THE INCORPORATION OF CYSTEINE-^{35}S, CYSTINE-^{35}S, and N-ETHYLMALEIMIDE-^{14}C INTO DOUGHS MADE FROM WHEAT FLOUR[1]

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ABSTRACT

The incorporation of cysteine-^{35}S, cystine-^{35}S, and N-ethylmaleimide-^{14}C into gluten and the soluble proteins of doughs made from wheat flour has been studied. The specific activities of the proteins have been compared. The gluten complex has been fractionated by ion-exchange chromatography and the amino acid composition and specific activities of the gluten fractions reported. The bearing of these results on the theory of disulfide-sulfhydryl interchange in dough is discussed.

Disulfide-sulfhydryl interchange was suggested by Goldstein (1) as the mechanism underlying the rheological properties of dough. Since that time, much evidence (2–9, 19), largely indirect, has been accumulated to support this mechanism. The development of a technique for the fractionation of dough (10, 11) enabled the direct demonstration of disulfide-sulfhydryl interchange to be made by following the distribution of cysteine-^{35}S added during mixing of the dough (10, 12). The present work extends these observations to doughs made from a variety of flours by using both cysteine-^{35}S and cystine-^{35}S and compares the specific activities of the various protein fractions.

Materials and Methods

Flour Samples. White flours from four Australian wheats were used. The properties of these have been described earlier (11).

Isotopes. L-Cystine-^{35}S, specific activity 41 mc/mM, and N-ethylmaleimide-^{14}C (2,3^{14}C), specific activity 15.9 mc/mM, were obtained from the Radiochemical Centre, Amersham, England.

L-Cysteine-^{35}S was prepared by electrolytic reduction of L-cystine-^{35}S in 0.5*N* sulfuric acid; a water-cooled, circulating mercury cathode and a platinum anode separated by a membrane were used. Electrolysis was carried out for 2 hr. at 6.4 amps and 12 volts. Previous experiments had demonstrated complete reduction of cystine to cysteine under these conditions with over-all recoveries of 87–93%. Both cystine and cysteine were stored, frozen, in 0.5*N* sulfuric acid, and neutralized just prior to incorporation in dough.

N-Ethylmaleimide-^{14}C (NEMI-^{14}C) was dissolved in ethanol 25μc/ml. The isotopically labeled NEMI (0.6 ml.) was mixed with 0.5 ml. of carrier NEMI (200 μM/ml.) before addition of dough.

Incorporation of Isotope in Dough. Doughs were mixed in a Brabender Farinograph fitted with a 50-g. stainless steel mixing bowl. The bowl was equipped with a tight-fitting Perspex lid carrying holes for inlet and outlet of

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gas and for addition of salt solutions. This permitted doughs to be mixed under oxygen-free nitrogen.

The flour (50 g.) was mixed under nitrogen for 5 min. before the addition of isotope in the salt solution. The salt solution was prepared by neutralizing cysteine-^{35}S or cystine-^{35}S and mixing with 0.5*M* sodium chloride. A portion of this solution was diluted and 0.20-ml aliquots were plated for counting; a second portion of the salt-isotope solution, warmed to 30°C. and containing 40 μc of isotope, and whose volume was equivalent to the water absorption figure of the flour, was then added to the flour and mixed until maximum consistency was obtained (2.5–8 min.). When two isotopically labeled compounds were used, the first was included in the salt solution and the dough was mixed for 4 min. and rested 10 min.; the second isotope was then added and mixing continued for a further 3 min. In these cases the final total volume of isotope plus salt-isotope solution equaled the water absorption figure.

Dough Fractionation. After resting for 45 min. at 30°C., the doughs were fractionated by preparative ultracentrifugation at 105,000 *g* (av.) for 60 min. in a Spinco Model L ultracentrifuge as described earlier (10,11).

After fractionation, the lipid layer was removed and the dough liquor decanted into tared flasks and weighed. Sufficient 0.5*M* sodium chloride was then added to effect a fivefold dilution (sp. gr. dough liquor = 1.06).

Samples of the diluted dough liquor were further diluted for plating and counting; others were percipitated by mixing with an equal volume of 10% phosphotungstic acid or were used for equilibrium dialysis against 5 volumes of 0.5*M* sodium chloride or for exhaustive dialysis against salt or water.

Albumin and Globulin Fractions. These were originally assayed by exhaustive dialysis of diluted dough liquor against both 0.5*M* sodium chloride and water with removal of insoluble material by centrifugation at 8000 x *g* for 30 min., the clear supernatants then being assayed for both protein and radioactivity. It was found, however, that the water-soluble fraction which should have contained only albumin had a higher total activity than the salt-soluble fraction, which should have contained both albumins and globulins. Investigation showed that the higher activity of the water-soluble fraction was due to the solubilization of suspended gluten in the original dough liquor. Albumin and globulin fractions were therefore determined by dialysis against salt solution, followed by removal of insolubles and assay of the supernatant for protein and radioactivity. The salt was then removed from the remainder of the supernatant by dialysis against water, the precipitated globulins were sedimented, and the supernatant (albumin fraction) was again assayed for protein and radioactivity.

Chromatography of Gluten. The tubes containing the remainder of the sedimented dough were frozen and the main gluten layer, layer III (11), sliced free from contiguous layers II and IV. A weighed amount of the gluten layer (10–15 g.) was transferred to 36/32-in. Visking dialysis tubing along with 100 ml. of 0.01*N* formic acid, and the suspension was dialyzed for 2–3 days at 2°C. against frequent changes of 0.01*N* formic acid. Suspended starch was removed by centrifugation and the gluten separated from contaminating soluble pro-

teins by precipitation with salt followed by re-solution in 1*M* dimethylformamide (DMF).

