

PROPERTIES OF GLUTEN FRACTIONS  
PREPARED BY ION-EXCHANGE CHROMATOGRAPHY  
ON CARBOXYMETHYL-CELLULOSE

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*Summary*

Seven fractions obtained from wheat gluten by chromatography on carboxymethyl-cellulose were studied by ultracentrifugation, gel electrophoresis, chemical, and *N*-terminal amino acid analysis. On rechromatography, five fractions eluted by sodium chloride behaved as distinct entities. Ultracentrifuge experiments indicated that four of these were each undergoing rapid, reversible association. Several *N*-terminal amino acids were found in each of the fractions which, moreover, could be resolved by the gel technique into a number of electrophoretic bands, some bands being common to those of neighbouring fractions. Total nitrogen values showed the chromatographic samples to be essentially free from non-protein material.

I. INTRODUCTION

The proteins of wheaten flour principally responsible for the viscoelastic properties of dough are collectively called gluten. Osborne (1907) separated gluten into two components on the basis of solubility in aqueous alcohol (gliadin) and solubility of the residue in dilute alkali (glutenin). More recently it has been shown that chromatography and electrophoresis are capable of resolving gluten into a relatively large number of components. Simmonds and Winzor (1961) separated the acetic acid-soluble proteins (gluten) of flour into 11 fractions by chromatography on carboxymethyl-cellulose. This paper describes the partial characterization of these chromatographic fractions by rechromatography, sedimentation analysis, *N*-terminal amino acid analysis, gel electrophoresis, and chemical analysis.

II. MATERIALS AND METHODS

(a) *Flour Sample*

The flour sample used in these studies was obtained by milling Gabo wheat to 71% extraction in a Buhler laboratory mill.

(b) *Extraction of Flour*

Lipid and proteins soluble in neutral buffer were removed successively from the flour sample by preliminary extraction with water-saturated *n*-butanol, followed by extraction with 0.01M sodium pyrophosphate (adjusted to pH 7.0 with hydrochloric acid). The gluten proteins were then extracted with 0.05N acetic acid (Coates and Simmonds 1961). This extract was dialysed against 0.005M sodium acetate (adjusted to pH 4.1 with acetic acid) prior to column chromatography.

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### (c) Column Chromatography

The procedure of Simmonds and Winzor (1961) was scaled up some 25 times in order to prepare the fractions in sufficient quantities for subsequent investigation. Acetic acid-soluble proteins, containing about 2 g total nitrogen, were applied to a column (60 by 6 cm) containing 120 g carboxymethyl-cellulose (Whatman powder CM70) which had been equilibrated with 0.005M sodium acetate (pH 4.1). Elution of protein fractions was followed by measuring the absorption at 280 m $\mu$  (Fig. 1). The contents of the tubes corresponding to each chromatographic peak were pooled and the respective solutions dialysed against 0.005M sodium acetate (pH 4.1) prior to rechromatography.

Each of the chromatographic fractions eluted with salt (denoted as fractions B, C, D, E, and G in Fig. 1(a)) was freed from neighbouring fractions by repeating the above chromatographic procedure until a symmetrical peak was obtained. Fractions B and E required one, fractions C and D two, and fraction G three rechromatographic cycles. For the rechromatography of fraction G, a gradient from 0 to 0.5M sodium chloride replaced the usual combination of a 0.02M salt gradient and a 0.5M salt elution. The contents of the tubes corresponding to each purified peak were pooled, and the solution dialysed exhaustively against 0.05N acetic acid before freeze-drying.

### (d) Sedimentation

Each fraction was dissolved directly in the buffer used for ultracentrifugal studies (0.09M sodium chloride, 0.01M sodium acetate, 1.0M dimethyl formamide, pH adjusted to 4.1 with acetic acid) to yield a clear solution, which was then dialysed against the same buffer at 4°C with constant mild agitation for 24 hr. In the concentration-dependence estimations, solutions were prepared by weight dilution from these dialysed stock solutions, the concentrations of which were determined by the method of Lowry *et al.* (1951).

