

INSTITUUT VOOR GRAAN, MEEL EN BROOD TNO
INSTITUTE FOR CEREALS, FLOUR AND BREAD TNO
WAGENINGEN, THE NETHERLANDS

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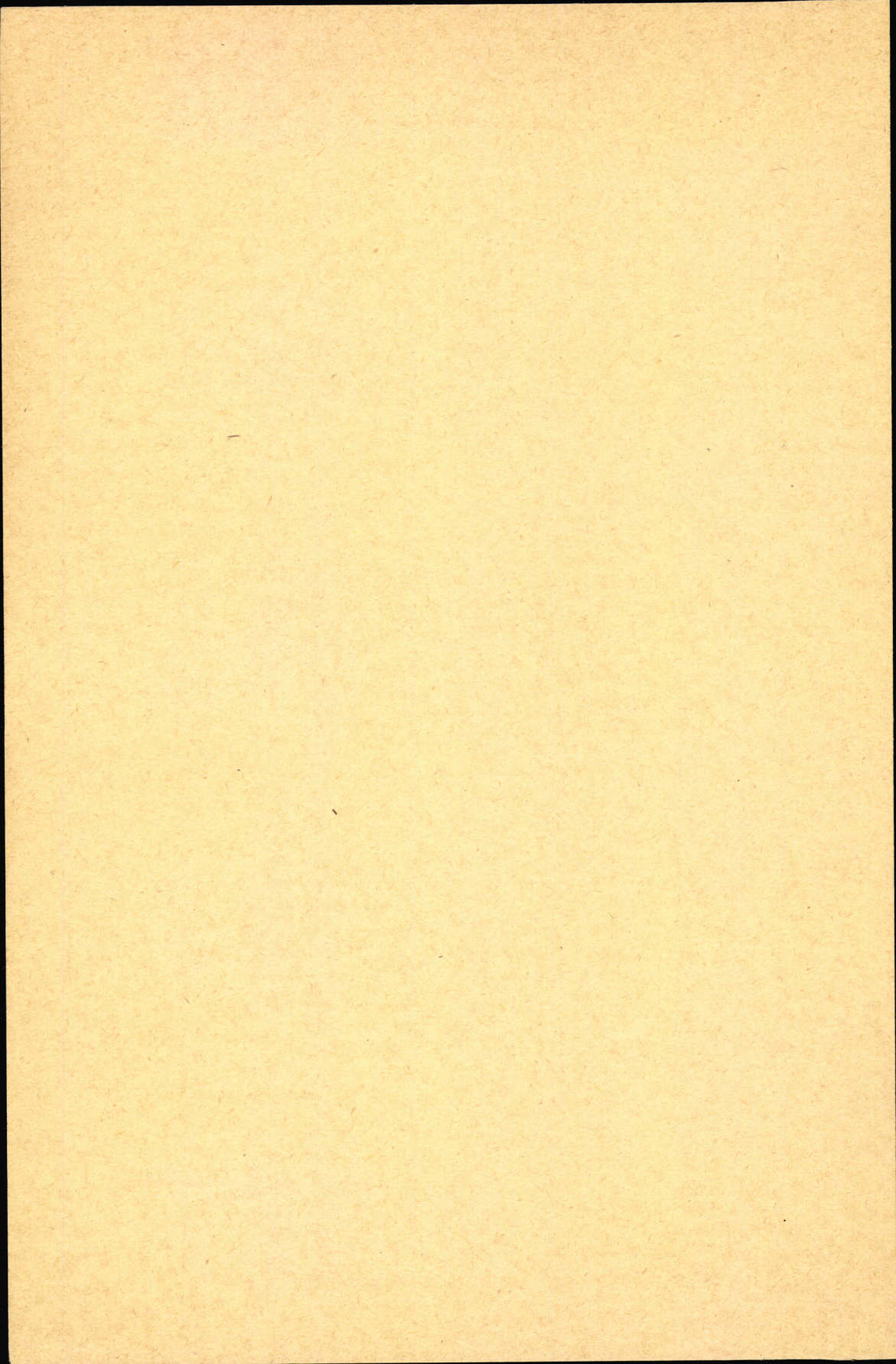
WHEAT GRAIN PROTEINS
analysis of varieties by starch-gel electrophoresis

by

G. J. DOEKES



OVERDRUK UIT PROEFSCHRIFT, LEIDEN, 1969
REPRINT FROM THESIS, LEIDEN, 1969



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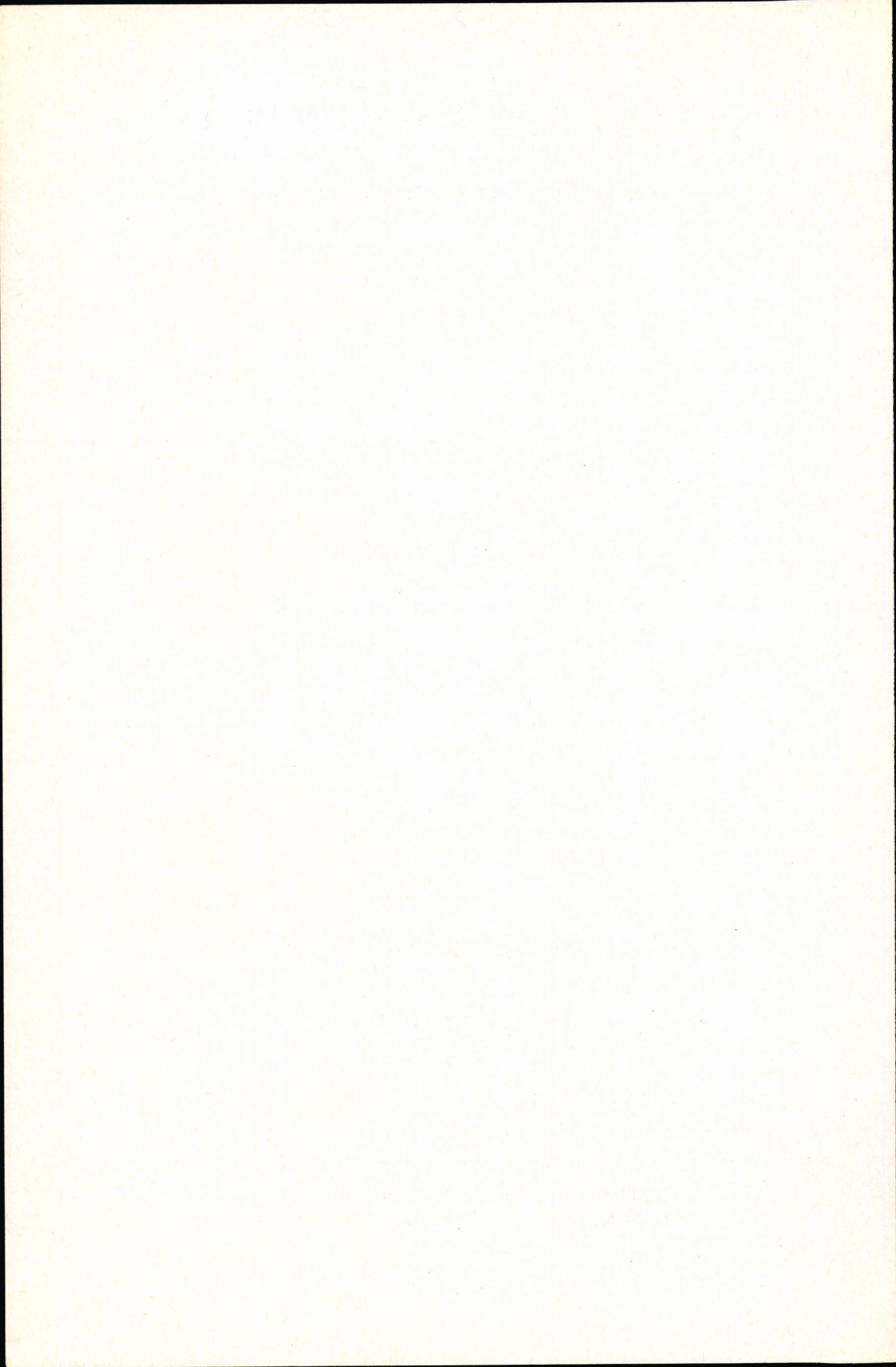


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I. INTRODUCTION

Lots of *Triticum aestivum* often display considerable differences in bread-making quality. Especially in the past few decades there have been constantly greater efforts to detect the causes of this phenomenon.

The view currently held is that the processing value is based mainly on the properties of the endosperm protein in the kernels. First of all, the protein content is important in this respect; in bread wheat a high protein content is a fair guarantee of good baking quality. However, there are lots which have roughly the same protein content and yet differ noticeably in quality, even if the grain is in good condition. The second determining factor would therefore have to be the nature of the protein, or protein quality.

The protein content can be controlled by nitrogen fertilization in the field, but it is open to question whether the protein quality can be influenced by exogenic factors. There are indications that the nature of the protein is largely determined by the genotype of the grain. As a basis for the discussion it seems justified to assume "protein quality" to be synonymous to the protein composition and to start with a possibly most accurate analysis of this.

When the starch and the soluble constituents of a wheat dough are removed by washing, a viscous mass with elastic properties, the gluten, remains. Beccari (1745) was the first to give a description of the phenomenon. The properties of the gluten are highly determinative for those of the dough, and since the baking quality of wheat is highly dependent on the viscoelastic properties of the dough, interest in gluten protein is understandable.

Osborne and Voorhees (1893) distinguished four types of protein in the endosperm of wheat:

- albumin, water-soluble immediately from flour;
- globulin, soluble in dilute salt solutions;

gliadin, soluble only in 70 % alcohol;

glutenin, soluble exclusively in dilute acid or in sodium hydroxide solution.

Gluten consists mainly of glutenin and gliadin. The term "gliadin" was introduced by Taddei (1820).

Since the article by Osborne was published, attempts have been made to study these fractions separately. Firstly, it was tried to find correlations between the amounts of the various fractions and the baking quality of a batch of flour. (Baking quality can be assessed quantitatively by means of standard baking tests, and may be expressed, for instance, as bread volume per 100 g flour used in the test). Guthrie (1899) determined the weight ratios of glutenin and gliadin preparations of various wheats. He did not find a good correlation with the baking quality of his material. Attempts in the same direction by later authors have likewise failed. In order to explain differences in baking quality between samples of wheat on the basis of protein composition apparently more precise methods of analysis are required.

The methods of column chromatography and electrophoresis that have been developed since then, have made it possible to divide Osborne's fractions into further protein components. Especially electrophoresis has been applied for this purpose with growing success.

The purpose of the present investigation was to explore the possibilities of electrophoretic analysis of wheat proteins as to the prognosis of quality factors in new varieties. First of all, a simple method was devised for identifying protein components separated by electrophoresis.

It appeared desirable to draw up new definitions for the classical wheat protein fractions of Osborne; the method designed appeared to be very suitable for this (see Section IV). The further investigations referred to the following three problems:

- Is the protein composition of the endosperm in *Triticum aestivum* determined entirely by the genotype, or is it also affected by agricultural conditions? The relevant literature demonstrates that the ratios between the components in wheat protein are largely dependent on genetic factors, but gives no definite conclusions as to the effects of external influences. It was therefore very important to deal with this problem (Section V) firstly.

- Can electrophoretic analysis provide information regarding systematic

relationships between species of the genus *Triticum*, or even between varieties or groups of varieties within a species? As the literature shows, the question of grouping and determination of wheat varieties on the basis of their electrophoretic patterns has so far hardly been considered. An analysis in this sense was performed (see Section VI).

The question of relationship between species was studied especially by Johnson and Hall (1965 and later), relative to the genetic origin of present-day bread wheat. Some questions have remained, however. In an attempt to answer these and also to define the results obtained by Johnson and Hall more precisely, this interesting subject was also included in the investigations (see Section VII).

- The main reason for studying the wheat proteins is the question, which has become almost an obsession among cereal chemists: is it possible to trace the main aspects of the complex notion "baking quality" back to chemical and physical factors?

Out of the various methods described, starch-gel electrophoresis was selected to be dealt with more extensively. The list of literature references (Section II) may partly account for this choice.

II. LITERATURE

For convenience, the results of the electrophoretic studies of proteins will be arranged according to the techniques used; at the end of each part an indication will be given as to how far the investigations have progressed at the particular moment.

1. Free electrophoresis

In free or "moving boundary" electrophoresis, the separation takes place in a U-shaped tube filled with a buffer solution. This technique was developed by Tiselius (1930), but was not applied to wheat protein until much later.

In 1959, Jones et al published electropherograms of gluten protein covering four varieties of *Triticum aestivum* (bread wheat) and two varieties *T. durum* (durum wheat); two of the bread wheats had good, two had poor baking properties. The composition of all four seemed to be the same; only that of *durum* wheat differed markedly.

Cluskey et al (1961) investigated the gluten of three "hard" *aestivum* wheats (with hard, vitreous grain contents, usually with good baking quality), each from three different fields, and the gluten of eight "soft" *aestivum* wheats (with soft, mealy kernel contents, mostly with poor baking quality). They calculated the relative contributions of the five visible components to the overall protein pattern, which enabled them to distinguish between hard and soft bread wheats.

Kelley and Koenig (1963) compared *T. durum*, *T. compactum* and varieties of hard, soft, red-seeded and white-seeded *T. aestivum*. The "salt-soluble" proteins and gluten proteins (albumins-globulins and gliadin-glutenin) formed

patterns characteristic of each type, except in the case of white-seeded *aestivum* wheat. One of the *aestivum* wheats had an extremely striking pattern. Otherwise the varieties could not be recognized individually.

Koenig et al (1964) examined the effect of the type of soil and/or climate on the ratio of components in the gluten of *T. aestivum*. It appeared that these ratios were determined both by the genotype and by external conditions.

A relationship between electrophoresis pattern and quality factors in wheat flour was first demonstrated by Mullen and Smith (1965): in one sample of hard *aestivum* wheat which had a long dough development time (which often combines with a good baking quality), the amount of gliadins with low molecular weight was smaller than in a sample of wheat which had a short dough development time (which to some extent correlates with poor baking quality).

Kloos (1966) found electrophoretic differences between the gliadins of four varieties of *T. aestivum* and, to a lesser extent, between the glutenin extracts. In 1968 he expressed the view, however, that climate and the condition of the soil would have even more influence on the protein composition than the variety.

