Analysis of Wheat Flour Proteins by Polyacrylamide Gel Electrophoresis[1]

JOHN M. LAWRENCE[2], HEDWIG E. HERRICK[2], and D. R. GRANT[3],
Washington State University, Pullman, Washington

ABSTRACT

A method for estimating the actual amount of protein within each of various groups of wheat flour proteins of similar electrophoretic mobility has been described. The principal steps are 1) a single extraction of the flour with an aluminum lactate buffer of pH 3.2 containing 3M urea to solubilize as much as possible of the total protein; 2) addition of lysozyme as an internal standard; 3) electrophoresis at pH 3.2 in a 4% polyacrylamide gel containing 2.7M urea, which has been previously equilibrated with aluminum lactate by overnight electrophoresis; 4) dyeing the gels with 0.1% amido black dye in 7% acetic acid; 5) conversion of the color of the protein bands to graphical form by densitometry; 6) measurement of the peak areas by planimetry and conversion to actual concentration units through use of factors representing the dye-binding capacity of the various protein groupings. Some examples of results of application of the method to flours from several wheat varieties are given.

During the past decade our concept of the protein composition of wheat flour has been undergoing a rapid evolution. Modern separation methods have shown that wheat gliadin, albumin, and globulin as defined by Osborne (1) are not single entities, nor do they represent exclusive groupings of proteins separable by the solubility methods he described. Elton and Ewart (2) have reviewed much of this work up until the advent of gel-electrophoresis methods. The latter methods have

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[2]Department of Agricultural Chemistry, Washington State University, Pullman.

[3]Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

made it clear that wheat proteins consist of numerous components, many of them present in significant amounts (3 to 8).

Many of the differences in quality of wheat varieties for various food purposes have been suggested to be due to more or less subtle differences in the proportions of the individual proteins in the flour (9). The most successful method for picturing these proportions is gel electrophoresis. Usually starch gels have been used in conjunction with an aluminum lactate buffer at pH 3.2 and various concentrations of urea (3,7,8,10). Very good separations are obtained in this way, but starch gels do not lend themselves well to densitometry (11), so that patterns have been compared by inspection without an attempt to obtain really quantitative estimations of amounts. Polyacrylamide gel electrophoresis is capable of yielding equally good resolution and gels that are transparent. Lee (12) has used polyacrylamide gel electrophoresis for separation of the acetic acid-soluble proteins of flour at pH 8.6 in the presence of 2M urea, and has examined the gels by densitometry. This paper presents a method for quantitative estimation of wheat proteins based on electrophoresis in polyacrylamide gels with the use of the more familiar and effective pH 3.2 buffer conditions.

METHODS

Preparation of Sample

Flour is suspended in aluminum lactate buffer, pH 3.2, 0.087 ionic strength (13), containing 3M urea, with a buffer-to-flour ratio of 6:1 to 8:1 (ml./g.), using the higher ratio for flours of relatively high protein content. The suspension is stirred at a moderate rate in the cold overnight and is centrifuged at about 100,000 × g (average) for 1 hr. A clear to somewhat opalescent center layer is removed with a syringe and used for electrophoresis.

A solution of lysozyme (3x crystallized, Sigma Chemical Co.) in water containing 18 mg. per ml. (3.0 mg. N per ml.) is used as an internal standard. It is mixed with the flour extract in the proportions of 1 part in 20 before electrophoresis. Both the extract and the lysozyme solution should be frozen if stored for more than a few days.

Gel-Electrophoresis Procedure

The gel-electrophoresis apparatus previously described (14) is used. The gel is made by dissolving 20 g. of Cyanogum (E-C Apparatus Corp.) and 72 g. of urea in 400 ml. of water, filtering the solution, dissolving in it 0.6 g. ammonium persulfate and 0.6 ml. dimethylaminopropiononitrile, and immediately pouring the solution into the gel mold in its container. (The solution thus contains approximately 2.7M urea and 4% polyacrylamide.) After the gel is set, the gel mold is removed from the container, the top electrode chamber is put into place, and the assembly is put in position for electrophoresis with 0.05 ionic strength aluminum lactate buffer in the electrode chambers, and with the cathodic connection at the top. An over-all potential of 300 v. (about 6 v. per cm. across the gel itself at the start) is applied for about 18 hr. The samples (including internal standard) are measured into the gel slots, and the gel mold is again put in position for electrophoresis with fresh aluminum lactate buffer in the electrode chambers and the cathodic connection now at the bottom. Electrophoresis at 650 v. (about 18 v. per cm. across the gel) for 1.5 hr. spreads the pattern over the gel so that the "globulin" (2,15) doublet

