PROTEINS OF WHEAT AND FLOUR. EXTRACTION, FRACTIONATION, AND CHROMATOGRAPHY OF THE BUFFER-SOLUBLE PROTEINS OF FLOUR[1]

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ABSTRACT

Six major protein fractions were identified following chromatography on DEAE-cellulose of the proteins extracted by 0.01*M* sodium pyrophosphate (at pH 7) from each of two flour samples differing widely in protein content and baking performance. The two flours appeared to differ in the relative amounts of certain of the components present. Two of the fractions were resolved further by rechromatography and by electrophoresis, giving a total of ten components so far recognized in these extracts.

One fraction (peak A), containing both protein and carbohydrate, passed unretarded through the DEAE-cellulose column. On hydrolysis, the carbohydrate component of peak A yielded arabinose and xylose, together with lesser amounts of galactose. Electrophoresis and further chromatography of peak A on carboxymethyl-cellulose (CM-cellulose) demonstrated it to be heterogeneous and to consist of at least three components. Peaks D, E, and F were eluted from the DEAE-cellulose column in the presence of increasing concentrations of sodium chloride. Electrophoresis experiments indicated the presence of at least five components in this group of proteins with the following approximate mobility values: A, $0–3 \times 10^{-6}$; D, $19–22 \times 10^{-6}$; E, $26–27 \times 10^{-6}$; F, $43–50 \times 10^{-6}$ cm^2 sec^{-1} $volt^{-1}$. Peaks D, E, and F were rechromatographed on DEAE-cellulose to yield single symmetrical peaks. Components J and K were eluted by 0.05*N* acetic acid and 0.1*N* sodium hydroxide, respectively. Peaks D and E, F and K have a lower amide and glutamic acid content than peak A and are higher in arginine, aspartic acid, glycine, leucine, and tyrosine.

As part of a comprehensive study of the proteins of developing and mature wheat grain, methods were required for the extraction, separation, and identification of individual proteins. Chromatography on columns of cellulose ion-exchangers was therefore investigated for this purpose. This paper describes the extraction and chromatographic procedures used and the results of electrophoretic studies on the products so obtained.

Materials and Methods

Two wheat samples, varieties Tichborne (sample 1) and Broughton (sample 5), were milled on a Buhler laboratory mill to 72 and 70% extraction, respectively. The resulting flour samples contained 2.61 and 1.60% nitrogen on a dry-weight basis. The two flours dif-

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fered considerably in baking behavior and in response to bromate addition when subjected to the standard baking procedure developed by the Bread Research Institute, Sydney. In this procedure two doughs are prepared from each flour consisting of flour (120 g.) to which are added 68 ml. of a liquor containing yeast (2.4 g.), sodium chloride (2.4 g.), and ammonium chloride (0.06 g.). To the unbromated dough 6 ml. water are added; the bromated dough is mixed with 0.02% potassium bromate solution (6 ml.; equivalent to 10 p.p.m. on the weight of flour taken). After mixing, proofing, etc., under controlled conditions, the loaves are baked at 510°F. (266°C.) and scored according to volume (possible mark, 36%), external appearance (20%), crumb texture (30%), and crumb color (14%). In this test flour 1 gave a medium-extensible dough, yielding an unbromated loaf of volume 550 ml. and a bromated loaf of volume 600 ml. Its quality rating was 63% without and 79% with the addition of bromate, and it therefore showed a normal response to this ingredient. Flour 5 yielded a strong, stable dough giving both unbromated and bromated loaves of volume 500 ml. Its quality score was 64% without and 65% with the addition of bromate, and it therefore showed a zero or negative response to this ingredient.

Preliminary Studies. Preliminary studies showed that the presence of lipids in dilute sodium pyrophosphate extracts (24) of disrupted wheat grain caused considerable interference during subsequent fractionation attempts with ammonium sulfate and ethanol precipitation. The lipids were successfully removed by preliminary treatment with water-saturated 1-butanol (17,23,24). Examination of the fractions so obtained by moving-boundary electrophoresis showed them to be markedly heterogeneous. Attempts were therefore made to fractionate them further on columns of cellulose ion-exchangers (33,37).

Extraction of Buffer-Soluble Flour Proteins. Both flour samples were extracted side by side according to the procedure summarized in Diagram I. All operations were carried out in a cold room at 4°C.

After preliminary treatment in a Waring Blendor with water-saturated 1-butanol, the flour samples were extracted several times with sodium pyrophosphate solution (0.01*M*, pH 7.0), followed by several extractions with acetic acid (0.05*N*, pH $\doteq$ 3.5). The nitrogen extracted from each flour at each stage is shown in Diagram I, expressed as a percentage of the total nitrogen in that flour.

The yellow-colored butanol extracts contained considerable amounts of lipid but only 0.4 to 0.5% of the total nitrogen. Since other studies (23,24) have shown that the nitrogenous constituents

Diagram I. Extraction Procedure for Flour Samples 1 and 5

100 g. FLOUR 1 AND 5

Homogenized 5 minutes with 200 ml. water-saturated butanol; centrifuged.