The results as discussed above may be summarised in the following points: Some species of *Triticum* can be distinguished with the aid of free electrophoresis. It is also possible to distinguish between hard and soft *T. aestivum*, although this requires some calculation and great technical accuracy. The method is not precise enough to identify *T. aestivum* varieties, however. Nor is there any definite proof of the protein pattern being determined exclusively genetically, or of this being partly influenced by agricultural conditions. There is some indication of a correlation between electrophoresis pattern and baking quality, but the available statistics are not sufficient to warrant a definite conclusion.

2. Zone electrophoresis

The term "electrophoresis" originates from Michaelis (1909), and "zone electrophoresis" from Tiselius (1930). In the latter the proteins do not move in the free buffer solution, but in a porous supporting medium, e.g. filter paper or a gel of agar, polyacryl amide, starch, etc. The filter paper method was developed by König (1937), that using agar gel as early as 1907 by Field and Teagre.

A drawback of zone electrophoresis is that the glutenin does not take

part in the process; this macromolecule is too big to pass through the pores of the supporting medium. This does not apply to free electrophoresis, in which the glutenin is even the fraction that migrates fastest.

Nevertheless, glutenin can be examined by zone electrophoresis if divided into smaller parts, viz. by splitting the disulphide bridges between the polypeptides. Nielsen et al (1962) performed such splittings by adding sulphite to protein suspensions; Woychik and collaborators (1964) subsequently followed this method in zone electrophoresis (on starch gels).

2.1. Paper electrophoresis

There is only one important literature item dealing with a study of wheat varieties using filter paper electrophoresis: Pence et al (1954) compared the composition of salt extracts (albumins and globulins) of five *aestivum* varieties and some *durum* varieties. Although the electropherograms each revealed only 9 or 11 vaguely defined spots, a marked correspondence between the five *aestivum* varieties and a clear difference with the *durum* varieties could be observed.

The resolving power (number of visible components) of paper as supporting medium is greater than that of free electrophoresis. Paper electrophoresis is moreover much easier to apply because the apparatus is very simple.

2.2. Agar-gel electrophoresis

This method is hardly anywhere referred to in the literature concerning wheat protein research. Poppoff and Iliev (1967) found six to eight components in aqueous salt extracts (albumins and globulins). In alcohol extracts (gliadins) they found one to three components per variety. The varieties showed all different patterns, however.

Agar-gel electrophoresis was used for immunochemical analysis of glutenin and gliadin by Grabar et al (1965), who thereby demonstrated the immunological relationship between the two protein fractions.

As a further illustration of the possibilities of electrophoresis, though not directly related to this review, reference should be made to the investigations by Zwartz (1967), who carried out agar-gel electrophoresis of the protein from potato tubers. The composition of this protein was found to be characteristic of the variety.

2.3. Polyacryl-amide-gel electrophoresis

This technique was developed by Raymond and Wang (1960) and improved for wheat protein analysis by Lee (1962) and other investigators. It has produced much better results than the other two, but so far found relatively little application.

Lee and Wringley (1963) obtained identical electrophoresis patterns of samples of one *aestivum* variety grown in two climatologically different places. The samples showed a difference in protein content. The protein compositions of eight *aestivum* varieties that were also examined by column chromatography, were characteristic enough for identifying some types: corresponding patterns were found in genetically related varieties. No relationship with the baking quality was established. The electropherograms consisted of eight to nine components. Nimmo et al (1963), however, found 15 to 17 of them and reported reproducible differences between some *aestivum* varieties. Johnson and Hall (1965 and later) investigating genetic origin of bread wheat, likewise used polyacryl amide gels. These studies are discussed in more detail in Section VII and VIII.

True, the resolving power of polyacryl-amide gels is greater than that of the media mentioned previously, but the problems referred to in the first section are by no means solved. Very little attention has been paid to the question of the influence of genus or variety compared with that of the ambient conditions on the protein composition. A definite conclusion is therefore not yet warranted, although it may be stated that the genotype is a dominant factor in the protein composition and that the influence of the ambient conditions, if any, is much less significant. There have been no further indications of correlation with quality.

2.4. Starch-gel electrophoresis

In an article on serum proteins, Smithies (1955) described a new medium for electrophoresis, viz. a gel of gelatinized starch, which had a resolving power vastly superior to the media known until then. The method was much improved upon by a development in free electrophoresis: Jones et al (1959) had found that a buffer solution of aluminium lactate with lactic acid (pH 3.1) was the best dispersing agent for wheat proteins. Elton and Ewart (1960) combined the advantages of this buffer and those of the starch gel. Although they were initially able to identify only eight components in the endosperm

protein, the number of articles written about this method has grown considerably, as well as the number of protein components discovered. They even completely overshadow the results mentioned previously.

Coulson and Sim (1961) found about 20 protein components in *T. aestivum*, Kaminski (1962) discovered about 30.

Elton and Ewart (1962) observed significant differences between the varieties in gliadin extracts of bread wheat, while the composition of albumins and globulins remained virtually the same for all varieties studied. This was also found by Bourdet et al (1963). Graham and collaborators (1963) compared albumin/globulin and gliadin preparations of seven *aestivum* varieties and one *durum* wheat. Apart from variety differences in the gliadin fraction of *T.aestivum* they found correspondence in it for three genetically related varieties. The differences with unrelated varieties were very slight, however.

In 1964 Coulson and Sim reported a study of *aestivum* varieties. Their results, in so far they are of interest for this review, were that:

- the most striking differences of the varieties are found in the gliadin fraction;
- the albumin/globulin electropherograms constantly reflect the same composition;
- differences in growing conditions of the plants (these were not extremely great) did not seem to cause differences in protein composition;
- no correlation was found between the protein patterns of the wheat and the physical properties of the flour such as they become manifest in the preparation of dough and bread;
- theoretically, electrophoretic determination of wheat varieties is possible.

For the latter purpose, however, the technique applied was not yet sufficiently accurate.

Feillet and Bourdet (1964) report (though they do not illustrate) that they could identify some *aestivum* varieties on the ground of gliadin electropherograms. Further they show by illustrations that it is possible to distinguish between species (in this case between *T. aestivum* and *T. durum*) by means of the albumin/globulin patterns. In 1967 they found gliadin to consist of 15 to 19 components, and also pointed out that the agricultural conditions did not seem to change the protein ratios. This was studied with reference to two *aestivum* varieties of two harvest years, four places and some greenhouse tests with the application of nitrogen fertilization.

Lee and Ronalds (1967) made a systematic study of the latter aspect in four *aestivum* varieties from various regions of Australia. The slight variations

in composition which they observed were attributed to test errors. Their conclusion was that the combined effect of growth place, soil type and season, which indeed influence the protein content of the grain kernels, leads to little change in the gliadin composition. These authors expressly point out, however, that starch-gel electrophoresis as a rule involves difficulties as regards reproducibility.

The study of protein by means of starch-gel electrophoresis has also failed to answer the questions raised in the previous section.

- As to the possible influence of the agricultural conditions, the authors using starch gels draw the same conclusion as the others: such influence, if any, is only very small. A decisive answer about its existence is not available, because the methods followed were inadequate to reveal the slightest differences in protein composition. It is obvious that higher demands will have to be made upon the reproducibility when the supposed influence of the conditions is smaller. In all electrophoresis methods, reproducibility is the weak spot. This subject was unjustly very often disregarded in the relevant literature; as a rule it was not mentioned at all. Lee and Ronalds (1967) are among the few who explicitly put in a proviso with respect to test errors affecting their results.

Apart from the technical difficulties, the number of wheat varieties subjected to such investigations was too small for exhaustive studies.

- It is true that the starch-gel method has a much greater resolving power than the polyacryl-amide-gel technique, but this greater power has not yet been sufficiently taken advantage of. The results obtained with starch gels have only confirmed those of tests made with other methods. It therefore seemed appropriate to use starch gels in the studies described below, in order to refine the method.

III. MATERIALS AND METHODS

When the author started the investigations the electrophoresis technique was already developed by Mr. A. Graveland in the same institute.

Preparing the flour.

The grains were moistened with water to 15%. Flour was made with a Brabender Quadrumat Junior laboratory mill. Extraction rate was approx. 70%.

Extraction of wheat proteins

To select the most appropriate solvent - i.e. the solvent that ensured the most distinct electropherograms - the following solvents were examined: aluminium lactate-lactic acid buffer (pH = 3.1), lactic acid solutions, acetic acid (0.05 N), sodium pyrophosphate buffer (pH = 7), 70-% alcohol, and water. These solvents were used with and without addition of urea (3M); the extracts were subjected to electrophoresis. It appeared that tailing occurred in the majority of cases, causing indistinct electropherograms. Dialysis of the extract effected only little improvement. Tailing was consistently associated with the presence of glutenin at the place of application in the gel. Alcoholic and aqueous extracts, without urea, gave the best electropherograms.

Water was finally chosen as solvent because this eliminated the need for dialysis of the extracts, which was time-consuming and often unreliable. However, a disadvantage was the low concentration of gliadins in the extracts, compared with those of the albumins and globulins.

Moureaux (1965) described the effect of gliadin solubility in water being reduced by all kinds of salts, especially phosphates (including phytate). Flour contains such salts in sufficient amounts to prevent the gliadins from dissolving (see also C.S.I.R.O. 1965-66). The salts themselves are water-soluble,

however, and if the residue from flour extraction with water is extracted further, the subsequent extracts contain more gliadins (see Fig. 1).

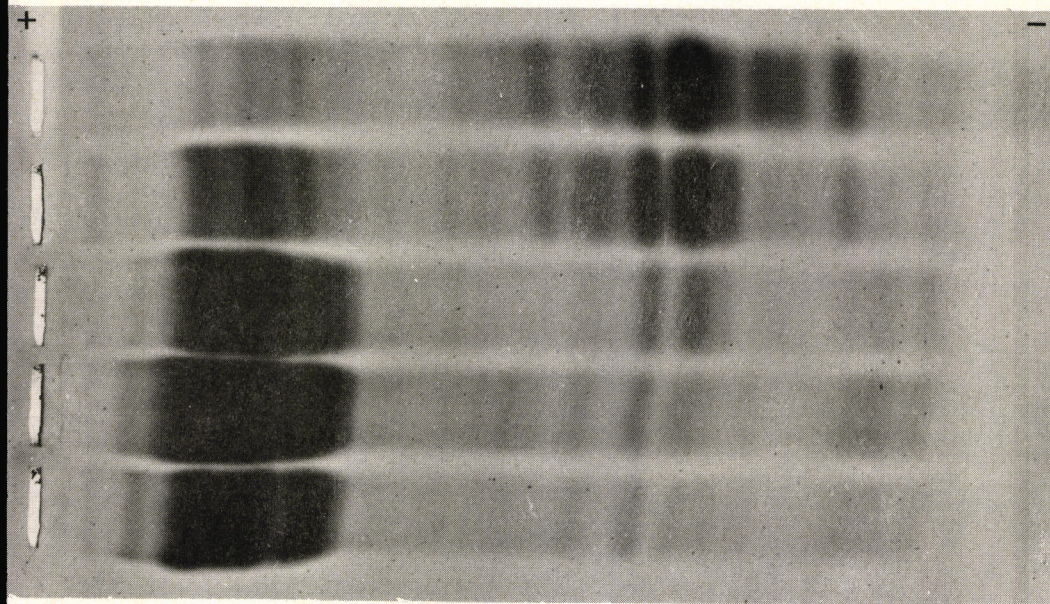


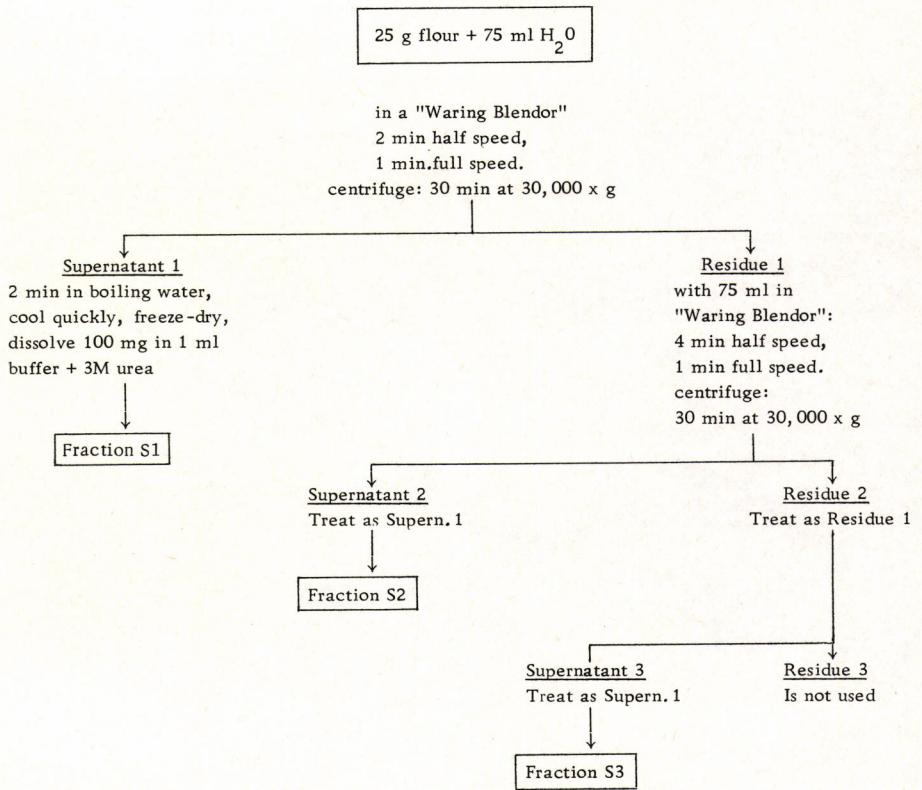
Fig. 1. Electropherograms of aqueous extracts of flour. Top to bottom 1st - 5th extraction.

Decline of albumin/globulin concentration and simultaneous increase of gliadin concentration, until this declines after 4 extractions.

A single extraction was sufficient for electrophoresis of albumins and globulins; in order to obtain a good representation of the gliadin pattern, the flour had to be extracted three times, and the third extract used for assessment.

After the flour-to-water ratio and the extraction time most favourable for yielding an ample amount of protein had been determined, the schedule shown in Table 1 was followed.

TABLE 1.



Fraction S 1 contains mainly albumins and globulins, fraction S 2 approximately equal concentrations of gliadins and albumins/globulins, and fraction S 3 mainly gliadins.