band is approaching the end of the gel. The ends of the gels are trimmed in different patterns for identification purposes. The gels are then removed from the gel mold and placed in trays, about 42 by 10 by 5.5 cm., to be dyed. They are covered with a solution of 0.1% amido black in 7% acetic acid, and allowed to stand for 20 min. The dye is removed by suction, and the excess background dye is removed from the gels by washing with four or five changes of 7% acetic acid in the trays placed on a motor-driven, slowly rocking platform. Additional details on some of the foregoing procedural steps are given in the accompanying paper (14).

Estimation of Protein Concentration from Gel Bands

The dye absorption by the protein bands is measured by scanning the gel strips in a densitometer shown in Fig. 1. We have modified the Photovolt model 501A densitometer, A, by use of a simple Plexiglas carriage to move the gel strip under the scanner, B, at a rate of 2 cm. per min. by a rack-and-pinion gear, C, driven by a 1-r.p.m. motor, D. The gel itself fits into a well, E, in a center section, F, of the carriage (shown in separate detail), whose position can be adjusted by means of a variety of spacer blocks, G, so that the scan can be made either along the center of the gel or along either side. The bottom of the well is a glass plate cemented to the Plexiglas carriage by a silicone rubber sealant. A few drops of water or 7% acetic acid are placed on the glass, the gel is put in place, air bubbles are pushed out, a few drops more are placed on the top of the gel, and another glass plate is slid over the top so as to exclude air bubbles. In the scanner two slits, approximately 2 mm. and 20 mm. above the top plate, both 0.6 mm. wide by 3 mm. long, control the length and width of the light beam striking the photomultiplier tube. For the scanner itself, a Gilford model 220 absorbance indicator is mounted in place of the Photovolt search unit. The signal from it goes to the Gilford Optical Density Converter, and is then recorded by a Heathkit Servo-Recorder, model EUW-20A.

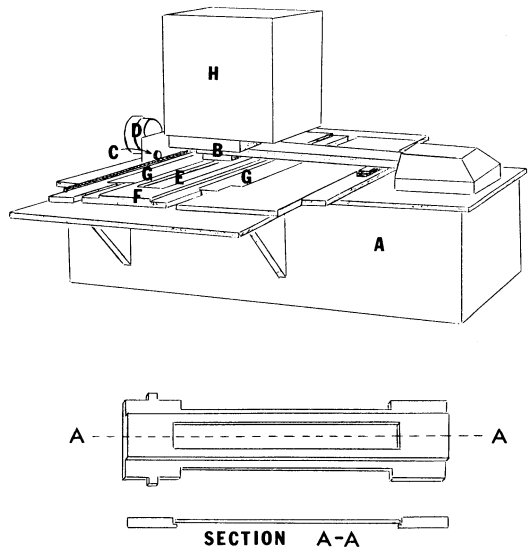

Fig. 1. Densitometer. The letter references are explained in the text. The lower drawings show in detail the part of the carriage which holds the gel under examination.

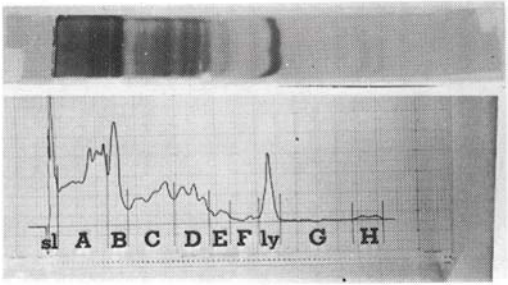

Fig. 2. A protein band pattern from polyacrylamide gel electrophoresis of an extract of Itana wheat flour, together with the corresponding densitometry recording. The vertical lines on the latter show the way the bands are divided into groups to correspond with the indicated letter designations. The starting slot and the lysozyme peak are labeled "sl" and "ly" respectively.