RESIDUE stirred 1.5 hours with 200 ml. 0.01*M*, pH 7.0 sodium pyrophosphate; centrifuged.

BUTANOL EXTRACT
(1): 0.50% (5): 0.36%

RESIDUE stirred 1 hour with 200 ml. 0.01*M*, pH 7.0 sodium pyrophosphate; centrifuged.

First 0.01*M* PYROPHOSPHATE EXTRACT
(1): 6.92% (5): 10.56%

RESIDUE: two further extractions with 0.01*M* sodium pyrophosphate; centrifuged between each.

Second 0.01*M* PYROPHOSPHATE EXTRACT
(1): 5.20% (5): 6.96%

Third 0.01*M* PYROPHOSPHATE EXTRACT
(1): 2.64% (5): 4.03%

Fourth 0.01*M* PYROPHOSPHATE EXTRACT
(1): 2.06% (5): 3.89%

Total N extracted into 0.01*M* sodium pyrophosphate
(1): 16.82% (5): 24.64%

RESIDUE stirred 1.5 hours with 200 ml. 0.05*N* acetic acid; centrifuged.

First 0.05*N* ACETIC ACID EXTRACT
(1): 32.76% (5): 27.35%

RESIDUE stirred 1 hour with 200 ml. 0.05*N* acetic acid; centrifuged

Second 0.05*N* ACETIC ACID EXTRACT
(1): 28.74% (5): 21.19%

RESIDUE: two further extractions with 0.05*N* acetic acid; centrifuged between each.

Third 0.05*N* ACETIC ACID EXTRACT
(1): 4.99% (5): 6.86%

Fourth 0.05*N* ACETIC ACID EXTRACT
(1): 1.50% (5): 2.92%

Total N extracted into 0.05*N* acetic acid:
(1): 67.99% (5): 58.32%

RESIDUE:
(1): 14.65% (5): 16.69%

Total N recovered:
(1): 100.7% (5): 99.3%

were mainly phospholipids, the butanol extracts were not further examined.

The combined sodium pyrophosphate extracts of each flour contained 16.8% (flour 1) and 24.6% (flour 5) of the total nitrogen. They were separately dialyzed for 2 days against three changes of glycine-sodium hydroxide buffer (0.006*M*, pH 9.5, 200 ml.) on a rocking dialyzer at 4°C.

Chromatography of Proteins. Diethylaminoethyl-cellulose (DEAE-cellulose)[3] was washed with 0.1*N* sodium hydroxide and then with distilled water. It was then poured into a glass or Perspex column to form a bed 2 cm. in diameter and 15 cm. high. The column was washed successively with 500 ml. glycine-sodium hydroxide (0.2*M*, pH 9.5) and 1,500 ml. glycine-sodium hydroxide buffer (0.006*M*, pH 9.5). The dialyzed pyrophosphate extract containing 25–50 mg. of protein nitrogen was applied to the top of the prepared column, and the effluent was collected in 4-ml. aliquots by means of a fraction collector. All operations were carried out in a cold room at 4°C.

Carboxymethyl-cellulose (CM-cellulose) was prepared by treatment of Whatman standard cellulose powder[4] with chloroacetic acid, followed by thorough washing with 10% acetic acid and distilled water (33). The ion-exchanger was then stirred with 0.1*N* sodium hydroxide (20 volumes) and 0.1*N* hydrochloric acid (20 volumes), followed by thorough washing with distilled water. It was suspended in acetic acid-sodium hydroxide buffer (20 volumes; 0.5*M*, pH 4.1) and poured into a column 2 cm. in diameter and 15 cm. high. Acetate buffer (0.5*M*, pH 4.1; 500 ml.) was passed through the column, followed by 1,500 ml. of acetate buffer (0.005*M*, pH 4.1). The protein solution (30 ml. containing 250 mg., peak A), dialyzed for 2 days against three changes of acetate buffer (0.005*M*, pH 4.1; 2,000 ml.), was then applied to the top of the prepared column. The column was washed successively with 200 ml. acetate buffer (0.005*M*, pH 4.1) and 200 ml. acetate buffer (0.005*M*, pH 4.1) containing urea (final concentration 4*M*), followed by a gradient to acetate buffer (0.005*M*, pH 4.1) containing urea (final concentration 4*M*) and sodium chloride (1*M*). The effluent was collected in 4-ml. aliquots on a fraction collector.

Analytical Methods. Absorbance values of the effluent samples were measured at 280 mμ with a Uvispek spectrophotometer. Nitrogen was estimated by a micro-Kjeldahl procedure. Hydrolysis of the samples with twice-redistilled, constant-boiling hydrochloric acid was

[3] Eastman Kodak Co., Rochester, N. Y.
[4] W. and R. Balston, Ltd., London, England.

carried out in evacuated sealed tubes for 20 hours at 110°C.