When an electropherogram of an S 2 fraction was made, either the gliadins, or the albumins and globulins were not separated distinctly enough, depending on the duration of the electrophoresis. As the gliadins apparently required a running time different from the other proteins, the S 3 and S 1 fractions respectively were used instead of the S 2 fractions.

Freshly prepared protein solutions gave slightly indistinct electropherograms, and the solutions were therefore kept for at least one night (at 3°) before they were used.

Buffer solution.

The buffer used in the gel and electrode boxes was the aluminium lactate-lactic acid buffer (pH = 3.1) described by Jones et al (1959). Aluminium lactate was prepared according to Jones & Cluskey (1963). The ionic strength of the buffer was kept at 0.06 as this value lies in the middle of the range (0.01 - 0.1) that offers optimum conditions both for obtaining sharp protein zones and for preventing undesirable heat development. To the stock solution of the buffer (5 l), 2 ml thymol (10% in 96-% alcohol) were added to prevent mould growth, and the solution was kept in the dark at 3°.

Gels.

As the electrophoresis results had been found to be largely dependent on the properties of the gel, the preparation of the latter was standardized as far as possible.

13 g "starch hydrolyzed" (Connaught Medical Research Laboratories, University of Toronto, Canada) were suspended in 100 ml buffer. The mixture was placed in a heating jacket and mechanically stirred for a fixed period of time. After complete gelatinization, urea was added to a concentration of 3M and heating was continued for some time. Dissolved gas was removed from the mixture by boiling it in the vacuum of a water aspirator. The time required for each operation was adhered to in subsequent preparations.

Preparing the gels in perspex troughs (Smithies, 1955, 1958) takes relatively large quantities of starch and too much time. The gels are difficult to manage and easily damaged in staining. These drawbacks are obviated if the gels are made on glass plates (in the present case 20 x 10 x 0.3 cm).

One hour after the gelatinized starch solution was poured off and cooled to room temperature, the gels were covered with PVC film to prevent drying, and kept at 3° for at least four days before they were used, because in gels that were too fresh the results of electrophoresis were not reproducible. This effect might be accounted for by the retrogradation of gelatinized starch, which seems to be completed after about four days (Pelshenke & Hampel, 1962).

Electrophoresis.

At one end of the gel plate a 4 cm strip of the PVC was cut off and, by suction through a small flattened piece of steel tubing, slots into which a few drops of the fractions were introduced were cut into the gel. The gel was placed with its ends on the edges of the electrode compartments, and connected with

the buffer solution by means of filter paper. Electrophoresis was consistently carried out at 13V/cm, and a standard current of 30 mA, for fractions S 1 for 80 minutes, for fractions S 3 for 160 minutes. The apparatus was installed in a room where the temperature was 3° and air was circulated, so that additional provisions for cooling the gels were not necessary.

Cutting, staining and scanning.

The topmost layer of the gel was sliced off by means of a steel wire 0.1 mm thick. This was necessary to eliminate interfering surface effects. In order to have a uniformly thick layer for subsequent operations the device shown in Fig. 2 was designed.

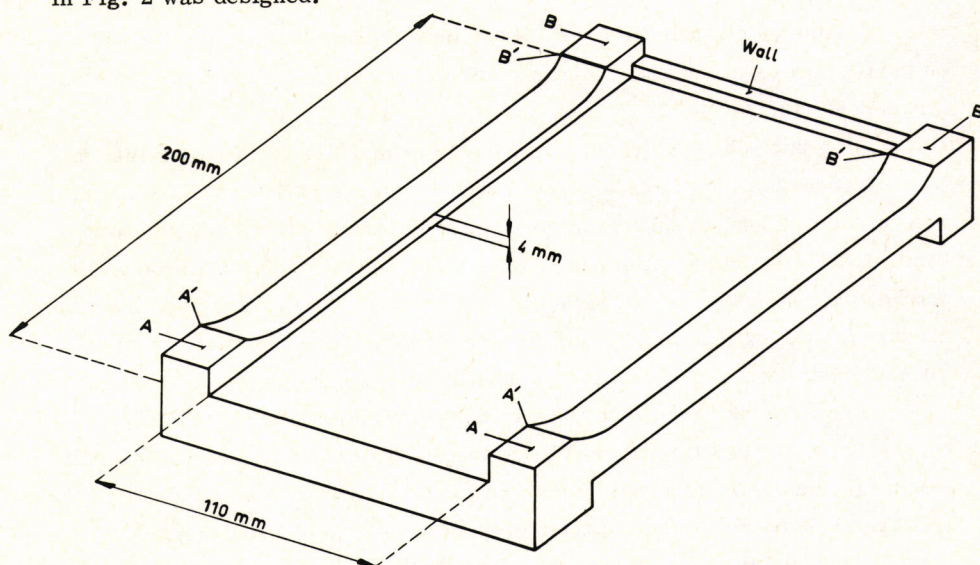


Fig. 2. Device for cutting the gel sheets. A taut wire is pulled along the edges from A to B. At A' it sinks into the gel and emerges at B'. The glass plates are 0.3 cm thick, so that there is always a gel 0.1 cm thick left on them. The raised edge ("wall") prevents the gel from sliding off when cut.

The gel was then placed in a tray containing the following staining solution: 1100 ml of methanol/acetic acid/water = 5 : 5 : 1, with 0.5 g water-soluble nigrosine (Amsterdamse Chininefabriek) and 0.25 g "Amidoschwartz 10B" (Merck, Darmstadt). In order to prevent irregular deposits of stain particles, the tray was placed on a platform which was slowly tilted up and down. This mixture of staining substances was chosen because the gluten proteins are stained better by nigrosine than by Amidoschwartz (Coulson & Sim,

1961). The latter stains the other proteins more intensively.

The correct degree of staining was judged by eye, and its duration turned out to be variable (10–30 min), depending on the freshness of the staining solution. The gel was then rinsed several times with 5 % acetic acid; after about 4 hours the background contrasted sufficiently with the protein bands. When a gel had to be kept overnight before it could be scanned, it appeared that the acetic acid caused the gliadin bands especially to run. Timely substitution of the acetic acid by a 5 % sodium chloride solution prevented this.

After the gel was washed densitograms were made of the protein tracks by means of a Vitatron Densitometer. Fig. 3 shows an example of the results obtained.

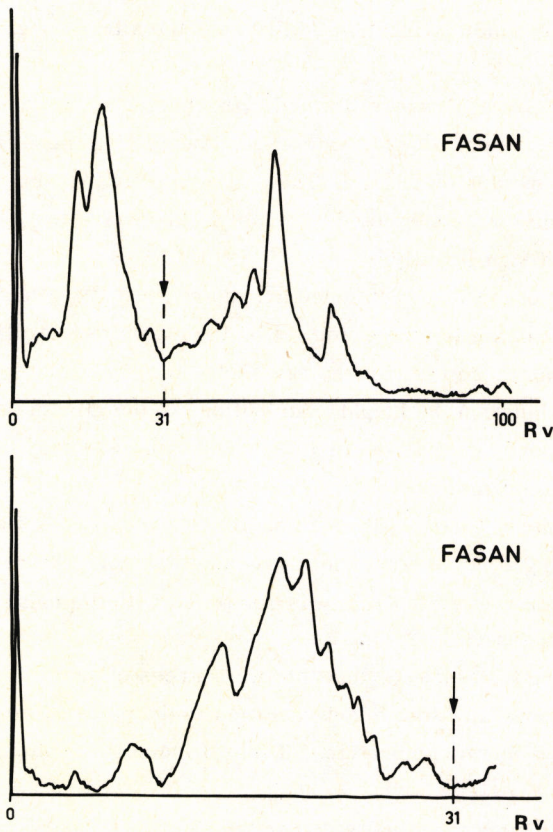


Fig. 3. Densitograms of Fasan variety.

Top: fraction S 1. Bottom: fraction S 3; increased concentration and longer electrophoresis leads for the sector to the left of the arrow to a more detailed curve.

Unless stated otherwise, the method described above was followed for the other cereals under review.

Reproducibility.

In order to gain an impression of the reproducibility of the results (densitograms), experiments were made in which the *extraction of the flour* and the *composition of the gel* were taken into account, as these are the main sources of variation.

In densitograms the concentrations of the components can be expressed in terms of the areas under the corresponding peaks. As the top angles of most peaks are virtually equal, the concentrations are simply reflected in the heights of the peaks. Thus, a measure for the relative concentrations can be found by converting the peak heights to percentages of their sum. This should be done separately for the gliadins (fraction S 3) and for the albumins/globulins (fraction S 1).

- The *extraction* effect (E) was examined by preparing an extract in threefold from a sample of flour - Opal variety - and depositing the 3 extracts (no. 1, 2, and 3) side by side on one gel. The 3 gliadin densitograms (each showing 18 protein peaks) were subjected 2 by 2 to a chi-square test, in order to try if they differed significantly.

In Table II the operations mentioned above are given for gliadin densitograms. The same operations were carried out on the albumins/globulins (with 13 components); as these revealed no new facts, they are not reported here. No difference between the height distributions of the gliadin peaks was statistically found at the $P = 0.01$ level. The same was true for the albumin/globulin densitograms.

- The *composition of the gel* (G) as a possible source of variations was examined by bringing a new extract (no. 4) on an other gel. The results (in which again the factor E was involved) was compared with the mean values of the former densitograms 1, 2, and 3).

The chi-square test showed, that the two sources of variation (E + G) did not even jointly cause any significant variation in the pattern, neither of the gliadins, nor of the other proteins. Table II includes a column for the estimation of E + G.

It follows from the results that in the examined cases reproducibility was very high, since neither extraction, nor gel composition separately, nor the combination of the two factors, caused any significant variation in the

protein pattern.

In addition to this *quantitative* aspect of reproducibility, the presence of all components in all cases was also kept in view. All densitograms of the same protein fractions showed to consist of the same series of components (18 or 13); so there was also a perfect *qualitative* reproducibility.

TABLE II. Estimation of the reproducibility of gliadin densitograms.

h = measured peak height.
h/S % = h as percentage of the sum of peak heights.
E = effect of extraction.
E + G = E + effect of gel composition.
S = sum.

Peak no. :	E						E + G		
	h_1	h_1/S_1 %	h_2	h_2/S_2 %	h_3	h_3/S_3 %	$h_{1, 2, 3/S}$ Mean	h_4	h_4/S %
1	9	2.11	7	1.55	9	1.74	1.80	7	1,50
2	4	0.94	4	0.88	5	0.97	0.93	3	0.65
3	5	1.18	5	1.10	5	0.97	1.08	4	0.87
4	6	1.41	7	1.55	5	0.97	1.32	10	2.17
5	3	0.70	4	0.88	3	0.58	0.73	3	0.65
6	4	0.94	5	1.10	5	0.97	1.00	4	0.87
7	5	1.18	6	1.33	6	1.16	1.22	4	0.87
8	10	2.36	12	2.65	12	2.32	2.44	11	2.40
9	59	13.88	61	13.47	70	13.54	13.63	66	14.35
10	31	7.29	32	7.06	40	7.74	7.35	33	7.17
11	67	15.76	73	16.12	87	16.84	16.24	77	16.74
12	77	18.12	84	18.54	94	18.16	18.27	80	17.40
13	39	9.18	43	9.49	48	9.29	9.32	41	8.91
14	36	8.46	38	8.49	44	8.51	8.45	32	6.95
15	31	7.30	32	7.06	42	8.12	7.49	32	6.96
16	20	4.72	22	4.85	24	4.64	4.74	34	7.40
17	11	2.59	11	2.43	11	2.13	2.38	11	2.40
18	8	1.88	7	1.55	7	1.35	1.59	8	1.74
S	425	100.00	453	100.00	517	100.00	100.00	460	100.00

IV. R_v VALUES

NEW DEFINITIONS OF BREAD WHEAT PROTEINS

A study of a number of densitograms led to the conclusion that all the samples of one variety had the same protein components, each holding its specific position among the others accurately. Consequently each component may be referred to by means of a number which represents its running speed.

For this purpose the term "electrophoretic mobility" is commonly used, expressed in cm/sec per Volt/cm, which indicates that the mobility depends solely on the voltage. There are other factors, however, which also have some effect, viz. the age, the viscosity and, perhaps, the temperature of the gel, as well as the age of the protein solution. The "electrophoretic mobility" is therefore by no means constant.

To eliminate these factors the relative mobilities of proteins were determined instead of the absolute ones, as follows:

The fastest component, clearly visible only in fractions S 1, was allocated the value 100 (see Fig. 3), the starting point, 0.

Between these limits, all components were allocated proportional values, referred to as R_v . It is comparable with R_f in chromatography if the fastest component in an electropherogram can be compared with the eluent front in a chromatogram. Fig. 4 shows an example of the use of R_v values.

Calculating R_v values from a large number of densitograms is very time-consuming, and it is therefore convenient to use an interpolation graph. This diagram is placed under the densitogram and light is projected through both; the R_v values are read from the diagram on to the densitogram.

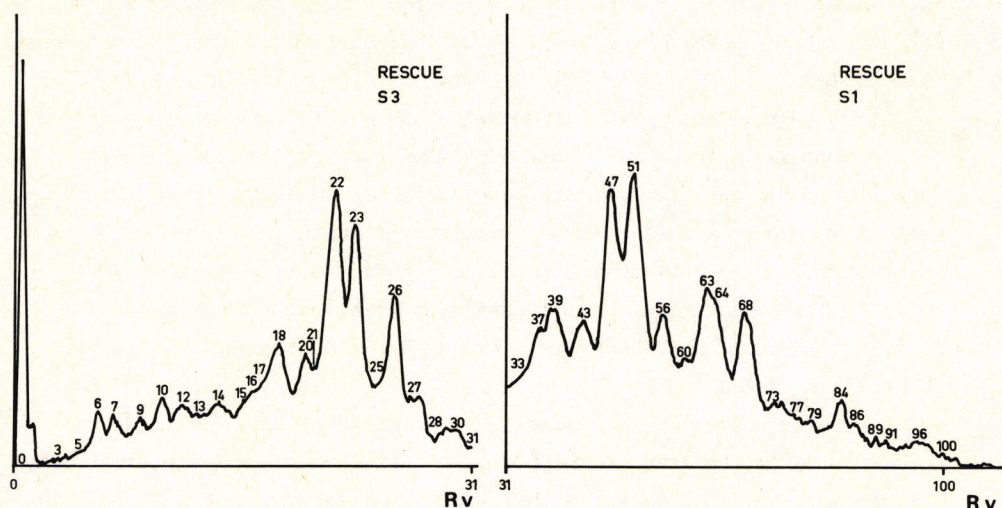


Fig. 4: Densitograms of Rescue variety with R_v values.