The equipment allows reproducible and sensitive recording of band absorbances with peak areas proportional to sample size and not dependent on base-line position. The recorder response is adjustable in four known ratios, so that reasonable peak heights can be obtained for bands of varying intensity. Other combinations of modules would probably serve as well. A typical densitometer record and the corresponding gel are shown in Fig. 2. We have used a planimeter to measure the combined peak areas between the divisions as shown. The base line is drawn from the level of a blank area preceding the slot to another beyond the last band. The peak area for the internal standard, lysozyme, will vary somewhat, primarily because of variation of sample size. The latter appears to result from slot width variations or deformations. These sometimes arise during withdrawal of the slot-formers. Also a small and variable widening may occur at times because of the weight of the gel in the running position. Assuming that factors affecting the area of the lysozyme peak will affect the flour protein peak areas similarly, one corrects the latter by a factor equal to the ratio of a standard lysozyme area to the measured lysozyme area. Numerous runs under our experimental conditions showed that 100 μl. of flour extracts containing lysozyme of the concentration previously mentioned gave an average lysozyme peak area of 14.7 units. Correcting the flour protein peak areas in this way gives the area per 100 μl. of flour extract plus lysozyme (95 μl. of flour extract only). These areas can then be converted to milligrams of protein by use of the estimated conversion factors previously determined (16) and given in Table I.

TABLE I. RATIO OF DYE COLOR INTENSITY TO FLOUR PROTEIN CONCENTRATION

	Band Group						
	A	B	C	D	E	F	H
Peak area per mg. of flour protein	130	200	260	310	330	380	280

TABLE II. NOMENCLATURE COMPARISONS

Band Group Designations Used Here	No. of Individual Bands Observed	Design. of Gehrke et al. (7)	Design. of Woychik et al. (3)
A	up to 7	S-zone	Beta-, gamma-, omega-gliadins
B	5	A_s	Alpha-gliadins
C	5	G_s	
D	5	Base	
E	4)		
F	3)	A_f	
G	2)		
H	2)	G_f	

RESULTS

A gel with its typical band pattern and the system of designating the band groups that we have used is shown in Fig. 2. We have preferred not to use names that imply an identification with the classic Osborne solubility classes, even though we recognize that some of the principal electrophoresis bands have been shown to be predominant in the pattern from one or another of those classes. All investigationss have shown that they are electrophoretically heterogeneous, and that a number of bands are common to several of them. The band patterns have a recognizable similarity to those shown by Gehrke et al. (7). We have attempted to list in Table II the corresponding designations insofar as the different electrophoresis conditions and the limitations of photographic reproduction permit identification. The correspondence to the designations of Woychik et al. (3) is more definite, since we have been able to make runs with samples of their alpha-, beta-, and gamma-gliadins made available through the courtesy of J. S. Wall. The band for the internal standard, lysozyme, appears between groups F and G in an area where there is rarely any sign of a wheat protein band. The second column gives the number of individual bands that we have observed in each group. All of these can seldom be detected in any one sample, or at a single sample concentration. With some samples the separation between groups A and B or between E and F will not be clear. The group G bands are usually undetectable. Except for these there is usually no difficulty in recognizing each group in any sample's pattern. There frequently is a problem, on the other hand, in establishing the correspondence between certain individual bands for different samples.

Two fractions of the proteins of flour are not accounted for by this gel-electrophoresis procedure; namely, those proteins not solubilized by extraction of the flour with aluminum lactate buffer, and those which, during electrophoresis, do not enter the gel structure but remain at the slot boundary.

As for the first of these fractions, 60 to 80% of the protein was actually solubilized by a single extraction with aluminum lactate-urea (see ref. 17)[4]. Average results for 12 varieties showed that an additional 12±6% can ultimately be solubilized by repeated extractions (see under "Discussion").

[4]Determined from analyses for protein by the Folin-Lowry method (17), standardized by comparisons with Kjeldahl analyses with aluminum lactate extracts (no urea).

The fraction of the total protein that remains at the slot boundary during electrophoresis can also be determined from the difference between the protein content (17) of the extract put in the slot and the sum of the calculated protein contents of the bands. It is clear that the precision of this figure is very low. Among the dozen varieties studied, from 4 to 35% of the dissolved protein did not enter the gel from the slot.

Some examples of the application of these methods to samples of flours from 12 wheat varieties are given in Table III. The data in this table represent only a single sampling of each variety. Statistical studies of the precision of different steps in the analytical method are under way, so that there seems to be no point in attempting to draw many conclusions about the varieties from these data.

There does seem to be a general inverse relation between the amount of protein unextracted and the amount which does not leave the gel slot. This is probably to be expected, since both would be high-molecular-weight protein material. If for some reason the extraction is less efficient, less of this material would be present in the sample placed in the slot.

Finally, it may be said that if the questionable identification be made of these two fractions with glutenin, of fractions A and B with gliadin, and of the remainder with the soluble proteins, the amounts reported here are similar to those often cited.