Ultracentrifugal experiments were performed at 20°C in a Spinco model E ultracentrifuge equipped with a phase-plate schlieren diaphragm, low-speed attachment, and R.T.I.C. unit. For molecular weight estimations the Klainer and Kegeles (1955, 1956) modification of the Archibald (1947) procedure was employed: measurements were made only at the meniscus. A value of 0.72 (Krejci and Svedberg 1935; Jones *et al.* 1961) was assumed for the partial specific volume of all fractions. Sedimentation velocity experiments were performed at 59,780 r.p.m., while a speed of 9,945 r.p.m. was used for the Archibald experiments. Diffusion coefficients were calculated from the sedimentation velocity and low-speed synthetic boundary runs by the method of Baldwin (1957).

### (e) Analytical Techniques

Quantitative protein determinations on bulked chromatographic effluents were performed using the method of Lowry *et al.* (1951). All other protein determinations were based on total nitrogen as determined by a microKjeldahl technique.

Chemical analyses of nitrogen, phosphorus, sulphur, ash, and loss in weight on drying were performed by the Australian Microanalytical Service, Melbourne.

The ignition temperature for ash determinations was 800°C, and the loss in weight on drying of the freeze-dried samples was determined after drying at 80°C over phosphorus pentoxide *in vacuo*. Nitrogen, phosphorus, sulphur, and ash values are given on a dry weight basis.