Left: S 3 with gliadins.

Right: S 1 with albumins and globulins only.

It is obvious that this method can be used to identify protein components separated by electrophoresis. The method is not confined to wheat proteins but can be used as well for all kinds of protein mixtures and under all experimental conditions.

Reference has been made to Osborne (see Sect. I), who distinguished four types of wheat protein, based on solubility characteristics.

This characterization, which has become traditional, is, however, open to objections on account of the results of electrophoresis. With regard to albumins and globulins electropherograms of aqueous extracts reveal the same protein components as those of extracts made with NaCl solutions. Any slight differences are within the range of errors inherent to the test. The differences found by Osborne were probably of a quantitative, and not of a qualitative, nature. In any case they have no practical significance for this study of wheat protein. With regard to the gliadins, these dissolve well in water provided this contains no salts. Electrophoresis moreover shows that with 70-% alcohol, albumins and globulins are extracted in addition to gliadins.

If the terms used by Osborne are to be maintained, new definitions will

have to be drawn up which agree with the electrophoresis results. For starch gel electrophoresis (at pH = 3.1) a definition based on the following observations is suggested:

Fig. 1 reveals two separate protein groups: a fast-moving group, found in large amounts in fractions S 1 and S 2, and a slow-moving group, contained especially in S 3 and subsequent fractions. A distinct absorption minimum between the two groups was observed in all densitograms (see arrow in Fig. 3). By reference to 44 densitograms (viz. those used in studying the effect of N fertilization) the position of this point was determined and found to be at $R_V = 31$. (In 7 out of the 44 samples it was $R_V = 30$, in 29 cases $R_V = 31$, and in 8 cases $R_V = 32$).

In accordance with this, it is suggested that the gliadins should be defined as the protein fraction which in starch-gel electrophoresis in aluminium lactate buffer (pH = 3.1) is segregated between the starting point and a distinct absorption minimum at $R_V = 31$. The albumins/globulins are defined as the proteins having R_V values between 31 and 100. Glutenin is the fraction the molecular size of which causes it to escape electrophoresis; it remains at the starting point in the gel.

V. EFFECT OF ENVIRONMENT ON PROTEIN COMPOSITION

Materials

The Foundation for Agricultural Plant Breeding at Wageningen supplied 18 samples of 3 varieties, Orca, Fasan, and Ring, which, under otherwise identical conditions, had been dressed with different amounts of N at various stages of growth, as follows: 30 kg N (as ammonium nitrate limestone) /ha at tillering; 0 or 30 or 60 kg/ha at stem extension; and 0 or 30 or 60 kg/ha at the beginning of flowering.

As the use of a N-fertilizer has the effect of increasing the protein content of the grains, samples of the three varieties showed ranges of protein contents.

Samples of the Carstens VI winter wheat variety were supplied by courtesy of the Institute for Soil Fertility at Groningen. This variety was grown for several years in:

Groningen (Netherlands), 1966 and 1967.

Gross-Gerau und Rauisch-Holzhausen (Giessen, W.Germany), 1966 and 1967.

Versailles (France), 1967.

Domžale (N.W.-Yugo-Slavia), 1967.

Linz (Austria), 1961 up to and including 1967.

The samples from these locations represented a diversity of possible effects of different soil and climate conditions and, within each location, a diversity of possible seasonal effects.

Effect of N fertilization on the protein pattern.

With regard to the first problem put in Section I, we were interested

to see any changes in the electropherogram with increases in protein content of the grain.

Flour samples made from Orca, Fasan, and Ring were examined and Fig. 5 shows the results obtained with the Fasan variety. The other varieties showed similar results.

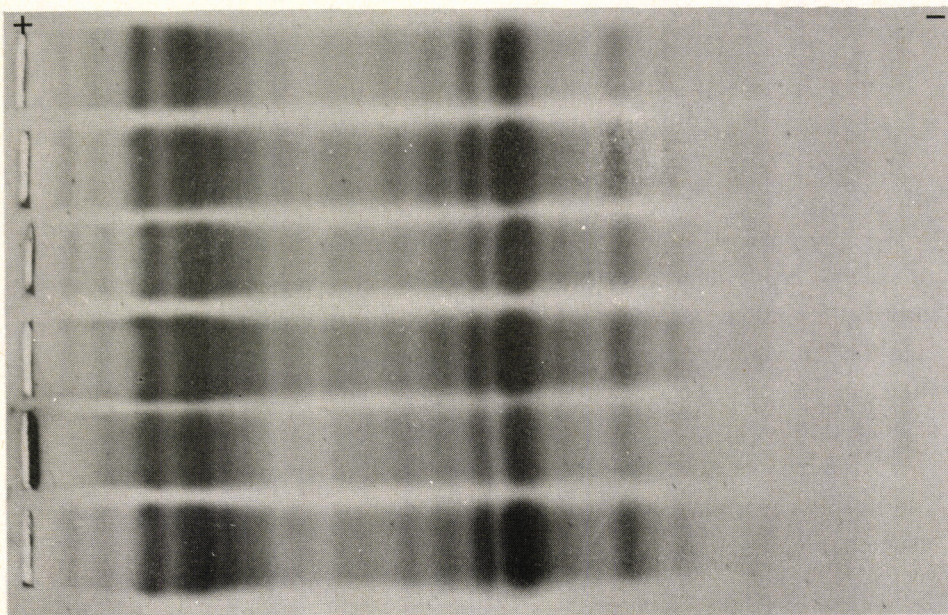


Fig. 5. Samples of Fasan variety.

Top to bottom: increasing N-fertilizer dressing, resulting in a protein content of the flour ranging from 9.2 to 12.1%.

This figure shows that N dressing had no demonstrable influence on the electropherogram, irrespective of the amount added or the stage of growth at which it was added.

Some effect was discernable, however, on the extraction yields: the yield of S 1 protein from samples of the same variety remained virtually constant, but the yield of S 3 protein (mainly gliadins) increased steadily. The reason this effect is not observed in the total intensity of the pattern is that in preparing the protein solutions the same amount of dry matter was dissolved each time.

Effect of weather, climate, and soil conditions on the protein pattern.

A possible effect of *weather conditions before harvest* was investigated with the aid of samples of the Carstens VI variety from one and the same growing region but from different crop years. For that purpose we used the samples from Groningen, Gross-Gerau and Linz. The densitograms obtained were operated in the same way as those of the reproducibility analysis (Section III).

The chi-square tests indicated that at the $P = 0.01$ level no significant difference existed between densitograms related to various crop years.

A joint effect of *climate and soil conditions* was examined by comparing the samples from Versailles, Linz, Groningen, Domzale, and Gross-Gerau with each other. The chi-square tests showed that even the combined factors did not cause any appreciable change in the protein pattern.

It may be inferred from this work that the composition of the endosperm proteins, as it is shown up by the technique used here, is not influenced by variations in environmental growing conditions.

Thus, the densitogram (or electropherogram) is a reflection of the genetically fixed protein composition of the endosperm.

VI. COMPARISON OF BREAD WHEAT VARIETIES.

Materials.

The Foundation for Agricultural Plant Breeding, the Institute of Research on Varieties of Field Crops and the Institute of Phytopathological Research, all at Wageningen, supplied varieties and selected lines from West, Central and East Europe, India and North and South America. The samples had been grown in Holland for quality testing.

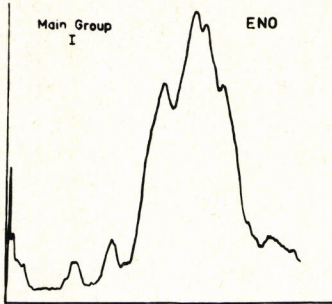
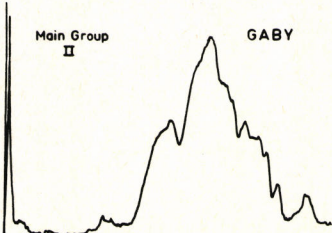
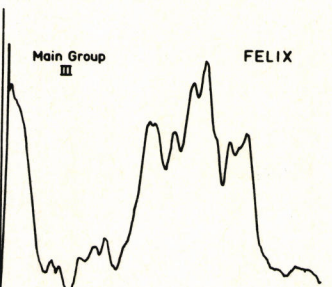
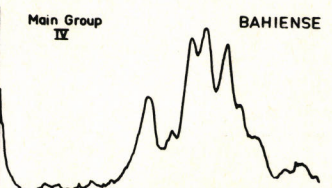
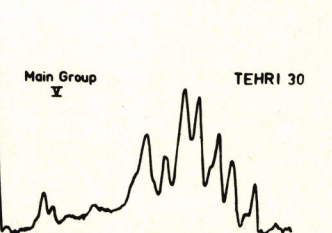
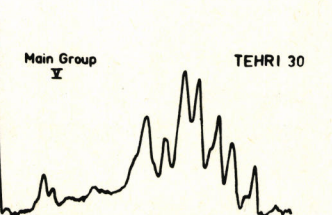
Classification of wheat varieties according to gliadin pattern.

The influence of the variety was studied by reference to densitograms of 80 varieties and selected lines. In the first instance five main groups were observed in the densitograms (see Fig. 6) viz. one with a compact gliadin pattern (I), one with a well spread pattern clearly divided up into components (V), and a series of three consecutive intermediate type (II - IV).

Each main group moreover consisted of sub-groups comprising varieties with very similar gliadin patterns. This arrangement (Table III) provided a morphological series consisting of a number of sub-groups in each main group.

From Fig. 6 it may be inferred that the very compact gliadin pattern is displayed mainly by winter wheat of main group I, whereas the well spread pattern is found mainly in spring wheat of main group V. Between these extremes transitional types are found among both spring and winter wheats. Generally, it might be said that: main group I contains soft-grain West-European winter wheats, having fairly poor baking qualities; main groups II, III and IV are varieties of varied origin and properties: winter wheats with large and small cold requirements, as well as true spring wheats; main group V contains

TABLE III. Morphological series of gliadin patterns.

 Main Group I ENO	Sub-Group	Winter Wheat	Spring Wheat
 Main Group II GABY	a	Eno *, Flamingo	
	b	Vaillant, Cambier 13307-14	
	c	Heine VII, Nord, Capelle	
 Main Group III FELIX	d	Rembrandt, Heine 3950	
	a	Carstens VI, Carstens 854, MGH 63-187, Doerfler W 16	Orca.MGH 60-293
	b	Sylvia, Tadorna	Gaby *, Jufy I, Nepal81
 Main Group IV BAHIENSE	c	Ibis, Flevina, Novi Sad 58, Dippe's Triumph, Dippe's 2076, Cleo, V CIV 7010	Peko, Fasan, Perso
	a	Felix *, Manella	Carpo, CIV 228-5
	b	Hybride 46, Stella, Marchal, Prof. Delos, Minister	Gaucho, Chili 3117-3
 Main Group V TEHRI 30	a	Kharkov (C.I. 1442), Novi Sad Banatka	Koga II, Cebeco 1009
	b	Kasni K.B., Obilje B	Buck Bolivar, Bahienze*, Vilela Mar.
	c		Thatcher, Saunders, General Roca
 Main Group V TEHRI 30	b		Opal, Doerfler 511
	c		Lee, Tehri 30 *, Kalimpong 2, Rescue

* Example of densitogram shown in Fig. 6.

Fig. 6: Morphological series of gliadin patterns.

predominantly spring wheats, characterized by hard, vitreous grains and very good baking qualities. The scheme presents only a tentative classification, on purely morphological grounds. It is therefore open to modification. For some of the varieties it was difficult to find the appropriate place in this sub-group system with certainty.

Notes on the sub-group arrangement.

More details can be given about the sub-groups listed in Table II, and a few examples are listed here.

IIb: Sylvia and Tadorna (Fig. 7) have very similar gliadin patterns.

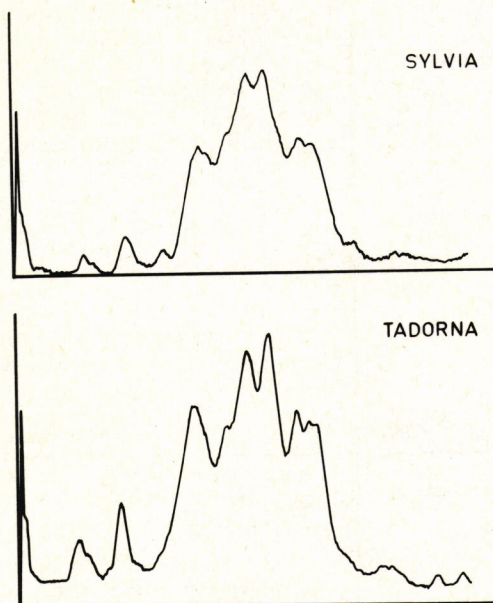


Fig. 7: S 3 densitograms of two IIb varieties.

These two varieties were selected from the descendants of the same cross, viz. (Chinese 165 x Panzer III) x Heine IV x (Teutonen x Hindukush 516) x Merlin.

IIc: Peko, Fasan and Perso (Fig. 8) also form a sub-group. They have the Peragis x Heines Kolben combination in common in their pedigree.

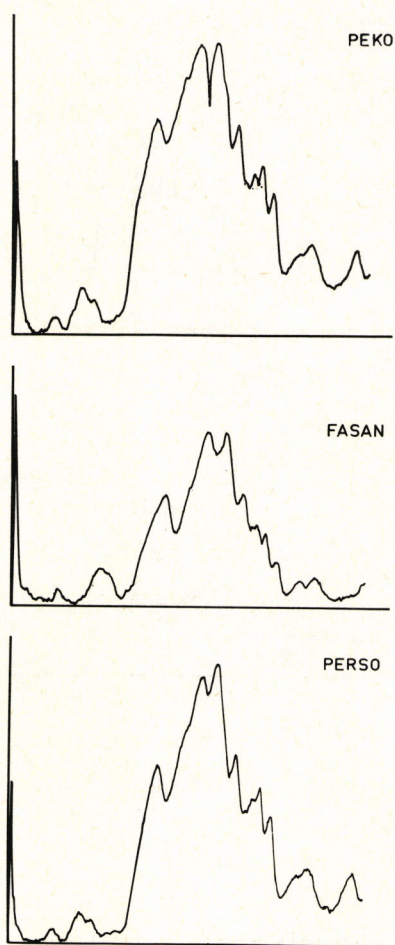


Fig. 8: S 3 densitograms of three IIC varieties.