Amino acids were estimated by the ion-exchange chromatographic procedure of Moore, Spackman, and Stein (22); the automatic apparatus of Simmonds and Rowlands (34,35) was used.

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (13), and also as micro-Kjeldahl-determined nitrogen multiplied by the factor 5.7.

Pentoses were estimated according to Dische (7) and hexoses by the method of McCready, Guggolz, Silvera, and Owens (16).

Electrophoresis Studies. Moving-boundary electrophoresis was carried out at 1.8° ± 0.05°C. using a Perkin-Elmer apparatus (model 38 A) with an open-cell assembly of volume approximately 2 ml. A standard glycine-sodium hydroxide buffer (ionic strength 0.1, pH 9.5) was employed as solvent. Protein solutions (approximately 4 ml.) were dialyzed against two changes of about 500 ml. of buffer at 4°C. for 48 hours before electrophoresis. The pH of both protein solution and external buffer was measured; the former was recorded as the pH at which electophoresis was carried out.

Refractive index gradients in both limbs of the electrophoresis cell were observed with a schlieren optical system in which the knife edge was replaced by a phase plate having a human hair attached along the phase border. The positions of the maximal ordinates of the schlieren patterns were measured relative to the shadow of a wire fixed across the cell mask. The cell current was measured with an avometer. All conductivity measurements were carried out at the temperature of electrophoresis. Mobilities are given for the descending limb only. Under the experimental conditions used, all proteins migrated toward the anode.

Ultracentrifuge Studies. A Spinco model E analytical ultracentrifuge was used.

Results

Chromatography of Flour Proteins. Figure 1 shows a typical trace of absorbance vs. effluent volume obtained when a pyrophosphate extract of flour 1 containing 55 mg. of nitrogen was loaded onto a 2-cm. by 15-cm. column of DEAE-cellulose.

Material designated peak A was not retained by the column and was washed out with a further 400 ml. of glycine-sodium hydroxide buffer (0.006*M*, pH 9.5). A linear gradient to 0.3*M* sodium chloride in glycine sodium hydroxide (0.006*M*, pH 9.5) was then started.

The retention of peaks D and E appears to be very sensitive to the concentration of sodium chloride in the eluting buffer; optimum

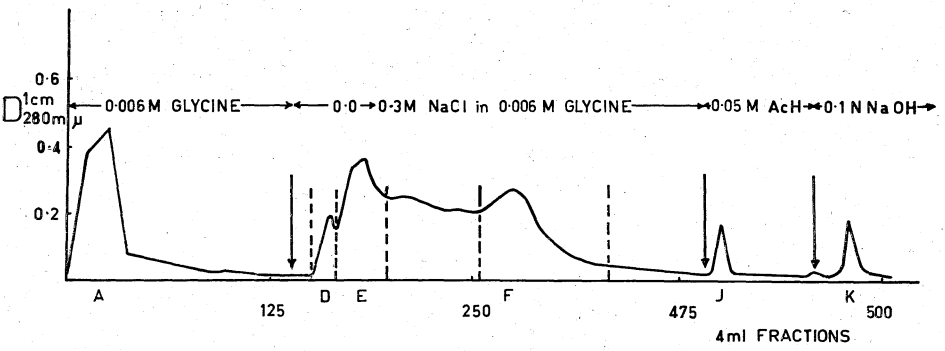

Fig. 1. Chromatography of 0.01*M* sodium pyrophosphate extract of flour 1 on DEAE-cellulose (5 g.; 2-cm. by 15-cm. column). Loading approximately 55 mg. of protein nitrogen in 50 ml. dialyzed solution. Column equilibrated with glycine-sodium hydroxide buffer (0.006*M*, pH 9.5).

resolution was obtained with a slow, continuous gradient in which 800 ml. of the starting eluent (glycine-sodium hydroxide; 0.006*M*, pH 9.5) and 800 ml. of the limit eluent (0.3*M* sodium chloride in glycine-sodium hydroxide, 0.006*M*, pH 9.5) were placed in each arm of the gradient device. Any discontinuity in the gradient was reflected by the appearance of artifact peaks in the effluent. This occurred if the gradient was changed in steps from 0.0 → 0.2*M* sodium chloride to 0.2 → 0.3*M* sodium chloride. No further components could be eluted by increasing the sodium chloride gradient to 1.0*M*. The effect of pH was not studied exhaustively, but poor separations were obtained at pH 8.5.

Regeneration of the column with 0.1*N* sodium hydroxide led to the desorption of two further components, J and K, one of which could be eluted with 0.05*N* acetic acid, or by running a further gradient from 0.006*M* glycine to 0.1*N* HCl prior to regeneration with sodium hydroxide (see Fig. 1). Flours 1 and 5 gave similar elution patterns, varying mainly in the relative proportions of peaks D, E, and F. As a guide, peaks A, D + E, and F represented about 30–40, 25–35, and 15–20% dry weight, respectively, of the pyrophosphate-extractable material. The remaining components were present in small amounts only.