DISCUSSION OF ANALYTICAL METHOD

Preparation of Sample

It has become abundantly clear that no simple fractionation of flour proteins on the basis of solubility will yield cleanly separated fractions. Even separation into gluten proteins and soluble proteins results in fractions, each of which shows gel-electrophoresis bands characteristic of the other. If estimations were made by studying such fractions, it would be necessary to sum up the amounts of such overlapping proteins from each fraction. The same has been largely true of the results of more complicated and refined fractionations by solubility methods (18), ion-exchange cellulose chromatography (19 to 21), or gel filtration (22 to 24); in any case, these methods are not simple enough for routine analyses. Hence, it was decided to use a single extract containing all the flour proteins which could be readily solubilized.

One of the objectives of the procedure is to obtain an extract with a protein concentration high enough for good electrophoresis bands. Extracts made in this way of flour of 12 varieties of all market classes listed in Table III contained from 2.2 to 3.6 mg. N per ml. Other gel-electrophoresis equipment may well require a higher concentration of protein in the extract, and hence a smaller relative amount of extractant.

In addition, the proteins dissolved in the single-extraction procedure should be representative of the proportions of ultimately extractable proteins in the flour. This has been said not to be the case for water extractions (6). We have tested this by comparing the products of single extractions with those obtained by exhaustive extractions of the same flours. In pooled extracts from multiple extractions the protein concentration is generally too low for quantitative electrophoresis. It was determined that the combination of three extracts, made with a 3:1 ratio of aluminum lactate-urea to flour, had a sufficiently high protein concentration, and

TABLE III. PROTEIN DISTRIBUTION OF FLOUR SAMPLES OF DIFFERENT WHEAT VARIETIES

Variety	Type	Total protein (14% water basis) %	Amount of Fraction								
			Unextracted[a] %	Remaining in gel slot %	A %	B %	C %	D %	E %	F %	H %
Baart	HWS	13.2	18	21	39	11	4	4	0.6	0.4	0.9
Burt	HWW	9.5	7	31	48	7	4	4	0.6	0.2	trace
Bezosztaja 2/selB	HRW	12.9	14	38	33	7	5	2	0.2	0.3	0.4
Cheyenne	HRW	8.6	18	13	41	11	9	4	1.5	1.1	0.4
Gabo	HWS	12.4	9	34	39	6	6	5	0.6	1.0	0.7
Henry	HRS	13.3	27	15	32	10	8	4	1.7	0.9	0.3
Idaed 59	SWS	13.4	19	23	33	11	8	5	1.0	0.4	0.3
Itana	HRW	9.8	32	12	30	11	7	5	0.9	0.6	0.6
Nugaines	SWW	8.1	14	27	33	9	8	6	1.4	1.2	0.3
Omar	SWW (Club)	10.1	13	24	41	11	4	4	0.8	0.2	1.3
Rio	HRW	13.6	21	21	36	9	8	3	1.1	0.6	0.2
Triplet	SRW	9.2	11	36	31	10	5	4	0.4	0.4	0.8

[a]The percent actually unrecovered in one aluminum lactate-urea extraction minus 12%. The latter figure is assumed to be the additional amount that would have been solubilized by repeated extractions.

contained nearly all the protein extractable by additional treatments. When such pooled extracts of eight flours of different varieties were compared with extracts from single extractions, it was found that there were no measurable differences in the gel patterns. Hence it seems reasonable to conclude that the method described does yield a solution representative of the total extractable flour proteins.

The extraction procedure should make possible an estimate of the fraction of the protein insoluble in the aluminum lactate-urea extractant. Multiple extractions are too tedious for routine analysis. A comparison of our data for single vs. multiple extractions indicates that a rough estimate of the total extractable protein can be made on the basis of the results of a single extraction. In the case of 12 wheat flour varieties, the mean difference between the percent extracted by three extractions and that extracted by one was 12%. The figure was highly variable, the standard deviation being ± 6%, but a very rough figure for the soluble protein is obtained by adding 12% to the amount actually found for one extraction. Other workers (25 to 28) by repeated treatments with various solvent systems have extracted 95% or more of flour protein. If our procedure had involved a less severe centrifugation, e.g., 12,000 × g, the apparent amount solubilized would have been greater. The extract under these conditions is turbid rather than clear or slightly opalescent as it is after centrifugation at 100,000 × g. However, our experience shows that this would result only in more glutenin-type material remaining at the slot boundary during the subsequent electrophoresis step. We suspect that when too much protein accumulates at the slot boundary, the entrance of other protein into the gel is hindered.