Va: Thatcher and Saunders are North American varieties, General Roca comes from Argentina (Fig. 9).

Saunders and General Roca are direct descendants of Thatcher.

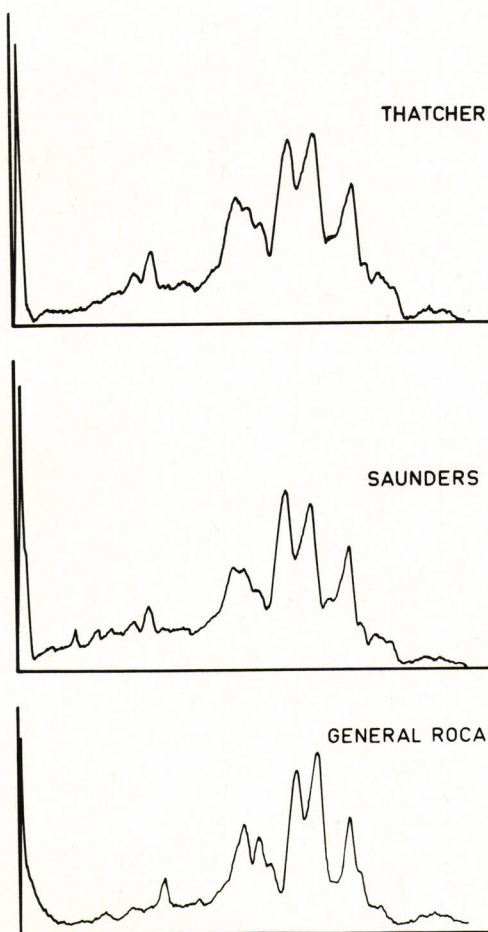


Fig. 9: S 3 densitograms of three Va varieties.

These three examples indicate that the sub-groups are based on genetic relationship.

Vc: Another interesting example is shown in Fig. 10.

According to the official American list of varieties Lee would be Hope x Timstein. Hope is a cross between *Triticum timopheevi* (from Eurasia) and

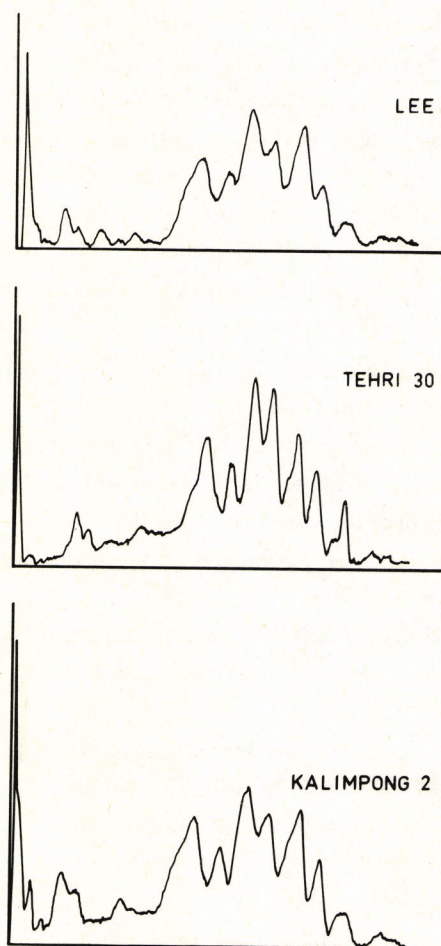


Fig. 10: S 3 densitograms of three Vc varieties.

Marquis, which in turn is a combination of Red Fife (Russia) and Hard Red Calcutta (India). Timstein is a cross between *T. timopheevi* and Steinwedel (Australia), which is a combination of Harvest Queen (North America) and Goldendrop (England). There are indications, however, that the official description of Lee is not correct and that its descent is not known with certainty,

A genetic relationship can be surmised between Tehri 30 and Kalimpong 2, both selected from Indian land populations, although the information

available does not include a description of their descent.

The gliadin pattern of Lee corresponds to that of Tehri 30 and Kalimpong 2, which suggests a genetic relationship of Lee with Indian varieties.

The relevant literature has disclosed hardly any information regarding the grouping of wheat varieties on the basis of electropherograms. Graham (1963), when examining 7 Australian varieties, found varietal differences between the slow-moving fractions of acetic acid extracts, and similarity of protein patterns between three genetically related varieties.

Closer characterization of wheat varieties by reference to the pattern of the albumins/globulins.

The albumins/globulins likewise reveal differences in variety through the electropherograms, although these differences are less marked than those shown by the gliadins. It was therefore also attempted to group the varieties according to corresponding features in the S 1 patterns. In general, however, this produced groups which could not be directly related to a genetic pattern. The provisional conclusion was therefore that there was little purpose in pursuing this approach.

On the other hand, the differences between the S 1 patterns of members of the sub-groups could be used for distinguishing between varieties (Fig. 11).

The Thatcher, Saunders and General Roca varieties are placed in main group V, sub-group a. Their gliadin patterns are identical, but they all have their individual characteristics in the albumin/globulin fractions, as indicated by the arrows.

It is suggested that a wheat variety can be identified through protein extraction of a sample of grains, and electrophoresis. The theoretical possibility has already been discussed by Coulson & Sim (1964). The procedure could be as follows: place the densitogram of an S 3 fraction in one of the five main groups and then determine the appropriate sub-group. This greatly reduces the range from which to select the variety. The final definition of the variety can then be made by reference to the albumin/globulin pattern.

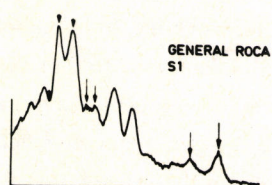
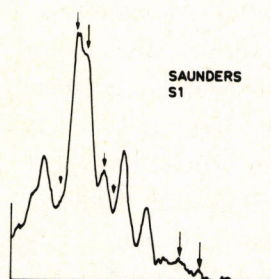
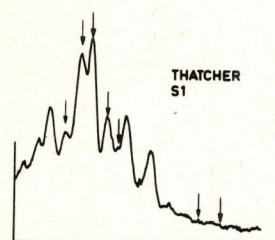


Fig. 11: S 1 densitograms of the wheat varieties of Fig. 9 for determination by combination of S 3 and S 1 densitograms.

The arrows indicate the differences between the members of the group.

PROVO

Let us digress for a moment to consider a practical aspect.

From a field of beardless wheat of the Felix variety adjoining a field of barley, a farmer had selected a few lightly bearded, long ears as he suspected there had been crossings of wheat and barley. He mixed the grains with barley grains, sowed the mixture and, after further selection and breeding, obtained a pure line of bearded wheat. He sent us a sample for further investigation. For the time being, the variety was named "PROVO", after the bearded group whose deliberate aim was to provoke action.

As a fertile cross between wheat and barley has never been realised even by experiment, the spontaneous occurrence of such a phenomenon must be considered very doubtful. Electrophoresis of an aqueous extract of the sample yielded a protein pattern virtually identical to that of Felix. There was no trace whatsoever of any barley protein, which could have become evident from components with characteristic R_v values and concentrations.

We were thus able to inform the farmer that PROVO was decidedly nothing but a selection from the Felix wheat variety.

VII. COMPARISON OF TRITICUM SPECIES

Introduction

The previous section gave an affirmative answer to the question whether electrophoretic analysis could provide information regarding relationships between *Triticum aestivum* (bread wheat) varieties. This section will enlarge upon the question whether electrophoresis will give an insight into the relationships between *Triticum* species. These include species which were formerly referred to as *Aegilops* but which Bowden (1959), followed by Morris & Sears (1967), classified under the genus *Triticum*.

T. aestivum is a hexaploid whose cell nucleus contains three different genomes. In addition, there are a number of other hexaploid, tetraploid and diploid species which all belong to the genus *Triticum*, by virtue of their morphology.

What is the genetic relationship between the hexaploid bread wheat and the other *Triticum* species and particularly, how has wheat evolved?

There have been various approaches to this problem. Especially cytogenetic research yielded important discoveries, expertly summarized by Morris and Sears (1967). The geographical distribution of the *Triticum* species likewise led to certain clues. The relevant investigations referred mainly to Vavilov's theory of the gene centres (Vavilov, 1926). Attempts were also made to analyse the endosperm protein of the *Triticum* species by electrophoresis as this protein at least partly reflects the expression of the genomes.

The following will briefly outline the developments which have resulted in our current knowledge of the subject.

Cytogenetic investigations

In 1918 Sakamura found that the wheat types einkorn (*T. boeoticum* and

T. monococcum), emmer (*T. dicoccoides*, *T. dicoccum* and others), and dinkel (*T. spelta*, *T. aestivum* and a few others) were diploid ($2n = 14$), tetraploid ($2n = 28$) and hexaploid ($2n = 42$) respectively. Kihara (1924) and Sax (1924) carried out crossing tests between the wheats and discovered simultaneously that allopolyploidy occurred in this series; the three types were given the genome formulae AA (einkorn), AABB (emmer) and AABBDD (dinkel). In other words, the einkorn genome A was assumed to be present in the emmer and dinkel wheats.

Subsequent investigations aimed at finding the diploid suppliers of the B and D genomes. The D genome was found first, viz. by Sax & Sax (1924) and by Gaines & Aase (1926), in the allotetraploid *T. cylindricum* (*Aegilops cylindrica*). Not until 1944 was the diploid carrier of the D genome: *T. tauschii* (*Ae. squarrosa*) found, viz. by Kihara and McFadden & Sears. This would therefore have to be the ancestor of both *T. cylindricum* and hexaploid wheats.

As to the B genome, Sarkar & Stebbins (1956) made morphological comparisons and indicated *T. (Ae.) speltoides* as the diploid donor. Cytological studies supported this (Riley et al., 1958): The B genome of the hexaploid contains two satellite chromosomes, and of the examined B donors only *T. speltoides* and *T. tripsacoides* (*Ae. mutica*) had two satellite chromosomes. All attempts at exact identification of the B genome of the hexaploids as originating from *T. speltoides* or *T. tripsacoides* have failed so far, however.

Also *T. bicornis* has been investigated as a possible B-donor (Kondo & Kamanoi, 1958, and other authors), because of the similarity of its genome with the B-genome of tetraploid wheat (AABB).

Studies of the geographical distribution

Alphonse de Candolle (1886) was one of the first who believed the area of origin of cultivated plants to be where the wild forms grow by preference. He was of the opinion that the possible origins of wheat were Palestine and Mesopotamia, and he called einkorn (*T. monococcum*) the oldest cultural form. Vavilov (1926) referred to gene centres, where the forms show the greatest variability, and whence the varieties spread and the diversity of forms gradually declines. Vavilov looked upon Anatolia as the primary origin of diploid wheats. Flaksberger (1939) argued that tetraploid and hexaploid wheats must have developed in western Asia. Gökgöl studied the entire Turkish wheat population and, working on the lines of Vavilov's theory, concluded that Anatolia had to be the area of origin of most *Triticum* species (Gökgöl, 1963).

It is furthermore remarkable that *T. tauschii* (*Ae. squarrosa*) and *T. cylindricum*,

carriers of the D genome, are found growing together only in the joint border area of Turkey, Transcaucasia, and Iran (Johnson, 1967) and that *T. (Ae.) speltoides* and *T. monococcum* (B? and A genomes respectively) grow mixed in great masses in Anatolia (Harlan & Zohary, 1966).

To these facts can be added the results of archeological investigations (Helbaek, 1959-1965): the oldest finds of wheat growing (tetraploid and hexaploid wheats) so far are North Iraq, North-West Iran and South-East Turkey. It can therefore be assumed that polyploid wheats likewise have their origin primarily in this area.

Electrophoresis of the endosperm proteins.

The first-mentioned methods of investigation use, for identification of genomes, the morphology of the wheat plants and the affinity of their chromosomes as estimated from the extent to which pairing of chromosomes occurs upon crossing. Neither method has led to exact identification of the donor of the B genome (Johnson, 1967), for

- (1) the morphology of the diploid, tetraploid and hexaploid wheats offers but little to go by, and
- (2) the chromosome affinity of the diploid A donor (*T. boeoticum*) and B donor (*T. speltoides*) with the tetraploid AABB (*T. dicoccum*) is incomplete.

This lack of correspondence has been ascribed to changes in the two genomes following after the amphiploidization (Kihara & Lilienfeld, 1932; Sarkar & Stebbins, 1956; Zohary & Feldman, 1962).

Electrophoresis is one more method of characterizing a genome. In this context the question is whether the electropherogram of an amphiploid does sum up the protein patterns of the ancestors. In other words: Does the pattern of each of the diploid genome donors show up in the resulting amphiploid?

The first attempt at studying this question covered wheat x rye hybrids or *Triticale*, viz. amphiploids of *T. durum* (AABB) or *T. aestivum* (AABBDD) and *Secale cereale* (RR). Moritz (1933), in pursuance of Kowarski's work (1901), found by immunological tests that the antigen composition of *Triticale* endosperm was the sum of the compositions of the two parents. Hall (1959) examined this by means of immuno-electrophoresis: all the demonstrable proteins of wheat were also present in *Triticale*, but some rye proteins were not found back. Using electrophoresis, Yong & Unrau (1964, 1966) showed that the protein pattern of *Triticale* was indeed a rough addition of the patterns of *T. durum* and *S. cereale*, but that new or hybrid proteins occurred as well. Amino acid analysis confirmed

these findings. The conclusion was that there had to be interactions between the genes, causing the protein synthesis in the hybrid to differ from that in the genome donors. On the other hand Hall & Johnson (1963) found the electropherogram of the amphiploid *Stiporyzopsis* to be made up of the complete patterns of *Stipa* and *Oryzopsis*.

As to the *Triticum* species, Johnson (1967), in identifying the parents of *T. cylindricum* with the aid of electrophoresis, found that addition of the protein patterns of *T. tauschii* (*Ae. squarrosa* DD) and *T. dichasians* (*Ae. caudata*, CC) yielded the pattern of *T. cylindricum* (CCDD).

The question whether within the genus *Triticum* the protein pattern of a diploid genome donor is clearly recognizable in the pattern of the resultant amphiploid has thus hardly been studied. This section will mainly refer to this question (see Results)

Material

We received approx. 150 samples of *Triticum* of the 1966, 1967, and 1968 crops, supplied by the Department of Field Crops and Grassland Husbandry and the Institute for Plant Breeding of the Agricultural College and also the Foundation for Plant Breeding (all at Wageningen).