Material for rechromatography, electrophoresis, ultracentrifuge studies, and amino acid analysis was obtained by combining fractions of the column effluent from various runs in a manner similar to that indicated in Fig. 1. Figure 2 illustrates the purification achieved by rechromatography (on DEAE-cellulose) of appropriately combined effluent fractions. After two further chromatographic separations,

single symmetrical peaks corresponding to peaks D, E, and F separately were obtained.

Electrophoresis of Fractions Obtained by Chromatography. Before

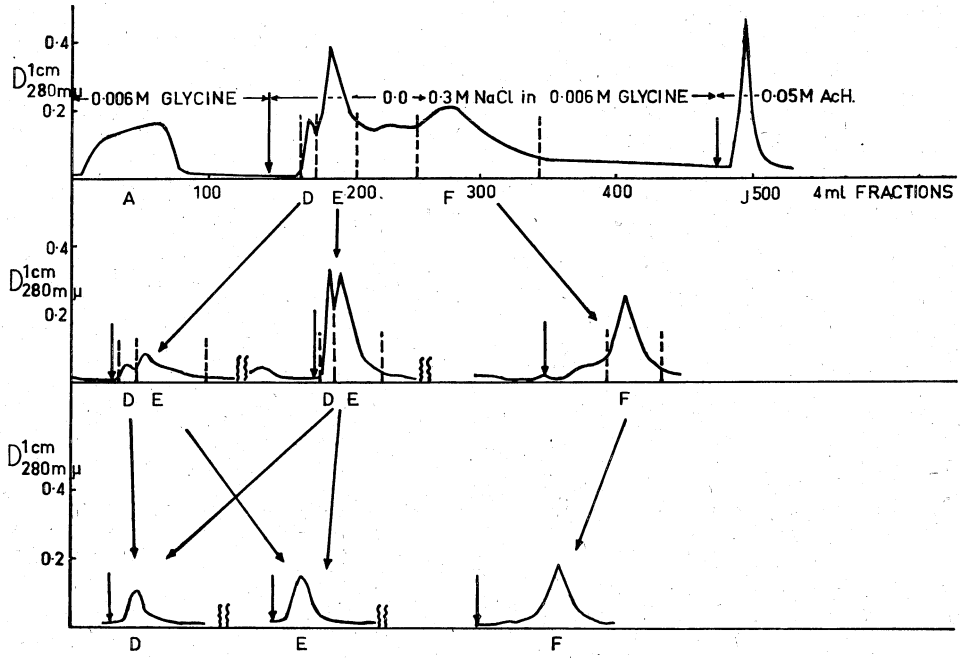

Fig. 2. Rechromatography of appropriately combined material from peaks D, E, and F on DEAE-cellulose. Column loading and buffers as in Fig. 1.

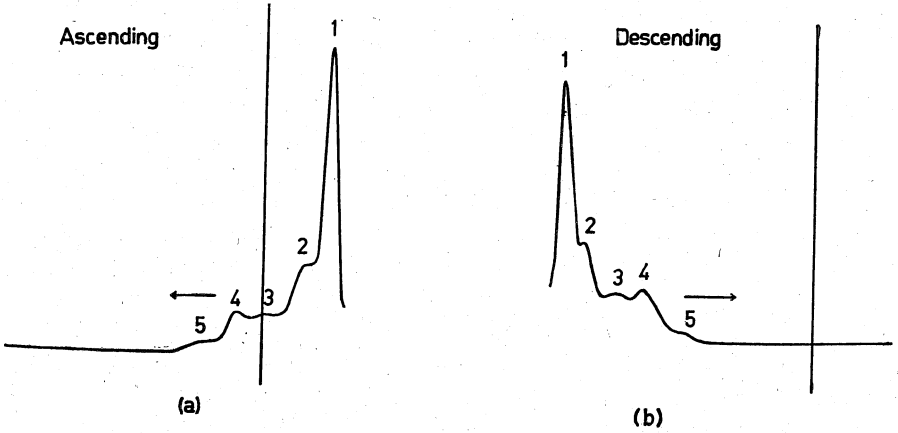

Fig. 3. Electrophoresis of sodium pyrophosphate extract of flour 1 in glycine-sodium hydroxide buffer, pH 9.98 (a) ascending limb, (b) descending limb after 2 hours. Field strength 3.62 volts per cm.

chromatography the pyrophosphate extract of flour 1 gave the electrophoresis pattern shown in Fig. 3.

The large peak close to the initial boundary moved so little that it was not possible to ascribe to it a meaningful mobility value. Four further peaks were observed having the mobilities summarized in Table I.