There is reason to believe that there are alterations in the proteins in the presence of aluminum lactate, perhaps due to complexing with the aluminum ion. The principal evidence is that dialysis of aluminum lactate buffer extracts of flour for extended periods and at various pH values reduced the aluminum content but only to a constant residual level. However, the distribution of flour proteins among the various band groups using aluminum lactate extracts in gels run with aluminum lactate buffer is essentially the same as for 0.05M acetic acid extracts, run in gels with acetic acid buffer. This, plus the fact 1) that use of aluminum lactate gives a highly characteristic pattern and promotes good resolution, and 2) that it appears to be the most efficient solubilizing agent, seems to warrant its utilization in a system for the separation of flour proteins.

Preliminary extraction of the flour with n-butanol has been advocated (7). We have found that much less protein is solubilized from butanol-extracted flour. Others (10,25) have reported similar findings. At first we avoided using urea in the extractant because of its well-known dissociating effect on proteins. We obtained much of the data in this report using extracts without urea (and with a 2:1 to 3:1 ratio of extractant to flour). However, in the case of wheat proteins, there is evidence that protein aggregates may form at the ionic strength used here, that urea prevents such aggregation (29), and that it causes negligible denaturation (30). We have been able to solubilize about 10% more protein when 3M urea was present in the extractant. Extracts of flours of four varieties, made either with or without urea in the extractant, gave gel patterns which were very similar both qualitatively and quantitatively. However, it should be noted that in all these experiments, urea was present in the gel, as will be discussed below. It seems reasonable to recommend that urea be included in the aluminum lactate buffer used for the extraction.

Flour extracts should be used for electrophoresis without undue delay. Significant decreases in the intensities of certain bands were observed after 8 months of frozen storage or 2 months under refrigeration.

It was considered that an internal standard would make the analyses more quantitative. Such a standard should be a protein readily obtainable in reliable purity, which gives a single band which does not overlap any significant band from wheat flour. Its presence should not influence the rate of migration of the flour proteins. The best such protein we have found is lysozyme. Its final position relative to those of the flour proteins is shown in Fig. 2. In the concentration recommended in the methods section above, about 100 γ is used in the 1.5 in. wide gels. There are indications that the lysozyme band coincides with a faint flour protein band in the case of some flour extracts.

Gel Electrophoresis of Flour Extracts

We have included urea in the gel formulation because the bands are more distinct, and more bands are distinguishable. Woychik et al. (3) reported that the presence of urea in a starch gel does not result in qualitative changes in the band pattern and has various advantages. Our observations have been similar, though some additional bands do become detectable, probably owing to improved resolution. Dissociating action of urea on some of the proteins cannot be ruled out. We have noticed, too, some differences in the relative intensity of certain bands. For example, the band patterns for a composited bread-type flour made with and without urea in the gel were similar with respect to distribution among the major band groups, but the patterns within group D were quite different (Fig. 3). We have referred previously to the somewhat conflicting evidence in the literature as to whether urea causes or prevents changes in the native wheat proteins.

We have found it preferable to use a formula for the gel which includes no buffer, and to introduce the aluminum lactate buffer to the gel by a preliminary overnight equilibration step. Under these conditions the resolution of the band pattern is much improved, and the migration rate is much greater. The gels set more reliably, and are stronger and more easily handled. A similar procedure has been used by Nimmo et al. (6) to study the water-soluble flour proteins at pH 6.0. Mitchell (31) has shown that artifacts caused by oxidation of a protein by the ammonium persulfate catalyst can be avoided by such a preliminary step.

The pH 3.2 buffer has been quite commonly used for gel-electrophoresis work with wheat proteins. We have shown that under these conditions all the proteins move toward the cathode, and none moves clear out of the gel in 1.5 hr. Runs on extracts of ^{14}C-labeled flour proteins resulted in recovery of only about 0.25% of

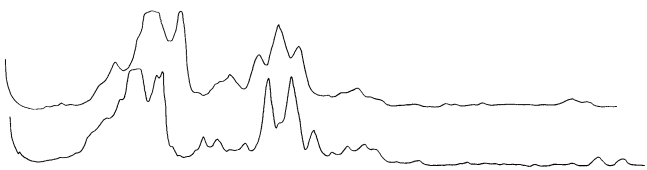

Fig. 3. Densitometry recordings of the protein band patterns made by gel electrophoresis with (upper curve) and without (lower curve) 2.7M urea in the polyacrylamide gel for a bread-type composite flour.

the radioactivity in the two electrode chambers. Experiments at other pH values can be useful for research purposes. For example, Nimmo et al. (15) found that the pair of fastest-moving bands are produced by exposure of the proteins to low pH, and are not present in the unaltered globulins. With longer running times these bands move out of the gel and the resolution of the slower-moving peaks is somewhat improved, so that often additional bands can be observed. However, these new bands are never sufficiently resolved for separate determination.