The material contained about 30 diploid, about 90 tetraploid and about 30 hexaploid samples, and also a few artificial polyploids, i.e. products with special genome combinations, such as hexaploid with AAAABB.

Threshing

The seeds of many *Triticum* species are firmly enveloped by the glumes so that conventional threshing methods cannot be used. Isolating the seeds by hand was very time-consuming, and the following procedure was therefore developed:

Ears were briefly beaten in a Waring Blendor and the product was mechanically vibrated on a few slotted screens (0.75 x 20 mm and 2 x 20 mm). The fractions containing whole and broken grains were separated from the chaff on a slightly inclined, flat vibrating table with tracks formed by zigzagging metal walls (paddy machine, Kvarmaskiner AB, Malmö, Sweden). The coarsest fraction, if still containing packed grains, was again treated in the Waring Blendor. This method implied considerable speeding up the further work.

Extraction

It appeared to be virtually impracticable to prepare flour (endosperm) and S1 and S3 fractions as well from the samples, which were mostly small (see Section III). Attempts were therefore made to find a method of extracting the protein direct from ground grains (whole meal). First of all, this required enlarging the extraction yield, the reason being that at identical extraction intensities the protein yield in an aqueous extract of whole meal is smaller than in an aqueous flour extract, owing to the high amount of non-endosperm contained in the grains. Instead of the Waring Blendor (see Section III) a homogenizer according to Potter-Elvehjem (a rotary teflon piston in a cylinder of solidex glass) was used, which made it possible to obtain approximately 50 per cent more protein from an equal amount of flour than with the Waring Blendor. This is undoubtedly attributable to the more intense contact between the solvent and the strongly comminuted flour particles,

In spite of the increased protein yield the gliadin electropherograms were indistinct; this was caused by the fact that whole meal extracts contain several times more salts than flour extracts (see Section III: Extraction). But extraction of whole meal with 70-% alcohol produced electropherograms which were virtually identical to those of aqueous flour extracts. The albumin/globulin pattern was slightly changed, however (see Fig. 12).

The following extraction procedure was used.

1 part whole meal with 3 parts 70-% alcohol (W/V) are treated in the "Potter" for 3 minutes; the suspension is centrifuged 30 min. at 30,000 x g; the supernatant is heated 2 min. in boiling water and cooled rapidly; the liquid is poured into a petri dish and the alcohol evaporated over a water bath.

A yellowish gel consisting mainly of lipids is formed. The gel is gathered by stirring carefully and the colourless liquid further evaporated until there is a very small amount left. Excess water is added, which causes clouding and formation of a new gel, which is also removed. In order to evaporate the remaining alcohol, the solution is frozen at -20°C and evacuated in a freeze-drier for 30 min. The dish is removed, the irregular surface allowed to melt and the solution thus obtained is freeze-dried. The intermediate phase of 30 min. is to prevent the occurrence of puffs, which would blow off protein. The dry substance is further processed as described in Section III.

Advantages of alcohol extraction of whole meal over aqueous flour extraction:

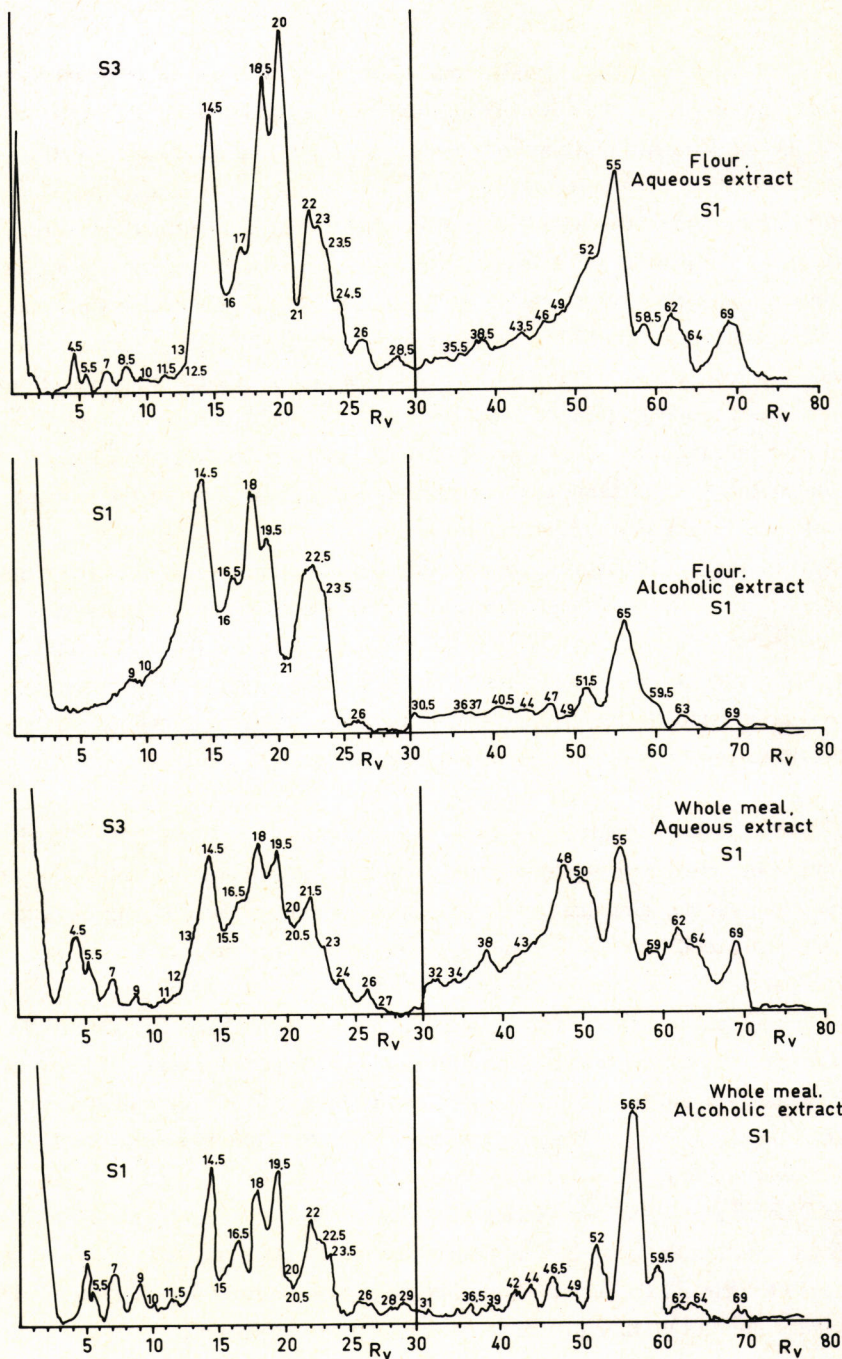


Fig. 12: Close resemblance in the gliadin compositions of aqueous extract from flour and alcoholic extract from whole meal (Opal variety).

1. The samples to be examined can be smaller, partly because the Potter is used, e.g. 3 g against 25 g in the Waring Blender.
2. According to the electropherograms a single extraction with 70-% alcohol (S1) produces sufficient protein to use the extract for both electrophoresis of albumins/globulins (1.20 h) and gliadins (2.40 h).

Electrophoresis

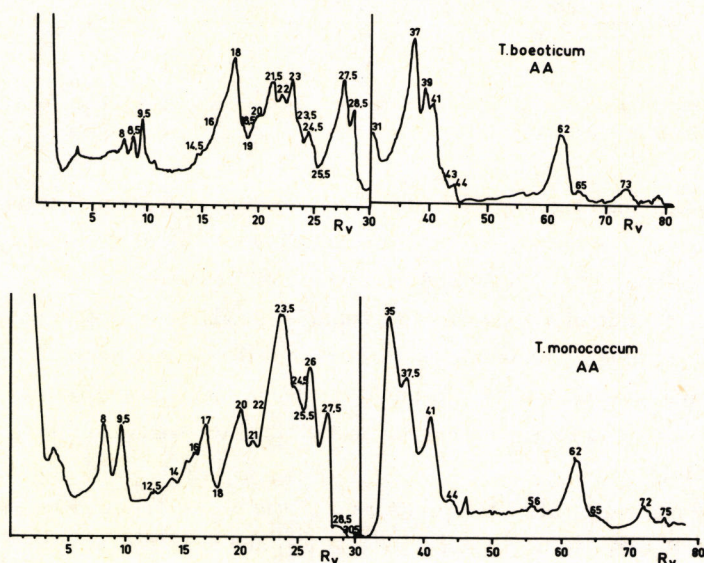
The material was processed as described. The densitograms were given R_V values (see Section IV) to facilitate comparison of the samples.

Results and discussion

We were of the opinion that the material was sufficiently representative for the species under study to attempt to answer the question whether the protein pattern of a diploid genome donor can be traced in the resulting amphiploid.

A. Diploids

Fig. 13 shows densitograms of some diploid *Triticum* species discussed in this section, viz. *T. boeoticum*, *T. monococcum*, *T. speltoides*, and two types of *T. tauschii*, formerly referred to as *Aegilops squarrosa* ssp. *eusquarrosa* (type 1) and ssp. *strangulata* (type 2).



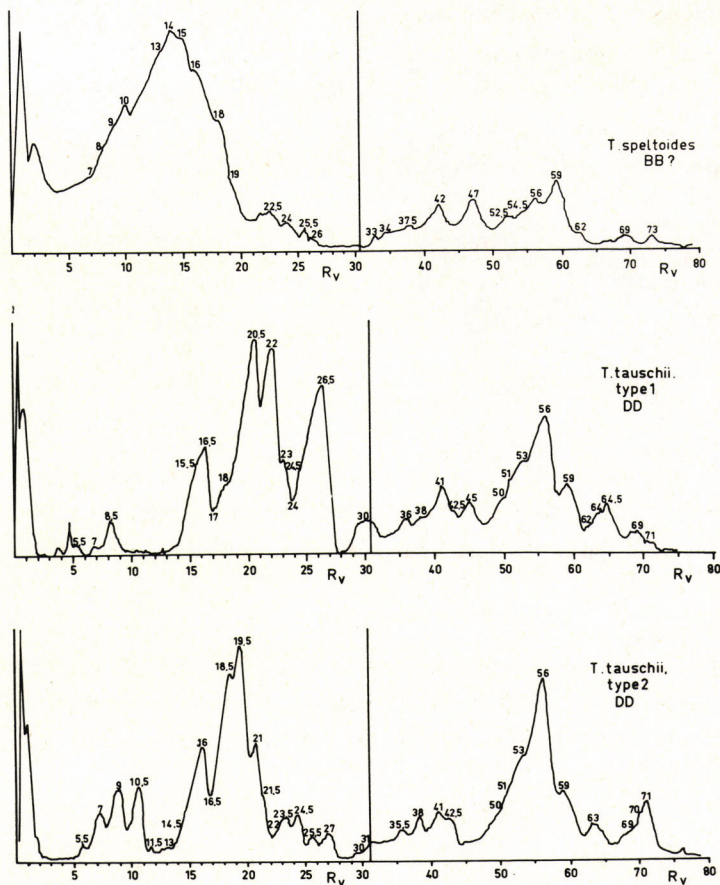


Fig. 13: Densitograms of some diploid *Triticum* species probably involved in the development of the *Triticum* amphiploids.

Comparing densitograms of *T. boeoticum* (the wild carrier of the A-genome) and of *T. monococcum* (einkorn) (probably raised from *T. boeoticum* through ancient breeding) did not reveal characteristic differences.

The sample of *T. speltoides* (B-genome?) produced a protein pattern that was indistinct in the gliadin region. Efforts to improve this by purifying the flour through sieving, and by repeated extraction, were unsuccessful. As a consequence we had to content ourselves with the densitogram of the short run (1 h. 20 min.).

We had material of two varieties of *T. tauschii* (= *Ae. squarrosa*, ssp. *eusquarrosa*, D-genome) type 1, viz. var. *Meyeri* and var. *typica*. Both varieties

had virtually the same albumin/globulin pattern, but a different gliadin composition.

Fig. 13 shows that diploid *Triticum* species differ throughout their protein patterns. The above observation suggests that within a diploid species the albumin/globulin composition is rather constant, while the gliadin composition is variable. Likewise, it was shown in Section VI that within one hexaploid species, *T. aestivum*, the albumin/globulin patterns are essentially identical, whereas the gliadin composition varies noticeably.

These facts justify the assumption that *Triticum* species are characterized by their albumin/globulin compositions and that the gliadin pattern provides only supplementary information (viz. regarding varieties within a species). Similar suggestions are made in literature, as by Feillet & Bourdet (1964).

As far as in the following discussions comparisons concern the species, we shall, for the reason explained above, omit the 2h.40 min. pattern (the extended gliadin pattern).

B. Tetraploids

We have tried to use variations in gliadin patterns to distinguish varieties or groups of varieties within each species. To facilitate the survey we shall confine ourselves to reporting the results; only a few densitograms will be shown.

We found that a number of tetraploid species had roughly the same albumin/globulin constitution, viz. *T. dicoccum*, *T. dicoccoides*, *T. durum*, *T. turgidum*, and *T. polonicum*. As an example representing this group we show a densitogram of *T. dicoccum* (AABB), Fig. 14.

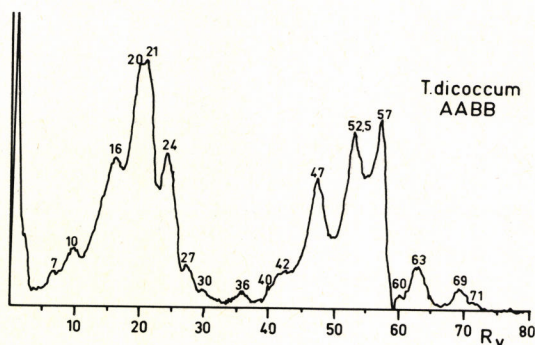
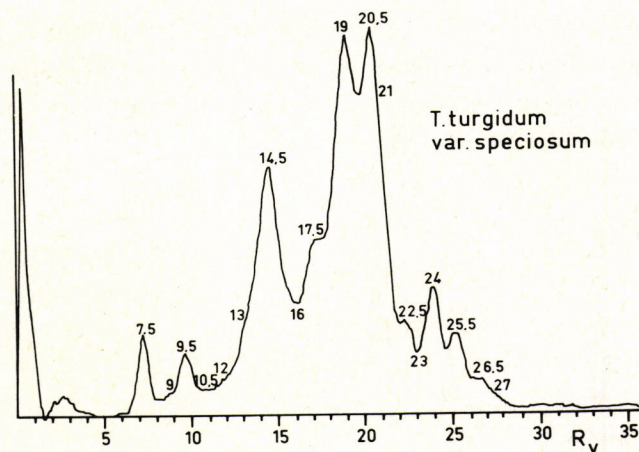


Fig. 14: Densitogram of *T. dicoccum* representative for a number of other tetraploid species.