TABLE I

MOBILITY VALUES OF THE MAJOR PROTEIN COMPONENTS SHOWN IN THE ELECTROPHORESIS EXPERIMENT OF FIG. 3[a]

PROTEIN COMPONENT	ASCENDING LIMB	DESCENDING LIMB
1	0.19	1.08
2	12.59	8.03
3	26.53	20.44
4	36.58	35.95
5	53.64	42.79

[a] Values are expressed as cm.^{2} volt^{-1} sec.$^{-1}$ $\times$ 10^{-6}.

Electrophoresis experiments were also carried out on the main fractions obtained after chromatography on DEAE-cellulose. The material which was not retained by the column (peak A) gave rise to two electrophoretic components with mobilities of 3 and 12 cm.^{2}

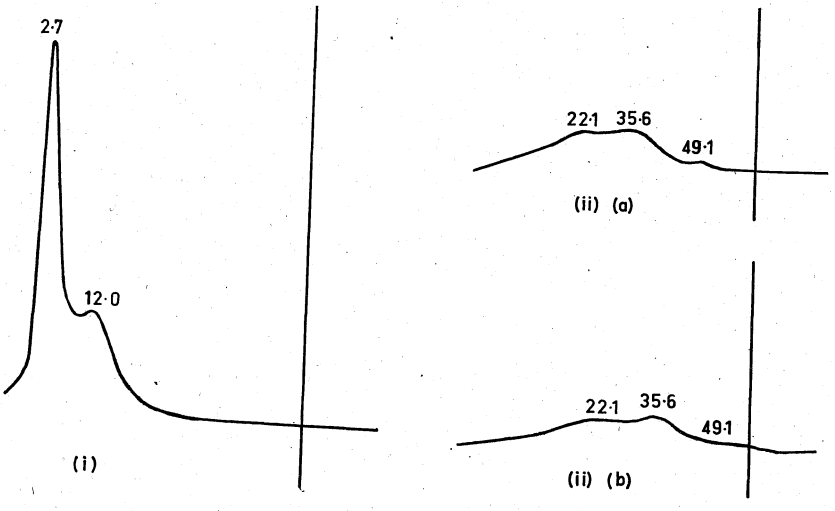

Fig. 4. Electrophoresis of sodium pyrophosphate extract of flour 1. (i) Material passing unretarded through 2-cm. by 15-cm. DEAE-cellulose column (peak A). Electrophoresis carried out in glycine-sodium hydroxide buffer. pH 9.40, descending limb after 5 hours. Field strength 3.67 volts per cm. (ii) Material eluted by 0.3*M* sodium chloride from 2 cm. by 15 cm. DEAE-cellulose column. Electrophoresis carried out in glycine-sodium hydroxide buffer. pH 9.28, descending limb (a) after 3 hours, (b) after 4 hours. Field strength 3.35 volts per cm.

volt^{-1} sec.$^{-1}$ $\times$ 10^{-6}, respectively, as shown in Fig. 4, i.

Electrophoresis of the material adsorbed by the DEAE-cellulose column and eluted by 0.3*M* sodium chloride (consisting of a mixture of peaks D, E, and F, Fig. 1) showed the presence of three components having mobilities of 22, 36, and 49 cm.^{2} volt^{-1} sec.$^{-1}$ $\times$ 10^{-6} (Fig. 4, ii).

When effluent fractions combined from the individual peaks marked D, E, and F in Fig. 1 were separately concentrated and examined by electrophoresis, a total of six components were detected as shown in Fig. 5.

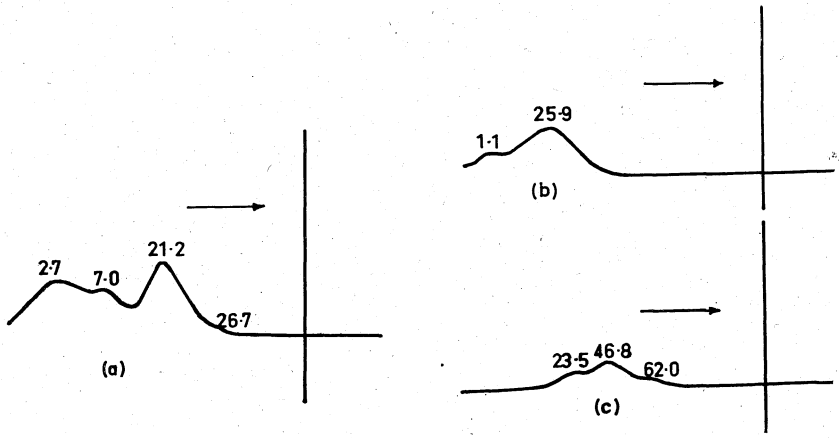

Fig. 5. Electrophoresis of sodium pyrophosphate extract of flour 1. Material eluted by 0.0–0.3*M* sodium chloride gradient from 2-cm. by 15-cm. DEAE-cellulose column as illustrated in Fig. 1. (a) Concentrate from area marked peak D (Fig. 1). Glycine-sodium hydroxide buffer, pH 9.46, descending limb after 5¼ hours. Field strength 3.64 volts per cm. (b) Concentrate from area marked peak E (Fig. 1). Glycine-sodium hydroxide buffer, pH 9.51, descending limb after 2¼ hours. Field strength 3.77 volts per cm. (c) Concentrate from area marked peak F (Fig. 1). Glycine-sodium hydroxide buffer, pH 9.42, descending limb after 2½ hours. Field strength 3.80 volts per cm.