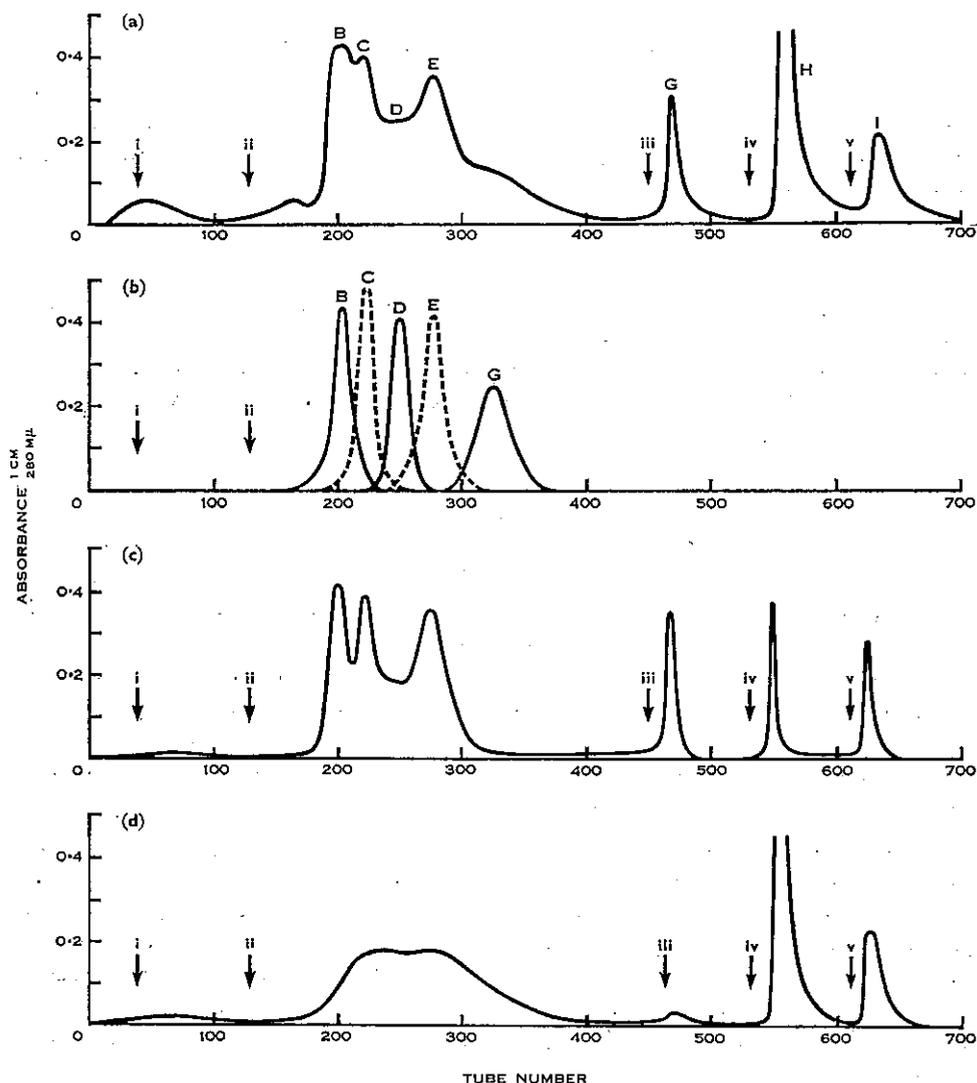


Fig. 1.—Elution patterns resulting from chromatography on carboxymethyl-cellulose. (a) Acetic acid extract of flour milled from Gabo wheat. (b) Fractions B, C, D, E, and G resulting from repeated rechromatography. For the rechromatography of fraction G a gradient from 0 to 0.5M sodium chloride was used. (c) Recombination of fractions B, C, D, E, and G. (d) First rechromatography of fraction H. Eluting solvents: *i*, 0.005M sodium acetate containing 1M dimethyl formamide (DMF) adjusted to pH 4.1 with acetic acid; *ii*, start of gradient from 0 to 0.2M sodium chloride in acetate-DMF; *iii*, 0.5M sodium chloride in acetate-DMF; *iv*, 0.005M sodium phosphate in 0.5M sodium chloride-1M DMF, adjusted to pH 12 with sodium hydroxide; *v*, 0.1N sodium hydroxide. Fraction size 30 ml.

Amide nitrogen determinations were performed by the method of Eastoe, Long, and Willan (1961). The sample was hydrolysed in 2N hydrochloric acid at 100°C for 1 hr and ammonia was measured in the hydrolysates by Conway's micro-diffusion technique.

#### (f) *N-Terminal Amino Acid Analysis*

*N*-terminal amino acids were determined by the fluorodinitrobenzene technique according to the procedure previously described (Winzor and Zentner 1962). In estimating the content of dinitrophenyl-amino acids, the values of Porter and Sanger (1948) were used to correct for losses during hydrolysis.

#### (g) *Acrylamide Gel Electrophoresis*

Electrophoresis was conducted in polyacrylamide gels containing 2M urea, pH 8·6, according to the procedure of Lee (1963). The voltage gradient was maintained at 50 volts/cm for 2 or 5 hr. Gels, after staining with water-soluble nigrosin, were scanned on a recording densitometer (Photovolt Corporation, New York) set on logarithmic response to give results essentially in terms of absorbance.

### III. RESULTS

#### (a) *Column Chromatography*

Figure 1(a) shows the protein elution profile obtained from the acetic acid extract of Gabo flour using the chromatographic procedure of Simmonds and Winzor (1961). The peaks that are labelled, namely B, C, D, E, G, H, and I, were subjected to repeated chromatography in an attempt to purify them of neighbouring fractions. Figure 1(b) shows that on rechromatography the salt-eluted fractions (B, C, D, E and G) appeared as symmetrical peaks at the same salt concentration as that at which they were originally eluted.

Fractions B, C, D, E, and G were recombined in the proportions in which they were originally eluted (Table 2). The chromatographic profile of this mixture (Fig. 1(c)) showed some marked differences from the original gluten pattern. The proteins eluted at the end of the salt gradient after fraction E were missing from the reconstituted gluten profile and the valley between peaks B and C suggests that a minor component, normally eluted between these two fractions, has been removed during their rechromatography. However, the five fractions applied were eluted at their characteristic salt concentrations in approximately the expected proportions.