In connection with the argument made under A a question now arises. If the albumin/globulin pattern is really characteristic for a species, are we then to distinguish five species here? Crossing experiments and cytologic examinations have gradually induced the belief that in these cases subspecies or mutants are involved (Morris & Sears, 1967). The morphologic differences that have formerly led to classify these species are apparently not reflected in the albumin/globulin constitution of the endosperm.

We have, nevertheless, been able to make a subdivision for each species on the base of the albumin/globulin constitution. In the *dicoccum* varieties two groups could be made; within each group the varieties could be recognized by details of their albumin/globulin patterns. In *T. dicoccoides* we were able to distinguish the four available varieties by their gliadin patterns. In *T. turgidum* five different gliadin patterns were found, one of which deviated considerably from the others. Fig. 15a shows the gliadin pattern of a *turgidum* variety chosen at random, viz. *speciosum*. Fig. 15b represents the gliadin pattern of the deviating variety *mirabile*. The name *mirabile* is based on the fact that this variety has branched ears. The albumin/globulin constitution of *mirabile* did not show remarkable differences with that of the other varieties.



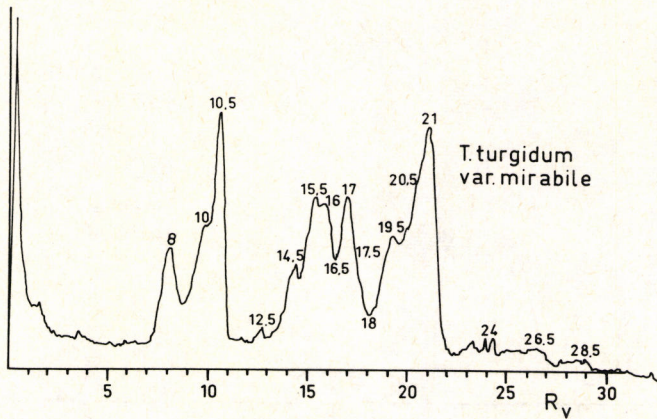


Fig. 15: a. Gliadin densitogram of a *T. turgidum* variety, *speciosum*.
 b. Gliadin densitogram of *T. turgidum* var. *mirabile*.

Varieties of *T. durum* could be classified in three groups, those of *T. polonicum* also. In both groups albumin/globulin patterns could be used to characterize varieties within each group.

C. Hexaploids

The hexaploid wheats of which we got samples, were *T. aestivum*, *T. spelta*, *T. compactum*, *T. macha*, and *T. sphaerococcum*. The firstnamed three species proved to have roughly the same albumin/globulin pattern. As an example we may therefore take *T. aestivum* (Fig. 12). In Section VI gliadin patterns of many varieties of *T. aestivum* were compared among themselves.

In *T. spelta* varieties the gliadin densitograms were so much like one another that no justified subclassification could be made. These varieties were probably closely related. Of *T. compactum* only two samples were available; they were different in their gliadin patterns.

Densitograms of *T. macha* and *T. sphaerococcum* are shown in Fig. 16a, b, as these species had an albumin/globulin constitution different from that of the firstnamed three species. Of *T. sphaerococcum* three samples were available; each of them had a characteristic gliadin pattern.

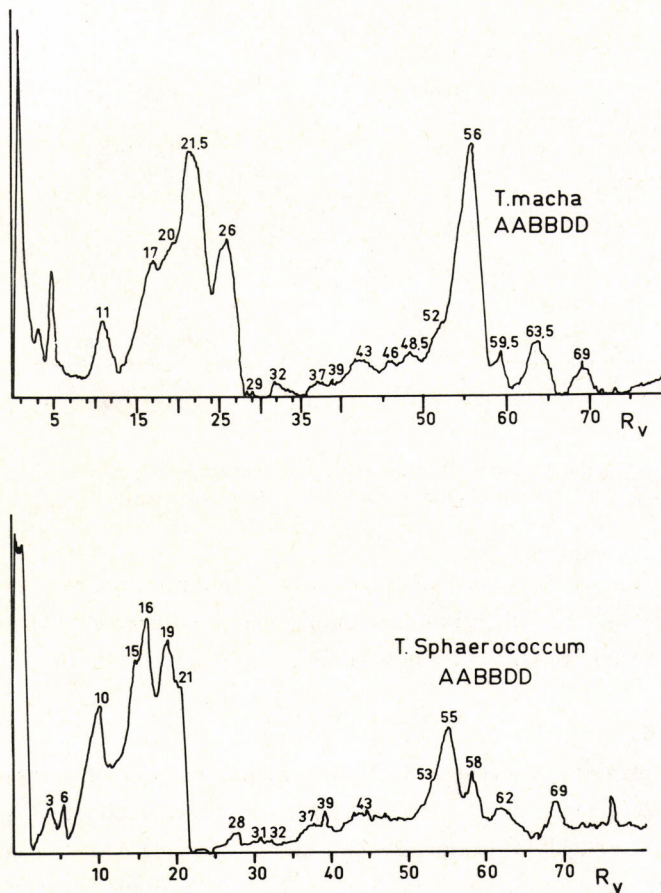


Fig. 16: Densitograms of *T. macha* (a) and *T. sphaerococcum* (b).

In the cases discussed here we had only a small number of varieties of each species available. It would be worthwhile to establish if by extending the number of varieties of each species similar systems as displayed in Section VI can be arranged.

D. Comparison of diploids with tetraploids

For the comparison of diploids with tetraploids we refer to Fig. 13 and Fig. 14. The latter shows a densitogram of *T. dicoccum* (AABB); this species may be considered to be representative of most tetraploids.

The protein pattern of *T. dicoccum* comprises albumin/globulin components

at R_V 36, 42, 47, 52 or 53, 57, 63 and 69; the highest gliadin concentration is found between R_V 19 and 23.

There is no evidence of the A-genome (*T. boeoticum* or *T. monococcum*, Fig. 13) except for a peak at R_V 62 or 63. This could equally well originate from *T. speltoides* or *T. tauschii*, type 2.

The pattern of *T. speltoides* (B-genome?) has 6 components in common with that of *T. dicoccum*, viz. R_V 42, 47, 52 or 53, 63 and 69, and there is also a certain amount of similarity in the concentration ratios. However, *T. speltoides* has R_V 38 and 59, which are not found in *T. dicoccum*.

T. tauschii (D-genome) also has components in common with *T. dicoccum*, viz. R_V 36, 41 ?, 52 or 53, 63, 69 and 71, whose concentration ratios differ strongly, however. Like *T. speltoides*, *T. tauschii* has R_V 38 and 59, but these are not found in *T. dicoccum*. *T. tauschii* also lacks the striking *speltoides* component R_V 47.

CONCLUSIONS

In those tetraploids that may be represented by a densitogram of *T. dicoccum* there is no trace of *monococcum*-proteins. Two possible explanations may be given: either

the A-genome of *T. dicoccum* does not originate from *T. boeoticum* or *T. monococcum*, or

it does originate therefrom, but it is not recognized as such owing to radical changes that have taken place after the amphiploidization.

Similarities in concentration ratios seem to indicate that *T. speltoides* or a closely related species is incorporated in these tetraploids. The gliadin pattern differs, though.

In view of differences in concentration ratios and the absence of R_V 47 the presence of the D-genome seems unlikely.

E. Comparison of diploids with hexaploids

For the comparison of diploids with hexaploids we refer to Fig. 13 and Fig. 12. The latter shows a densitogram of *T. aestivum* (AABBDD), Opal variety. This densitogram represents most of the hexaploid wheats, including *T. spelta* and *T. compactum*.

T. boeoticum and *T. monococcum* (A-genome) possess gliadins that are also

found in *T. aestivum*, but in different proportions. For, a striking protein concentration occurs at R_V 23 to 27, whereas *T. aestivum*, *T. spelta* and *T. compactum*, like the tetraploids, display their highest concentration at R_V 19 to 23.

The albumin/ globulin pattern is poor in components, compared with that of *T. aestivum*. Its characteristic features are the high protein concentrations at R_V 35 to 41, and components at R_V 62 and 73.

The gliadin pattern of *T. speltoides* (B-genome) resembles that of *T. aestivum* even less than those of *T. boeoticum* and *T. monococcum* (A-genome). The highest protein concentrations are found between R_V 13 and 17.

The albumin/globulin pattern is characterized by peaks having R_V 35 to 41 and 72 or 73 (which are also found, though in different concentrations, in the A-genome), and by peaks with R_V 42, 47, 56 and 59 (which do not occur in the A-genome). The latter four peaks are also found, though in different concentrations, in *T. aestivum*, where also a gradual increase of concentration from R_V 30 towards 40 is observed.

T. tauschii (D-genome) very much resembles *T. aestivum* as to its protein composition. For, most *aestivum* components are represented, and both in type 1 and in type 2 the highest gliadin peaks are found at R_V 19 to 23. Small deviations from the *aestivum* configuration occur between R_V 23 and 27. In this respect type 1 resembles bread wheat more than does type 2.

The albumin/globulin pattern lacks the *aestivum* component R_V 47, but it has R_V 41 which is not found in *T. aestivum*. Components R_V 42 or 42^5 , 56 and 59, observed both in *T. tauschii* and *T. aestivum*, are also found in *T. speltoides*. The latter is, therefore, only characterized by R_V 47. The pattern of type 2 furthermore displays a protein concentration at R_V 70 and 71 which is not found in type 1 and in *T. aestivum*.

CONCLUSIONS

The protein pattern of *T. boeoticum* or *T. monococcum* (AA) was not found back in *T. aestivum*. This may be attributed to two causes, similar to the case of *T. dicoccum*: either

the A-genome of *T. aestivum* does not originate from this diploid, or it does originate therefrom, but owing to some cause or other it has become unrecognizable.

Of the albumins/globulins of *T. speltoides* (B-genome) we found component R_v 47 back in *T. aestivum*, as well as a gradual concentration increase from R_v 30 towards 40. The latter feature may, however, also be attributed to the influence of the D-genome. The lack of conformity between the concentration ratios as they are found in *T. speltoides* and in *T. aestivum* is responsible for the fact that we are still uncertain about the origin of the B-genome.

The D-genome of *T. tauschii* (= *Ae. squarrosa*) is undoubtedly present in *T. aestivum* and apparently dominates the protein synthesis. The resemblance with *T. aestivum* was greatest in type 1 (formerly ssp. *eusquarrosa*), somewhat smaller in type 2 (formerly ssp. *stragulata*).

Partly owing to the dominant effect of the D-protein on the protein pattern, it was not possible to establish by electrophoresis whether the A-genome A-genome does in fact originate from *T. boeoticum* and the B-genome from *T. speltoides*.

T. macha and *T. sphaerococcum* display also the pattern of the D-genome, but it seems to have been slightly altered.

F. Artificial polyploids

1. Tetraploid *T. monococcum* (AAAA)

The protein composition of this autotetraploid (created by colchicine treatment) appears to be identical with that of the diploid basic material AA (cf. Fig. 13).

There is no question of any increase of protein concentrations.

2. *T. dicoccum* + *monococcum* (AAAABB)

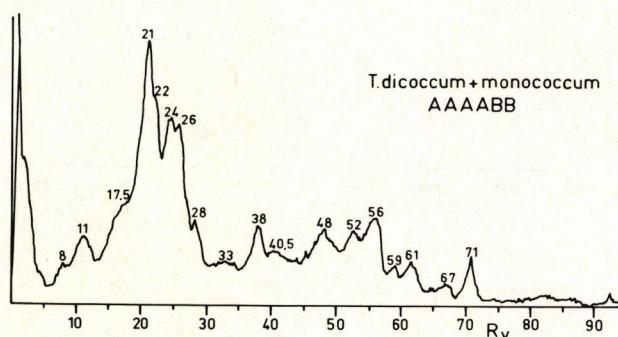
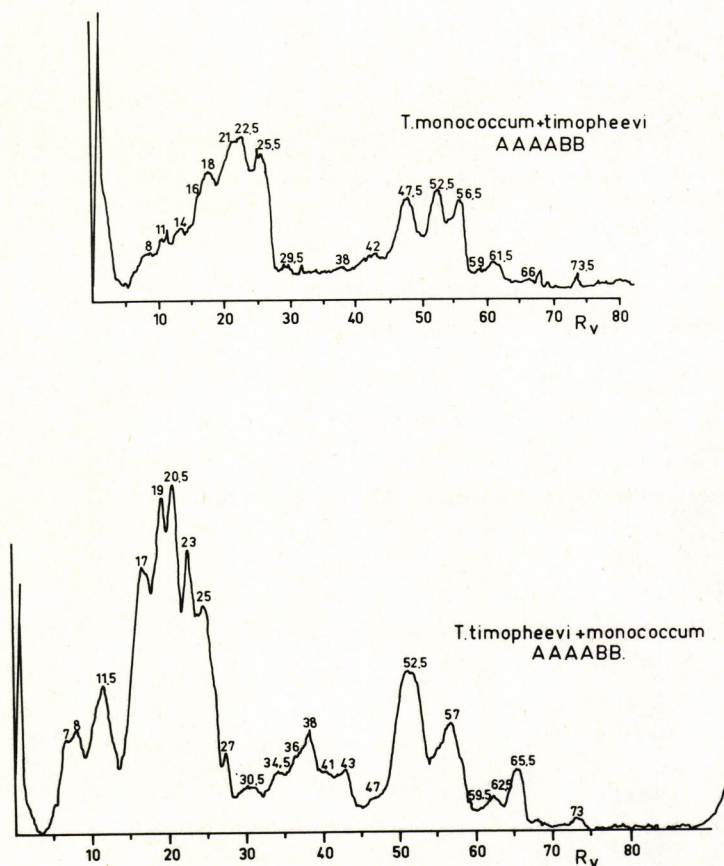


Fig. 17: Densitogram of the artificial polyploid *T. dicoccum* + *monococcum* showing *monococcum* proteins at R_v 38 to 41.

