- 0.19, 0.33, 0.59, 0.67 and 0.71 of rye.

Group c — Protein components similar to rye and Triticale --- 1.01, 1.38 and 1.57 of rye.

Protein components similar to durum and Triticale --- 0.80, 0.89, 1.06, 1.44 and 1.70 of durum.

Group d — Protein components present in rye and durum but absent in Triticale --- 0.93, 1.10 and 2.02 of rye.

Group e — Protein components present in rye but absent in Triticale --- 0.13, 0.53, 0.76 and 0.85 of rye.
Protein components present in durum but absent in Triticale --- 0.29, 1.20, 1.33, 1.52, 1.64, 1.82 and 2.32 of durum.

Group f — Protein components present in Tritipyron but absent in durum --- 0.38, 0.50, 0.78, 0.86, 0.99, 1.16 and 1.96 of Tritipyron.
Protein components present in Kharkov but absent in durum --- 0.08, 0.40, 0.52, 0.63, 0.77, 1.18 1.28, 1.96 and 2.27 of Kharkov.

Group g — Protein components present in Triticale but absent in rye and durum --- 1.15, 1.28, 1.48 and 1.96 of Triticale.

In splitting the mobilities of the protein components into similar arithmetic groups (as shown in Table IV) on the basis of allowing a maximum variation of 0.03 (experimental error) within each group, it was noted that some protein components possessed mobilities which were intermediate between the mobilities of two presemably different groups of proteins. Examples of this were 0.42 of rye, 1.28 of Triticale, 0.63, 0.77, 1.18 & 1.28 of Kharkov, and 0.38, 0.78, 0.86 & 1.67 of Tritipyron.

Protein patterns as classified in group (a) (b) and (c) indicated a direct responsibility of the parent genomes for the synthesis of these particular groups of proteins in the species hybrid.

TABLE IV. OVERALL MOBILITIES OF THE CEREAL SPECIES ($\times 10^{-5}$ $cm^2 sec^{-1} V^{-1}$)

Rye (Prolific)	Durum (Stewart)	Triticale (6A190)	Wheat (Kharkov)	Tritipyron (6A58)
0.0	0.0	0.0	0.0	0.0
			0.08	
0.13				
0.19	0.17	0.20		
	0.29			
0.33	0.36	0.36		
			0.40	0.38
0.42	0.44	0.44	0.45	0.44
0.53			0.52	0.50
0.59	0.57	0.60		0.57
			0.63	
0.67	0.65	0.68		0.68
0.71	0.71	0.72	0.70	
0.76			0.77	0.78
	0.80	0.80	0.83	0.81
0.85				0.86
	0.89	0.89		
0.93	0.93		0.92	0.92
1.01		0.97		0.99
	1.06	1.06	1.03	1.04
1.10	1.11		1.09	1.11
		1.15	1.18	1.16
	1.20			
1.24	1.25	1.24	1.25	1.22
		1.28	1.28	
	1.33			1.31
1.38		1.36	1.36	1.38
	1.44	1.44	1.43	1.44
		1.48		
	1.52		1.54	
1.57		1.56		1.59
	1.64		1.62	
	1.70	1.71		1.67
	1.82			
		1.96	1.96	1.96
2.02	2.05			
			2.27	
	2.32			

Group (d) and (e) were protein components detected in either or both the parent species (T. durum and S. cereale) but they were absent in Triticale. The absence of these protein components in the hybrid species may be attributed to some type of interaction or inhibition resulting in some manner from the new association of the alien genomes.

Protein components in group (f) were probably contributed by the genomes of another parent species, which, in the case of Tritipyron (6A58) was A. elongatum, and in the case of T. vulgare (Kharkov) was A. squarrosa. Since the seed protein complements of A. elongatum and A. squarrosa were not readily available for study, little more can be suggested at this point. Alternatively, these "unaccounted" protein components might be new proteins synthesized through some unknown interactions of the alien genomes in the species hybrid.

As observed in the comparison of the mobilities in homologous protein extracts, the patterns of contribution of the "AABB" genomes of T. durum to those of the three hexaploid cereal species (Triticale 6A190, Tritipyron 6A58 and T. vulgare) were not constant or comparable. This variability may be attributed to the dominance or repression of certain genes in the "AABB" complement when they were in intimate contact with one another in the synthetic species.

Protein components classified in group (g) were those found only in Triticale but not in either of the parent species. A careful comparison of these "new" protein components (1.15, 1.28, 1.48 and 1.96) with the neighbouring protein components of the parent species showed the same pattern of relationship between them as observed in the homologous fractions. These "new" protein components were found to be present in the mobility range where protein components of the parent species had no complementary ones in the hybrid. For example, 1.15 of Triticale is an intermediate of 1.10 (rye), 1.11 (durum) and 1.20 (durum). Mobility 1.28 (Triticale) appears to be more closely related to 1.25 (durum) than to 1.33 (durum) whereas, 1.48 of Triticale is probably an intermediate of 1.44 (durum) and 1.52 (durum). The protein component of 1.96 mobility (Triticale) may be a "hybrid product" of 1.82 (durum), 2.05 (durum) and 2.02 (rye).