When fraction H was subjected to rechromatography (Fig. 1(d)), the bulk of the protein was eluted by a gradient to 0·2M sodium chloride. During the first rechromatography of fraction H, 25 and 5%, respectively, of the total protein were eluted by the phosphate buffer and by 0·1N sodium hydroxide. After a second and third rechromatography of peak H, only about 3% of the original gluten protein persisted as material eluted by the phosphate buffer. On rechromatography

of fraction I, again a large proportion of the adsorbed protein was eluted by the salt gradient, leaving some 40 and 5% for elution by the phosphate buffer and by 0.1N sodium hydroxide, respectively. The protein profile obtained with a salt gradient on rechromatography of fraction H (Fig. 1(d)) bore little resemblance to the original gluten pattern (Fig. 1(a)). This change probably resulted from the prolonged contact of the fraction H material with the alkaline-eluting solvent after initial chroma-

TABLE 1  
SEDIMENTATION DATA FOR WHEAT PROTEIN FRACTIONS IN ACETATE-CHLORIDE-DIMETHYL-  
FORMAMIDE BUFFER, pH 4.10, IONIC STRENGTH 0.10

Fraction	Initial Concentration (g/100 ml)	$S_{20,w}$ (svedberg)	$(D_{app.})^*$ $\times 10^7$ ( $\text{cm}^2 \text{sec}^{-1}$ )	$(D_{app.})^\dagger$ $\times 10^7$ ( $\text{cm}^2 \text{sec}^{-1}$ )	Apparent Molecular Weight‡
B	0.57	2.4 <sub>0</sub>	6.2 <sub>0</sub>	3.8 <sub>1</sub>	59,000 ± 2,000
	0.34	2.3 <sub>1</sub>			
	0.14	2.2 <sub>7</sub>			
	0.08	2.2 <sub>4</sub>			
C	0.65	2.4 <sub>7</sub>	7.6 <sub>2</sub>	4.2 <sub>6</sub>	55,000 ± 2,000
	0.16	2.2 <sub>2</sub>			
	0.09	2.2 <sub>0</sub>			
D	0.82	2.2 <sub>7</sub>	7.6 <sub>9</sub>	4.1 <sub>1</sub>	51,000 ± 2,000
	0.56	2.2 <sub>5</sub>			
	0.29	2.3 <sub>0</sub>			
	0.09	2.1 <sub>5</sub>			
E	0.66	2.7 <sub>9</sub>	10.3 <sub>0</sub>	4.0 <sub>4</sub>	63,000 ± 2,000
	0.45	2.5 <sub>7</sub>			
	0.30	2.3 <sub>2</sub>			
G	0.51	1.3 <sub>8</sub>	4.6 <sub>1</sub>	4.5 <sub>3</sub>	28,000 ± 1,000 (24,000)§
	0.34	1.4 <sub>4</sub>			
	0.21	1.4 <sub>1</sub>			
	0.07	1.4 <sub>2</sub>			

\* Apparent diffusion coefficient calculated from sedimentation velocity experiment.

† Apparent diffusion coefficient calculated from diffusion experiment performed in the ultracentrifuge at low speed.

‡ Derived from synthetic boundary area and Archibald plate measurements. The range of calculated values is indicated in each instance.

§ Molecular weight found by combining  $S$  and  $D$  in the Svedberg equation.

tography. On elution with a salt gradient a similar protein profile was exhibited by a gluten extract that had been dialysed against 0.1N sodium hydroxide for several days prior to chromatography.

After rechromatography of each of the salt-eluted peaks approximately 5% of the adsorbed protein in each case remained on the column after salt elution and could only be desorbed by alkaline solvents. Furthermore Figure 1(c) shows that,

on rechromatography of a recombination of fractions B, C, D, E, and G, a small amount of protein remained on the column after salt elution to appear as peak H (6% of adsorbed protein) and peak I (2%).