The technique used here permits one to cast gels of widely varying polyacrylamide concentrations. Both 3 and 8% gels seemed to give better resolution of flour protein bands in certain regions, but the former were so soft and weak as to be difficult to manipulate, and the migration rate was much slower in the latter.

The recommendation of dyeing the gels for 20 min. in 0.1% amido black derives from experiments showing no differences with dye concentrations as low as 0.05% and dyeing times from 12 min. to 1 hr. Besides amido black, three other dyes were tested. A number of reports have stated that nigrosine is superior to amido black for starch gels (8,10,32) and polyacrylamide gels (12). This is not the case for polyacrylamide gels under our conditions. Gels were dyed with water-soluble nigrosine in 5:5:1 methanol-water-acetic acid at various concentrations and for various times. Dye uptake was comparatively slow for group A and B proteins, though eventually all the bands were darker relative to lysozyme than were the corresponding bands dyed with amido black. The washing rate was much slower than for amido black-dyed gels, and the resulting background color was always much more uneven, resulting in less regular densitometry curves. This latter finding was also made for Coomassie brilliant blue R250 (33,34) which was tried under various conditions. A few experiments with Procion brilliant blue RS (33) indicated no advantages over amido black.

With continued washing with 7% acetic acid, the intensity of the amido black bands decreases, but densitometric measurements show that the background color decreases at essentially the same rate up until at least 2 days, at which time the background absorbance is very small. Beyond that time real fading of band intensity occurs, particularly for the slower-moving peaks. The greater stability of nigrosine-dyed bands is one of the main advantages cited (10,11), and our experience is in agreement. However if it is necessary to hold amido black gels for longer periods before densitometry, they can be held in the refrigerator between sheets of plastic film for considerable time without fading. The gels can be dried (35)[5] to obtain a permanent record.

Estimation of Protein Concentration from Gel Bands

The source and limitations of the conversion factors given in Table I are described in an accompanying paper (16). It might be pointed out here that they were derived from analyses using flours of a soft white spring variety, Federation, and a HRS variety, Thatcher. A given factor represents a weighted average of the ratios of all the individual proteins within a band group. The proportions of these will certainly vary with variety and many other influences, so that the factors given here have an inherent arbitrariness. Nevertheless, the estimated protein contents obtained with their use will be much more realistic than otherwise.

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[5]If the gels are dried at room temperature rather than at 70°C. as previously recommended (35), one can avoid the splits and checks that occasionally occur.

Frequently, groups A and B do not become separated to any useful degree, perhaps because of the presence of intermediate-mobility proteins, the predominance of one group over another, or interactions. Determinations made on flours in which they do separate suggest that there is always considerably more protein of group A than of group B. A combined conversion factor of 145 has been calculated; it is based on the assumption that the proportions are approximately the same whether the groups are separated or not. Table I gives no factor for a group G. This designation refers to the observation in certain cases of at least two very faint bands between lysozyme and the high-mobility doublet H. They were not present in sufficient quantity to allow an estimate of the conversion factor. One might guess that they are proteins binding a relatively large amount of dye. In the area just beyond the slot and before the first strong band, there is always much background dye color, often tending to obscure several bands that occur there. This section, and the proteins contained in it, are included in group A.

Since the mixture of the bands within a group, and the consequent contour of the peaks representing it, may differ considerably with different varieties of wheat, one often is faced with a difficult decision as to where to make the division between groups. This is particularly the case for the divisions between groups D, E, and F. The area measured for a peak grouping will be strongly influenced by this decision. When one is dealing with flour samples of a similar type, the similarity of the patterns makes this decision less difficult. Experience here is the best teacher.

A quantitative assessment of the relative areas representing different bands within a group is also a problem. Occasionally flours show obvious differences of this kind, even though the relative amounts in the various band groups are very similar. In most cases, we see no solution better than a qualitative description of the shape of the densitometry curve. Within group D, the two principal bands, each of which seems to include at least two overlapping bands, are often sufficiently resolved that at least an estimate of the relative amounts can be made, based on respective peak heights.

Acknowledgment

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