Since the relationship between the patterns of protein components of the parent species and those of the hybrid were consistent regardless whether comparing the mobilities of the proteins in the homologous extracts or when the over-all general mobilities of the proteins of each species were considered, it seems reasonable to suggest that influence (probably in the form of genome interactions) was exerted when the alien genomes were in intimate cellular contact. It appeared, as suggest previously that the alien genomes were unable to maintain rigorously their biosynthetic integrity.

v) Discussion

From the results of the electrophoretic analysis, it is evident that some protein components present in the different fractions of S.cereale were absent in the comparable protein fractions of T.durum or if present, occured only in very small quantities. Conversely, it was evident that some protein components present in the various extracts of T. durum were absent in the comparable S.cereale extracts or if present, occured only in very small quantities. It was also evident that some of the protein components were electrophoretically similar or common to both species. This confirms the statement made by Kowarski (1901) and by Moritz (1933) that serologic similarities existed between wheat and rye (1,2). Hall (3) later showed that the proteins which were found similar in their immunologic reactions were electrophoretically identical.

As evident from the electrophoretic studies in this investigation, not all the protein components detected in either or both the parent species were present in Triticale. Furthermore, it was also observed that not all the protein components present in Triticale were present in the parent species. These observations are therefore not in complete agreement with those reported by Hall (3) who stated that nearly all the protein components extracted from rye and all of those from wheat were present in the extracts of the hybrid rye-wheat species. Hall (3) has

mentioned that one protein component was observed in the rye-wheat extracts which was not present in either of the two parent species. However, he was reluctant to identify this particular component as a "hybrid substance". The fact that very few, if any, of these "hybrid substances" were detected by Hall but which were apparent in these investigations might probably be due to the different techniques employed in electrophoresis of the protein preparations. It is known that agar gel electrophoresis has a much lower resolving power than that of starch gel (48). This is also evident by comparing the number of protein components resolved by starch gel in this investigation to those resolved by agar gel electrophoresis which was employed by Hall (3). Further evidence indicating the more complex nature of the cereal proteins has been provided by column chromatographic fractionation (25), polyacrylamide gel electrophoresis (27,28) and fractionation by means of gel-filtration (30).

The absence in the hybrid species of certain protein components, which were present in either or both of the parent species, may possibly indicate the inability of the alien genomes, in the new cellular environment of the hybrid species, to maintain their individual biosynthetic integrity. The observation that the unaccounted protein components in Triticale (possibly new proteins) were generally found in

the mobility range where some corresponding protein components of the parent species were absent, lends further evidence to the above suggestions. A plausible explanation for the inability of the alien genomes to maintain their synthetic integrity may be due to some form of interaction between them which presumably caused inhibition or inactivation of a certain portion of the genes responsible for the replication of certain specific proteins. These assumptions are given some further enlightenment when considering the results of Rees (49) who reported that the mean DNA content in the hexaploid wheat T. aestivum (vulgare) (AABBDD) was found to be essentially the sum of the two parent species, T. durum (AABB) and A. squarrosa (DD). If the DNA content of the individual genomes were maintained in the cell of the synthetic species, a plausible explanation for the absence of certain proteins derived from the parent species, in the hybrid, is the suggestion elaborated above.

If the alien genomes did have interactions in the nucleus, giving rise to a new expression of the combined genomes, there should be new proteins formed at the expense of the original ones. Interestingly enough, proteins with slightly different mobilities were observed to be present in the different extracts of Triticale at the expense of proteins of quite similar mobilities in the parent species.

Kerber (50) reported that the reconstituted tetraploid component (AABB) of two hexaploids differed widely in morphology from T. durum (AABB) that existed in nature. The process of reconstitution was accomplished by extracting the AABB complement from an original cross between Canthatch (AABBDD) and Stewart durum (AABB), resulting in a pentaploid which was then backcrossed seven times to Canthatch (AABBDD) finally giving, after selfing, the reconstituted tetraploid (AABB). His observation lends further support to the postulations elaborated here that some forms of interactions were at play when these alien genomes existed together in intimate contact in the hybrid species.

A point of caution which must be mentioned here is the fact that those proteins which are considered identical under the criterion of similar electrophoretic mobility, may, in fact, not be exactly identical. Because of the apparent characteristic that a protein can be resolved into a spectrum of bands in the starch gel is presumably by virtue of the component's molecular size and net electrical charge, it is plausible that a molecule smaller in size and carrying lower net electrical charge may have the same mobility as one which is slightly larger in size and carrying higher net electrical charge. Such differences can only be resolved by immuno-electrophoretic procedures.

If Mendel's law of dominance also holds in the sub-cellular level, the difference in the patterns of contribution of the "AABB" genomes of T. durum, compared to the three hexaploid cereal species (Triticale 6A190, T. vulgare and Tritipyron 6A58) as seen by comparing their mobility patterns, may be attributed to dominance or suppression of certain genes in the "AABB" genomes when these are in intimate contact with an alien genome and under different cellular environments. In conclusion, it may be stated, considering the experimental evidence presented in this investigation, that the synthetic hexaploid cereal species do possess certain abilities to synthesize proteins which are quite different from those present in the parent species. Whether this new synthetic ability is inherited through interaction at the code level of the alien genomes when in intimate contact in the cell is not definitely known but it may be so postulated. The inability of the new hybrid species to synthesize certain S. cereale proteins was confirmed. Such characteristics were also observed for proteins derived or which might be expected to be derived from the tetraploid wheat genome, T. durum. The influence of the "AABB" genomes of this tetraploid wheat on the protein synthesis of the three hexaploid cereal species which contain the "AABB" complement varies to a considerable extent.

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