A further example of the apparent interchange of protein between fractions H and I and the salt-eluted fractions was observed when the chromatographic separation was performed at a range of temperatures. When the separation was conducted routinely (at a room temperature of about 25°C), the alkali-eluted fractions accounted for about 30% of the total gluten proteins. However, at 5°C, fractions H and I represented 55% of the gluten proteins, and at 45°C, these two fractions

TABLE 2  
CHEMICAL ANALYSIS OF CHROMATOGRAPHIC FRACTIONS

Fractions B, C, D, E, and G denote the preparations resulting from rechromatography. Fractions H and I refer to preparations that have not been subjected to rechromatography. The fraction denoted "H-salt" is the material eluted by salt during the first rechromatography of fraction H. Fraction H-phosphate is material that, after repeated rechromatography, is eluted by the phosphate buffer

Fraction	Percentage of Gluten Extract	Percentage Loss on Drying	Nitrogen (%)	Sulphur (%)	Amide Nitrogen (as % of total N)
B	15	7.0	17.9	1.5	26.5
C	12	6.5	17.7	1.5	24.6
D	7	7.9	17.2	1.8	23.8
E	15	5.5	17.1	1.5	22.9
G	6	7.8	16.4	2.0	12.2
H	25	8.7	16.9	1.8	23.7
I	5	7.7	15.9	1.9	—
H-salt	(17)	6.6	16.9	1.7	25.1
H-phosphate	(3)	6.5	15.1	1.6	21.6
Pyrophosphate extract		9.5	9.0	1.4	10.7
Acetic acid extract		6.7	17.3	1.6	22.0

accounted for 15% of the adsorbed protein. Despite these fluctuations in fractions H and I, the shape of the protein profile obtained with a salt gradient remained approximately the same throughout this range of temperatures.

#### (b) Ultracentrifugal Experiments

All of the results are summarized in Table 1. The sedimentation coefficients (column 3) have been determined from the movement of the maximum ordinate of the peak, since fairly symmetrical boundaries were obtained in all of these experiments. Where tested, location of the boundary position by using the square root of the second moment (Baldwin 1957) yielded essentially identical sedimentation coefficients. Positive concentration dependence of sedimentation coefficient, evident

from columns 2 and 3 of Table 1 for fractions B, C, D, and E, suggests that they are interacting systems involving rapid association-dissociation equilibria (Schwert 1949; cf. however, Rupley and Neurath 1960; Harmison, Landaburu, and Seegers 1961). The large differences between the apparent diffusion coefficients calculated from high- and low-speed ultracentrifuge experiments (columns 4 and 5) further suggest that each of these fractions is a chemically reacting system (Field and O'Brien 1955; Field and Ogston 1955), although these differences may be due in part to the heterogeneity of the fractions, shown by gel electrophoresis and end-group analysis. The results obtained with fraction B are treated more fully elsewhere (Winzor 1963). No evidence of association behaviour was observed with fraction G as is shown by the agreement between the two diffusion coefficients and the lack of any observable concentration dependence of sedimentation coefficient. Molecular

TABLE 3  
N-TERMINAL AMINO ACID COMPOSITION OF CHROMATOGRAPHIC FRACTIONS

Fraction	Content of <i>N</i> -Terminal Amino Acid ( $\mu$ moles dinitrophenyl-amino acid/g dinitrophenyl-protein)							
	Valine	Aspartic Acid	Glutamic Acid	Histidine	Threonine	Serine	Alanine	Methionine
B		5.4				trace		trace
C	7.0		3.3	2.5				
D		3.1		5.0	trace	trace		
E	6.4	trace	trace		trace			
G	trace	0.6			0.8	trace	trace	
Acetic acid extract	2.9	1.6	0.7	0.7	trace	trace	trace	

weight estimations on the fractions at the concentrations indicated are shown in column 6. Although the values obtained from the later stages of these experiments were generally slightly lower than those from earlier exposures, the trend was only very slight, and throughout the 3-hr duration of each experiment all the values were within the stated range.