The mobility values calculated from these electrophoretic runs are given in Table II.

Preliminary analysis of peak E and peak F material in the ultracentrifuge has shown in both the presence of only two components, having sedimentation coefficients of approximately 5S and 2S, respectively. In view of the greater resolution attainable by electrophoresis, further ultracentrifuge work has been deferred until electrophoretically homogeneous components have been prepared.

Material eluted from the DEAE-cellulose column by 0.05*N* acetic acid, or by 0.1*N* hydrochloric acid at pH 1.5–2 (peak J), contained

TABLE II

MOBILITY VALUES OF COMPONENTS IN FRACTIONS D, E, AND F ELUTED FROM DEAE-CELLULOSE[a]

SAMPLE	MOBILITY VALUES[b] AND APPROXIMATE PROPORTIONS[c]						
	0.3	7-13	19-22	26-27	36	43-50	60
Total extract	+++	++	++	−	++	+	−
Eluted by 0.3*M* NaCl	+	−	++	−	++	+	−
Peak A	+++	++	−	−	−	−	−
Peak D	+	+++	++	+++	−	−	−
Peak E	+	−	−	+++	−	−	−
Peak F	−	−	−	++	−	+++	+
Peak J	++	−	−	−	−	++	−

[a]Calculated from electrophoretic analyses illustrated in Fig. 5.
[b] cm.^{2} sec.$^{-1}$ volt^{-1} $\times$ 10^{-6}.
[c]+, small amount only; ++, moderate amount present; +++, main component; −, not detected.

two electrophoretic components with mobilities of 4 and 43 cm.^{2} volt^{-1} sec.$^{-1}$ $\times$ 10^{-6} respectively.

Further Purification and Chemical Properties of Peak A. Rechromatography of peak A on DEAE-cellulose equilibrated at pH 9.5 with 0.005*M* borate buffer yielded two major components, A_1 and A_2; the former passed unretarded through the column, and the latter was eluted in the position of peaks D, E, and F (Fig. 1) by 0.005*M*,

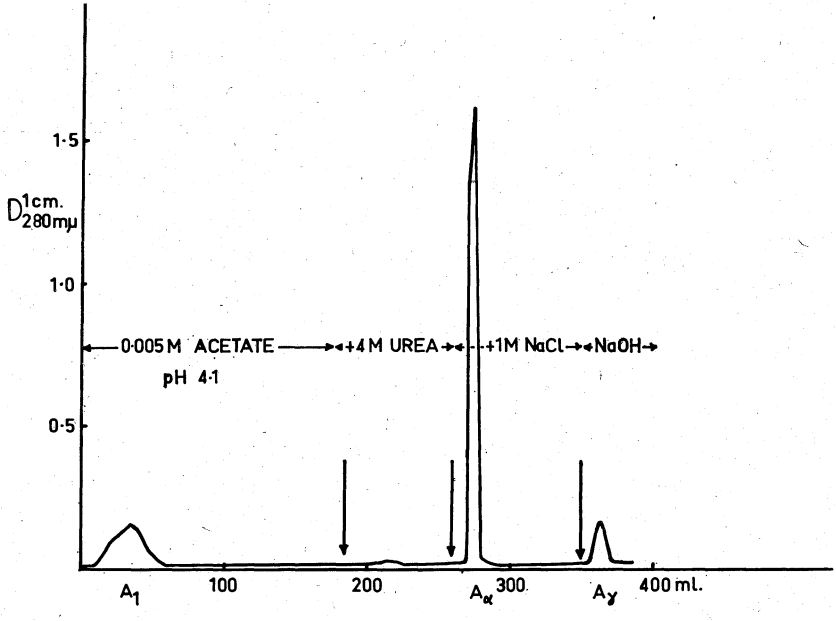

Fig. 6. Rechromatography of peak A on CM-cellulose equilibrated with acetate buffer (0.005*M*, pH 4.1). Loading: 250 mg. of lyophilized peak A in 30 ml. dialyzed solution.

pH 9.5 borate buffer containing 0.3*M* sodium chloride. Peak A_1 gave positive tests for protein (13) and carbohydrate (Molisch); Peak A_2 contained protein and gave only a very weak Molisch test. The borate treatment has therefore separated from peak A, a component corresponding in chromatographic mobility to peaks D, E, and F in Fig. 1.