This protein pattern is compared with the densitograms of the parent types (Figs. 13 and 14). The presence of *monococcum* genome A, which was not demonstrable in *T. dicoccum*, becomes apparent in this polyploid at R_v 38 to 41. This is the first evidence of the *monococcum* genome being recognizable in a polyploid. The pattern of the other parent, *T. dicoccum*, remained largely unaltered.

3. *T. monococcum* + *timopheevi* (AAAABB)



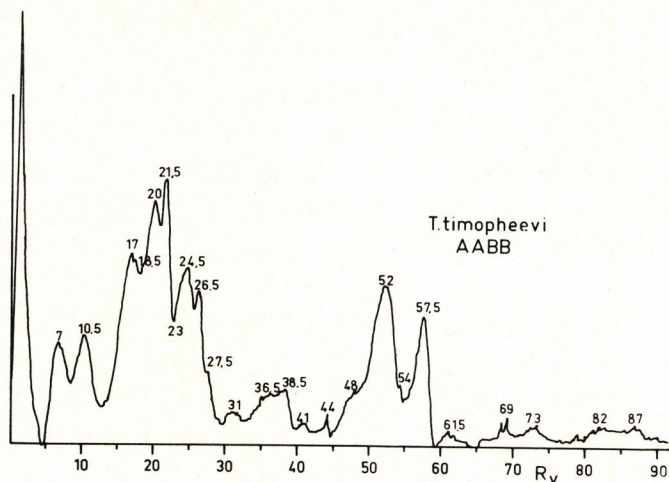


Fig. 18: Densitograms of the artificial polyploids *T. monococcum* + *timopheevi* (a), the reciprocal polyploid *T. timopheevi* + *monococcum* (b), and *T. timopheevi* itself (c). In a the pattern of most tetraploid wheats is recognized, b shows the *timopheevi* pattern, in c the *monococcum* proteins are indicated at R_V 36⁵, 38⁵, 41, 61⁵, and 73.

The densitogram of the aphiploid is compared with that of *T. monococcum* (Fig. 13b) and that of *T. timopheevi* (Fig. 18c).

First of all, however, we must discuss *T. timopheevi* itself: its pattern differs clearly from the tetraploids represented by *T. dicoccum* (cf. Fig. 14). *T. timopheevi* is endemic only in Georgia, S.W. Caucasus; there is no certainty at all about its origin. We do know, however, that in addition to the A-genome there is another in *T. timopheevi* which slightly resembles the B-genome. It is referred to as B, but also as β or G (Isenbeck & von Rosenstiel, 1950). On cytogenetic grounds the formula AABBB for *T. timopheevi* is accepted, though (Wagenaar, 1961).

In *T. timopheevi* the presence of the *monococcum* genome A is perceptible at R_V 37, 39, 41, 62 and 73 (cf. Fig. 13). The expression of the A-genome is reduced, however. There was no trace of the A-genome in the *dicoccum* tetraploids.

What other diploid contributes to the *timopheevi* pattern is not known; the component R_V 47 which is characteristic of *T. speltoides* (Fig. 13) is but an inconspicuous detail in the densitogram.

The amphiploid *T. monococcum* + *timopheevi* (AAAABBB, see Fig. 18a) has a remarkable feature: the A-genome, supplied by both parent types, should be

clearly visible. But the range from R_V 35 to 41 is virtually empty, and the amphiploid displays the same pattern as found in the *dicoccum* tetraploids (Fig. 14). This points to a strong relationship between *T. timopheevi* and the *dicoccum* group.

The reciprocal amphiploid *T. timopheevi* + *monococcum* (AAAABB) (see Fig. 18b) has largely retained the pattern of *T. timopheevi*, only R_V 69 is absent. R_V 63 apparently originates from *T. monococcum*; R_V 66 is found in neither parent.

Also in this amphiploid the A-genome is recognizable, but in spite of the fact that it is present in fourfold, its components do not occur in concentrations higher than they are found in *T. timopheevi*. This phenomenon is in keeping with what we observed in the autotetraploid *T. monococcum*: doubling the A-genome seems not to lead to an intensified expression, as far as reflected by the densitogram.

CONCLUSIONS

The A-genome was found to be present in the artificial hexaploids *T. dicoccum* + *monococcum* and *T. timopheevi* + *monococcum* (AAAABB), as well as in the tetraploid *T. timopheevi* (AABB).

In the reciprocal amphiploid *T. monococcum* + *timopheevi* no trace of the A-genome was observed, nor of the *timopheevi* pattern. The close resemblance of the protein compositions of this amphiploid and *T. dicoccum* points to a close relationship between *T. timopheevi* and the *dicoccum* tetraploid. This suggests that *T. timopheevi* was involved in the development of those tetraploids.

The difference in the densitograms of the two reciprocal amphiploids justifies the assumption that the ovule cytoplasm influences the endosperm protein composition. As we had no material available of other reciprocal amphiploids like *T. monococcum* + *dicoccum*, we were unable to furnish additional arguments for this assumption.

Final Remarks

The results of these discussions may be summarized as follows.

Investigations into the recognizability of the *boeoticum* or the *monococcum* genome disclosed contradictory phenomena: *T. boeoticum* or *T. monococcum* protein was found neither in the albumin/globulin pattern of *T. aestivum*, nor in

that of the so-called "*dicoccum* tetraploids", nor in that of the amphiploid *T. monococcum* + *timopheevi*. *Boeoticum* or *monococcum* components were unmistakably located in the patterns of the amphiploids *T. dicoccum* + *monococcum*, *T. timopheevi* + *monococcum*, and in the pattern of *T. timopheevi* itself.

For the absence of *monococcum* protein two explanations are offered: The A-genome of *T. aestivum* or *T. dicoccum* is not derived from *T. boeoticum* or *T. monococcum*, or it does originate therefrom, but some cause or other has changed the A-genome after the amphiploidization to such an extent that it is no more recognizable in the densitograms of the above named amphiploids.

As to the latter possibility it is remarkable that the A-genome is not found in *T. dicoccum* and other members of its group, but that it is present in the amphiploid *T. dicoccum* + *monococcum*. A further question is why in one case the A-genome should have been altered, while in the other it was not. On the ground of these considerations we are inclined to think the first explanation the most satisfactory.

Similarities in concentration ratios of the albumin/globulin patterns of *T. speltoides* (B-genome) and *T. dicoccum* led to the assumption that the B-genome of the *dicoccum* tetraploids originates from *T. speltoides* (or a closely related species) though its gliadin composition does not suggest so. In the hexaploids (with the exception of *T. macha* and *T. sphaerococcum*) only one component of the *speltoides* protein was found back, viz. R_V 47, which seems to be characteristic for this diploid.

The D-genome of *T. tauschii* finds undoubtedly expression in *T. aestivum*, *T. spelta* and *T. compactum*; it dominates protein synthesis in these species. Among the two types of *T. tauschii* that we were able to examine, the similarity to *T. aestivum* was most pronounced in type 1, formerly referred to as *Aegilops squarrosa* ssp. *eusquarrosa*.

VIII. SUMMARY AND DISCUSSION

This thesis deals with the analysis of wheat endosperm proteins by means of starch-gel electrophoresis.

In the introduction it is explained why one is interested in the investigation of wheat endosperm proteins. Three questions are raised:

- Is the protein composition of the endosperm in bread wheat (*Triticum aestivum*) affected by differences in agricultural and/or climatological conditions during growth?

- Is it possible to establish, by means of electrophoretic analysis, relationships between the various species of the genus *Triticum* or between varieties or groups of varieties within one species?

- Does electrophoresis provide information about the background of the concept baking quality?

In the literature survey, Section II, the various electrophoresis techniques that have been employed for the examination of wheat proteins are discussed. Emphasis is laid on the potential value of starch-gel electrophoresis for that purpose.

Section III is devoted to a description of the techniques of protein extraction and starch-gel electrophoresis as applied by us, and to a survey of the reproducibility of the method. Results proved to be reliable.

Section IV describes a simple method to identify the protein components separated by electrophoresis. In the light of this method new definitions are suggested of the wheat endosperm protein fractions formerly described by Osborne (1893).

Section V deals with the first of the above named questions. It is shown in *T. aestivum* that the endosperm protein composition, as far as it is reflected in the electrophoretic pattern, is not affected by variations in agricultural or climatological conditions during growth.

Differences between *T. aestivum* varieties were most pronounced in the composition of the gliadin fractions. This is reported in detail in Section VI. Gliadin densitograms of 80 varieties and selections from various regions of the world could be classified in five main groups forming a morphological series. Main group I comprises varieties having a compact gliadin pattern. Main group V includes varieties having spread out patterns. Main groups II, III, IV show successive intermediate patterns. Each main group comprises sub-groups of varieties having identical gliadin patterns. As far as we were able to ascertain from lists of varieties, the sub-groups are formed by genetically closely related varieties. In each sub-group the varieties could be individually recognized by details of their albumin/globulin patterns. This means a partial answer to the second question formulated in the introduction.

It appeared also that this classification is roughly correlated with technological properties (including baking quality) of the varieties. Main group I comprises soft Western European wheats of poor baking quality. Main groups II, III, IV include varieties from a diversity of origins, and considerably varying in kernel hardness and baking quality. Main group V comprises largely hard non-European wheats having excellent baking quality.

It should be recommended to classify a greatest possible number of *T. aestivum* varieties, which the breeders have available, according to the above system. This will make it possible to read from the densitograms data that may help selecting the parents for breeding new varieties.

Under the title "Provo" an example is demonstrated of the practical applicability of electrophoresis for recognizing a wheat variety by its gliadins.

In Section VII different species of *Triticum* are compared. Technical problems with "threshing" the kernels (which are, especially in diploid species, very tightly enveloped by the glumes) and with extracting the protein from small wheat samples, could be solved satisfactorily.

A literature survey is given as an introduction to this section. The theory of the origin of the *Triticum* amphiploids comes to the following: either

the diploid *T. boeoticum* or the diploid *T. monococcum* (both AA) must have been one of the parents of the oldest tetraploid, presumably *T. dicoccoides* (AABB). There is no complete certainty, yet, about the origin of the B-genome, but especially *T. speltoides* would be eligible as the diploid donor of the B-genome. The majority of the recent tetraploids would have originated from the oldest tetraploid, partly growing wild, partly cultivated. The first hexaploid, presumably *T. spelta* (AABBDD) would, on its turn, have originated from a tetraploid by incorporating the D-genome of the diploid *T. tauschii*; the other hexaploids would have developed from *T. spelta*.

A new question was raised: The proteins of diploid genome donors are they traceable in the resulting amphiploids?

It is explained that species differences are largely reflected in the albumin/globulin patterns, whereas gliadin patterns provide only supplementary information about varieties within a species. Along this line of comparison we found the following relations.

T. speltoides may very well have been the donor of the B-genome in most tetraploids (AABB). In *T. aestivum* (AABBDD) there is only one albumin component to indicate the presence of the *speltoides* genome B. This may be accounted for by masking through proteins from *T. tauschii* (DD), which species was found to be much in evidence in the pattern of *T. aestivum*. Of the A-genome, which is assumed to be derived from *T. boeoticum* or *T. monococcum* (AA), nothing could be traced in most of the tetraploids, nor in the hexaploids. Only in the tetraploid *T. timopheevi* (AABB) the protein of the diploid was observed.

We have further looked for the recognizability of the A-genome by studying some artificial polyploids, viz. the hexaploids *T. dicoccum* + *monococcum* and *T. timopheevi* + *monococcum* (both AAAABB). In these hexaploids, just as in *T. timopheevi* (AABB), the protein of the *monococcum* genome A was found. This was not the case in the reciprocal amphiploid *T. monococcum* + *timopheevi* (also AAAABB), nor could the presence of the *T. dicoccum* complex be detected. We found, however, in this reciprocal amphiploid the albumin/globulin pattern of the tetraploid *T. dicoccum*. In a summarizing paragraph we concluded that the presence of the *monococcum* genome in tetraploids like *T. dicoccum* and in hexaploids is doubtful.

In those tetraploids and hexaploids of which we had several samples of each species, we found that the varieties of each species are characterized by their gliadin pattern and their albumin/globulin pattern together. This situation is analogous to that in Section VI for *aestivum* varieties. Classifications of each

of the species according to their gliadin composition as described in Section VI, will still have to be made with the aid of electrophoretic patterns of many more varieties. This will probably provide new information regarding the genetic relationships between the varieties within the *Triticum* species and on the origin of the valuable characteristics of wheat, including its baking quality.

Among the tetraploids similar albumin/globulin compositions were found in *T. dicoccum*, *T. dicoccoides*, *T. durum*, *T. turgidum*, and *T. polonicum*. Among the hexaploids the same situation is found in *T. aestivum*, *T. spelta*, and *T. compactum*. The tetraploid *T. timopheevi* showed a characteristic albumin/globulin pattern, just as the hexaploids *T. macha* and *T. sphaerococcum*.

In Sections I and II reference was made to a similar study made by Johnson & Hall (1965 and later). These authors found *monococcum* proteins (A-genome) in tetraploids and hexaploids. The presence of the D-genome protein in the hexaploids was looked for by comparison of ABD with AB electropherograms; only a few components were found that might originate from the D-genome, however. In our study the genome of *T. tauschii* (DD) appeared to express itself very clearly in the hexaploids.

Johnson (1967) and Johnson et al. (1967) found in the tetraploids the albumin/globulin composition of a large group of species (AABB) to be similar to that of *T. dicoccoides*, which species seems to have been the forerunner of that group. We were able to confirm this.

We are of the opinion that comparing wheats by means of electrophoretic protein patterns will elucidate more on relationships and possible origins of the *Triticum* species, and especially so if artificial polyploids can more often be included in the study.

The results of Sections VI and VII provide an affirmative answer to our second question, viz. establishing relationships between *Triticum* species and between varieties within one species. (Part of this work has already been published. Doekes, 1968).

As to an answer to the third question we got from this study an indication in what direction the work will have to be continued. A relation appeared to exist between the gliadin composition of *aestivum* varieties and the technological properties (baking quality and other characteristics) of the grains (see Section VI). This is at present being investigated further.

By means of baking tests and chemical analysis of flour from diploid

and tetraploid species, it is being tried to establish which of the three genomes, A, B, or D, is responsible for the baking quality of *T. aestivum*, the "bread wheat".

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