(c) *Chemical Analysis*

Table 2 shows the results of chemical analysis of the freeze-dried chromatographic fractions. The low total nitrogen content of the pyrophosphate-soluble material (dialysed against 0.05N acetic acid before freeze-drying) is probably due to a gross contamination of the protein with high molecular weight polysaccharide material. The sulphur content of the gluten preparations varied only from 1.5 to 2.0%. (If the sulphur of the pyrophosphate-soluble material was associated with the protein only, the value of 1.4% based on the dry weight would represent about 2.7% sulphur based on protein only.) The ash contents of all chromatographic

fractions were low ( $<0.3\%$ ) as were the levels of phosphorus ( $<0.01\%$ ). The low amide nitrogen content of fraction G was also found in the pyrophosphate-soluble material.

(d) *N-Terminal Amino Acid Analysis*

Each of the salt-eluted fractions contained a number of *N*-terminal amino acids. A quantitative estimate of the major end groups is given in Table 3, but each fraction also contained traces of further *N*-terminal amino acids.

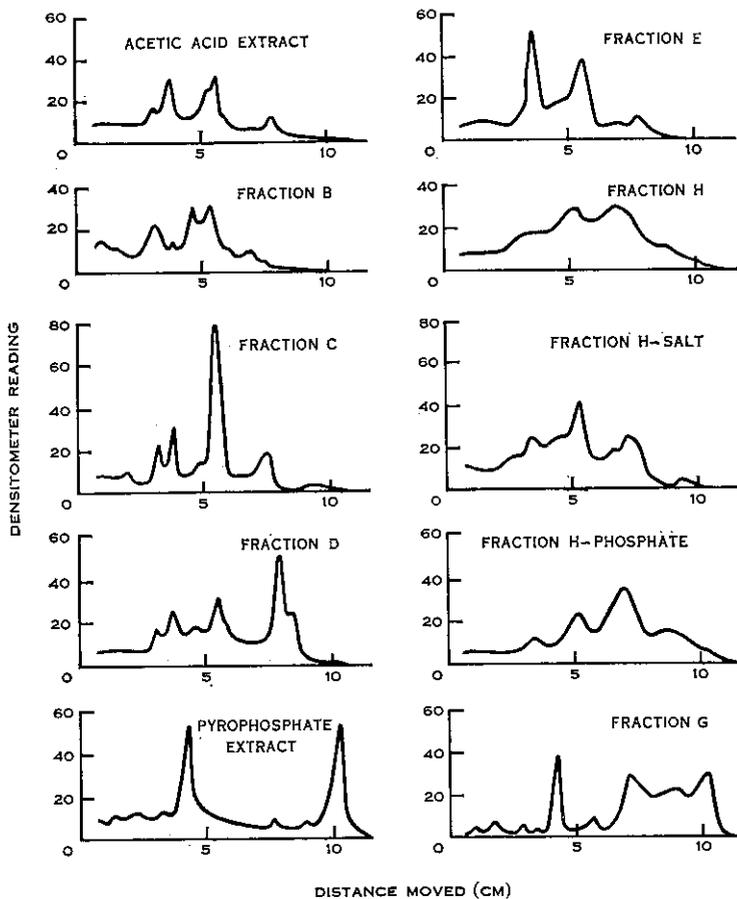


Fig. 2.—Densitometer scans of polyacrylamide electropherograms of the chromatographic fractions (denoted as in Table 2). Time of running for pyrophosphate-soluble protein and fraction G, 2 hr at 50 volts/cm; all other gels, 5 hr at 50 volts/cm. Gels were stained with water-soluble nigrosin and the densitometer set for logarithmic response.

(e) *Gel Electrophoresis*

Densitometer scans of stained polyacrylamide electropherograms of each of the chromatographic fractions are shown in Figure 2. It is obvious that all of the fractions show the presence of a number of protein bands and there is considerable overlapping of bands between adjacent chromatographic fractions.