Further evidence for the heterogeneity of peak A has come from rechromatography on columns of CM-cellulose. At pH 4.1 in 0.005*M* sodium acetate containing 4*M* urea, three main components were observed, as shown in Fig. 6.

Peak A_1 passed straight through the column; peak A_α was eluted by 1*M* sodium chloride dissolved in 0.005*M* sodium acetate containing 4*M* urea; peak A_γ was eluted by 0.1*N* sodium hydroxide. All peaks gave positive protein and Molisch tests and contained the amounts of protein (N × 5.7) and carbohydrate (expressed as xylose, Dische, 7) shown in Table III.

TABLE III

COMPOSITION OF PEAK A AND ITS SUBFRACTIONS PREPARED FROM FIGURE 1[a]

COMPONENT	PROTEIN (N × 5.7)	CARBOHYDRATE (DISCHE, 7)	TOTAL ACCOUNTED FOR
Original peak A (from DEAE-cellulose column)	40.6	49.6	90.2
Peak A_1 from CM-cellulose column	6.4	87.2	93.6
Peak A_α from CM-cellulose column	69.0	15.4	84.4
Peak A_γ from CM-cellulose column	++++	+	—

[a] Results are expressed as percentage dry weight.
[b] Insufficient material available for analysis. + indicates relative amounts of protein and carbohydrate present.

The carbohydrate component of peak A and its derived fractions A_1, A_α, and A_γ appears to be mainly pentose in nature as determined colorimetrically with orcinol (7). It gave comparatively little color with anthrone (16). The absence of starch was indicated by its failure to give any color with iodine. Paper chromatography of a dilute acid hydrolysate of peak A (1*N* sulfuric acid, 110°C., 6 hours in a sealed tube) demonstrated the presence of arabinose and xylose in the approximate proportions 1:2, together with lesser amounts of galactose and traces of glucose. The carbohydrate component of peak A therefore corresponds to the pentosan fraction of wheat flour previously investigated by Pence, Elder, and Mecham (27), Perlin and co-workers (8,31,32), Simpson (36), and Smith and co-workers (10,11,18–21). It is likely that the traces of glucose observed are due to slight contamination with starch. The presence of galactose confirms a similar observation made by Simpson (36) and Ford and Peat (9).

Fractions separately combined from the areas marked A, D + E, F, and K in the chromatographic experiment of Fig. 1 have also been subjected to amino acid analysis. The results, determined on protein fractions from flour 1, are summarized in Table IV.

TABLE IV

AMINO ACID COMPOSITION OF PEAKS A, D + E, F, AND K DERIVED FROM FLOUR SAMPLE 1 [a]

AMINO ACID	PROTEIN FRACTIONS			
	A	D + E	F	K
Alanine	3.3	6.2	6.1	4.9
Amide	14.8	10.7	8.3	12.4
Arginine	11.0	18.6	19.6	17.1
Aspartic acid	4.1	6.4	7.1	6.3
Glutamic acid	16.1	12.5	10.7	8.7
Glycine	3.6	6.8	7.0	6.2
Histidine	3.9	6.2	6.4	6.1
Isoleucine	2.4	2.9	3.2	3.1
Leucine	4.0	6.3	7.1	5.3
Lysine	4.4	6.4	6.7	6.7
Phenylalanine	2.9	1.8	2.1	2.7
Serine	3.4	4.5	3.8	3.4
Threonine	2.7	3.0	3.4	2.7
Tyrosine	1.2	2.6	3.6	2.1
Valine	2.6	5.4	5.2	4.3

[a] Amino acid nitrogen is expressed as a percentage of the total nitrogen present in the sample after hydrolysis.

It will be noted that peak A, containing approximately 45% of protein, differs markedly from the other three protein fractions in having an amide and glutamic acid content approaching that of gluten (28). Its content of alanine, arginine, aspartic acid, glycine, histidine, leucine, lysine, tyrosine, and valine is less than that of the other peaks.

Discussion

Moving-boundary electrophoresis was used in this study as a qualitative tool to evaluate the effectiveness of substituted cellulose ion-exchange columns for the separation of soluble wheat proteins. Figure 3 shows that the patterns obtained in both ascending and descending limbs are enantiographic within the limits imposed by the electrophoretic technique (1). This supports the assumption that these proteins migrate in an electric field in a manner uncomplicated by interactions and permits identification of the macromolecular components of the column effluents with specific components in the electrophoretic patterns. Such components may or may not correspond to discrete protein species in terms of other physicochemical criteria. Additional components may well be detected by further electrophoresis studies over a wider range of pH and ionic strength than

those reported here. The mobility values quoted in Table II show reasonable agreement between ascending and descending limbs. Because of the complexity of the pattern, first-moment calculations for each peak could not be carried out, and apparent maximum ordinates were therefore used. This led to exaggerated differences in mobility values between ascending and descending limbs. This lack of precision in the mobility measurements was shown in the plots of distance moved against time and also in the lack of complete reproducibility of mobility values from run to run. For this reason, the ranges of values observed have been quoted in Table II. However, the over-all shapes of the electrophoretic patterns were fully reproducible.