## IV. DISCUSSION

Simmonds and Winzor (1961) showed that, on rechromatography, each of four of the salt-eluted fractions from carboxymethyl-cellulose columns gave essentially a symmetrical peak, each being eluted at its characteristic salt concentration. Each of the five salt-eluted fractions described in this paper also appeared on rechromatography at its characteristic salt concentration. Each of these fractions, then, represents a distinct, though not necessarily homogeneous, entity. On rechromatography, the material eluted by phosphate buffer (fraction H) behaves differently, the major part being eluted by the salt gradient and a smaller part appearing as "true" phosphate peak. The reason for this behaviour of fraction H is not understood. Possibly part of the original sample is adsorbed onto the carboxymethyl-cellulose column by some type of non-ionic binding, or alternatively some of the material which is adsorbed is modified by the alkaline (pH 12) phosphate buffer giving rise to salt-gradient material and true phosphate fraction. It is of interest that both the electrophoretic mobilities and amide nitrogen levels of fraction H and of the material eluted by the salt gradient are of the same order.

The sedimentation data indicate that under the conditions used fractions B, C, D, and E, comprising the greater part of the gluten fraction of flour, are each undergoing rapid reversible association. The failure to observe any marked time-dependence of molecular weight values in the Archibald experiments with these systems is due to the fact that the concentration at the meniscus remained essentially unaltered because of the slow rate of sedimentation of the gluten fractions. The molecular weights of the unassociated species of fractions B, C, D, and E cannot be deduced from this study but they will obviously be lower than the values given in Table 1.

The results of the nitrogen determinations in Table 2 show that each of the chromatographic fractions was relatively free from non-protein material. Although there is little variation in the level of sulphur between fractions, results are included because of the importance of the thiol-disulphide groups in the physical behaviour of doughs. The amide nitrogen constitutes a slightly decreasing proportion of the total nitrogen with each successive fraction eluted from the chromatographic column by the salt gradient. Fraction G eluted by 0.5M sodium chloride shows a much lower proportion of amide nitrogen than do the preceding fractions. The value of 12.2% for fraction G is much closer to the value of 10.7% found for the pyrophosphate-soluble protein than to the higher values found in what might be termed "true"gluten.

The main conclusion to be drawn from the electrophoretic patterns of the fractions is that each is grossly heterogeneous. With the exception of fraction G all fractions showed a characteristic low mobility, probably caused by the fact that the acidic amino acids are largely present as their amides and thus being essentially uncharged at the pH of the experiment. Not only is each fraction heterogeneous but there is considerable overlapping of protein bands from neighbouring fractions. Fraction G has a much higher electrophoretic mobility than the other fractions and its scan is similar to that of the pyrophosphate-soluble proteins. It may be that

the presence of fraction G in the chromatographic profile is indicative of contamination of the acetic acid-soluble proteins with material incompletely removed by the preliminary pyrophosphate extraction. Starch-gel electrophoresis in aluminium lactate buffer at pH 3.2 has also shown heterogeneity for all the chromatographic fractions. Although results are not presented in this paper fraction G again appeared very similar to the pyrophosphate-soluble proteins on starch-gel electrophoresis.

There is further evidence of heterogeneity in the chromatographic fractions in the studies of their *N*-terminal amino acids. Calculated from the molecular weight of each fraction listed in Table 1 the yield of any one dinitrophenyl-amino acid from dinitrophenyl-protein is considerably less than equimolar, and since most of the fractions appear to be reversibly associating systems, the yield of *N*-terminal amino acid per monomeric protein molecule is even lower. This presumably indicates greater hydrolysis and other losses than have been allowed for in the calculations. At this stage it is difficult to associate any *N*-terminal amino acid with any electrophoretic peak but the results do emphasize the heterogeneous nature of the chromatographic fractions.

Despite the heterogeneity of the fractions, chromatography of gluten still appears to be a useful technique in that reproducible profiles are consistently obtained from the same variety of wheat and these profiles vary from one variety to another (Lee and Wrigley 1963). Moreover, of the techniques available, it is one of the most suitable for preparative work.

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