During concentration of the column effluent fractions by freeze-drying, a small quantity of insoluble material, possibly denatured protein, was formed. However, its removal by centrifugation of the final solutions used for electrophoresis is unlikely to have substantially affected the proportions of components observed. Further factors that complicated the interpretation of the results are the small but noticeable variations in pH, which affect the electrophoretic mobility values calculated on similar protein fractions from different chromatographic separations, and the tendency for any discontinuities in the salt gradient to be reflected as small peaks in the elution pattern.

Column chromatography on DEAE-cellulose has clearly split the pyrophosphate-soluble flour proteins into three main groups: 1) Material not retarded by the column — peak A; 2) material adsorbed by the column but eluted by low concentrations of sodium chloride — peaks D, E, and F; and 3) material eluted from the column only under extremes of pH — peaks J and K.

Electrophoresis of peak A consistently showed the presence of two components: a major component which remains practically stationary, and a minor peak with a mobility of about 12 cm.^{2} volt^{-1} sec.$^{-1}$ $\times 10^{-6}$. It is possible that the stationary peak corresponds to the carbohydrate-rich material which passes unretarded through a CM-cellulose column (peak A_1, Fig. 6) and the minor component to the protein-rich peak A_a (Fig. 6). It seems significant that rechromatography of peak A on borate-DEAE-cellulose splits from the original mixture a component which runs in a position similar to that of peaks D, E, and F. It is possible that the pentosan interacts with members of this group to yield products which are stable to chromatography. The stability and strength of this interaction are matters requiring further investigation.

With respect to the second group of proteins, those adsorbed by the DEAE-cellulose column and eluted in the presence of sodium chloride, examination of Table I and Fig. 1 shows that, in general, components with low electrophoretic mobilities are eluted by low salt concentrations, whereas components with large electrophoretic mobilities are eluted at higher salt concentrations. The ultracentrifuge evidence indicates that the protein molecules comprising peaks D, E, and F do not differ greatly in their sedimentation properties. If their molecular weights are also similar, they will, in addition, have frictional coefficients of the same order. Under these conditions, the mobility of a given electrophoretic component will be approximately proportional to its effective charge under the conditions of pH ($\simeq$ 9.5) and ionic strength (0.1) used. Since the chromatographic analysis is conducted at a constant pH up to the elution of peak F, neither the adsorbed proteins nor the DEAE-cellulose will gain or lose hydrogen ions. However, the effective charges of both the column material and the adsorbed proteins will be reduced by the collapse of their ionic double layers as the ionic strength increases. If the adsorption of the proteins is purely electrostatic, the slow decrease in effective charge should result in the species of lowest charge desorbing from the column at lowest salt concentrations; whereas those of greater charge will require a higher ionic strength to reduce their effective charge to the point of desorption. If a particular protein has a very high charge at the pH of the experiment, increase in ionic strength may never collapse the ionic double layer sufficiently to permit desorption. Such appears to be the case with peaks J and K which can only be eluted by raising the pH until the removal of hydrogen ions from the DEAE-cellulose removes its charge completely.

It is interesting to compare the procedures and results described in this paper with the many methods which have been proposed for the extraction and separation of the proteins contained in the wheat grain and its milled products (2–4,6,12,14,15,25). Many albumin components were observed by Pence and co-workers (26,29,30) with the use of paper electrophoresis, and Danielson (5) separated two different globulins in the ultracentrifuge. In our hands, paper electrophoresis has yielded streaky patterns inferior to, but supporting in general results, the patterns obtained by solution electrophoresis. The few ultracentrifugal analyses carried out supported the electrophoresis results, but again the resolution into individual components was inferior. However, all results indicate that a complex mixture of proteins is extracted from flour by salt solutions at neutral pH values. Replicate chromatographic analyses on DEAE-cellu-

lose have given consistently reproducible patterns showing the presence of some six components. Electrophoresis and rechromatography have increased this total to at least ten, and further studies may well reveal the presence of yet additional minor components. Much work will be required to determine the exact relationship of these components to those reported elsewhere in the literature.

The authors' experience has been that wheat proteins are particularly sensitive to denaturing influences such as high temperatures and pH values. The extraction procedure developed (Diagram I) attempts to overcome these difficulties. Extraction with sodium pyrophosphate followed by acetic acid allowed a clear distinction to be made between the two main groups of proteins present. Prompt fractionation of the pyrophosphate extract on DEAE-cellulose gave protein fractions more clearly resolved than those attainable by salt fractionation. Furthermore, the techniques can be scaled up or down to suit the quantities of material available for examination. Thus, microscale studies are being initiated on developing wheat endosperm and to investigate differences in the protein composition of different flours. Large-scale preparation of individual proteins is also being undertaken, so that their physical and chemical properties can be examined and a study of their role in the baking process can be made.

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