The gluten complex was fractionated by chromatography on carboxymethyl cellulose (CMC) by the procedure of Simmonds and Winzor (13) as modified by Wrigley (14).

The columns, which had a packed bed volume of 100 ml., were water-jacketed and operated at 40°C. The gluten solution (approx. 200–300 mg./100 ml.) in 0.005*M* sodium lactate, lactic acid buffer pH 4.1 and 1.0*M* with respect to DMF, was absorbed onto CMC which had been equilibrated with the same buffer.

The gluten was eluted by the following successive steps: (a) sodium chloride gradient 0–0.1*M* in 0.005*M* sodium lactate pH 4.1 and 1*M* DMF; (b) sodium chloride gradient 0.1–0.3*M* in 0.005*M* sodium lactate pH 4.1 and 1*M* DMF; (c) stepwise elution with 0.005*M* potassium pyrophosphate (pH 11.8) in 1*M* DMF; and (e) stepwise elution with 0.05*M* sodium hydroxide. The volumes of the fractions collected varied slightly from run to run, but were usually between 5 and 7 ml. per tube.

Protein Estimations. The protein content of fractions was determined both by direct spectrophotometry at 280 mμ and by the modified Folin-biuret procedure of Lowry *et al.* (15).

After suitable dilution, samples, 0.20 ml., were plated in quadruplicate onto 1-in. circles of Whatman No. 1 filter paper in 1-in. stainless-steel planchets. All planchets were counted 4 to 5 times to a minimum count of 5,120; a Nuclear Chicago Model 183 B scaler with automatic sample-changer and print-out was used. Under these conditions 1.0 μc of cystine-^{35}S gave 1.52×10^5 c.p.m. Radioactivity measurements were corrected for background and decay sulfur-^{35}S.

Gluten fractions from column chromatography were first dialyzed against 0.01*N* formic acid to remove DMF, and 1.0 ml. samples (x 2 or 4) were then plated onto 1¼-in.-diam. stainless-steel planchets with concentric rings.

Amino Acid Analysis. Gluten fractions for analysis were dialyzed against 0.01*N* formic acid and lyophilized. Samples, usually 10 mg., were dissolved in 2.0 ml. of constant-boiling, glass-distilled hydrochloric acid, then frozen, sealed *in vacuo,* and hydrolyzed for 22 hr. at 110 ± 0.5°C.

The hydrolysates were dried *in vacuo* over solid sodium hydroxide and dissolved in an appropriate volume of buffer; the amino acid composition was determined by the method of Spackman, Stein, and Moore (16), with a Spinco Model 120 B amino acid analyzer.

Calculations. Four radioactivity measurements were used to calculate the distribution of added isotope in the various dough fractions. These were the activities of 1) the original salt solution, 2) the dough liquor, 3) the diffusate from equilibrium dialysis, and 4) the filtrate from phosphotungstic acid precipitation.

The activity of the salt solution, added to the flour, was corrected for dilution and for the moisture content of the flour. This gave the theoretical activity of the dough liquor if no absorption by gluten or other insolubles had

taken place. The dough liquor activity was corrected for dilution and expressed as a percentage of this theoretical, original activity from which the percentage activity bound to gluten could be obtained.

The diffusible material and phosphotungstic acid-soluble material were similarly expressed as a percentage of the original activity. The difference between the percentage activities of dough liquor and diffusible material gave the percentage of isotope bound to soluble proteins. Similarly, the percentage of isotope in the phosphotungstic acid-insoluble material was obtained by the difference beween diffusible and phosphotungstic acid-soluble figures. The experimental basis of these calculations has been justified earlier (12).

Where double labeling with cysteine-^{35}S and NEMI-^{14}C was used, the planchets were counted on three separate occasions at 6-week intervals. From the decay of standard ^{35}S, it was possible to calculate the activity (and hence distribution) of both ^{14}C and ^{35}S at the first time of counting. Unfortunately, this method of counting magnifies any counting errors and, while the standard deviation of counting on a large series was only $\pm$ 3%, the activity of the NEMI-^{14}C was not as high as originally stated by the Radiochemical Centre and therefore the initial ratio of ^{35}S/^{14}C was greater than intended. The results from the double labeling experiments should, accordingly, be interpreted with some caution.

Specific activities of protein fractions were calculated on the basis of count/min./unit absorbance at 750 mμ of the color developed in the Folin-biuret procedure.

Results

One of the aims of the present study was to compare the incorporation of cysteine-^{35}S and cystine-^{35}S into proteins of dough. For this reason, all doughs were mixed under nitrogen, for it had been shown earlier (12) that oxidation of cysteine-^{35}S to cystine-^{35}S could be effectively minimized, but not eliminated, by mixing under nitrogen.

The distribution of added cysteine-^{35}S and cystine-^{35}S in fractions of doughs made from four different flours is shown in Table I. The four flours used

TABLE I
COMPARISON OF THE PERCENTAGE DISTRIBUTION OF ADDED CYSTEINE-^{35}S AND CYSTINE-^{35}S IN DOUGH FRACTIONS FROM DIFFERENT FLOURS

Flour	Isotope Added	Percentage of Added Isotope in Fraction				
			Soluble Protein		Diffusible Peptides	
		Gluten	A[a]	B[b]	PTA-Soluble	PTA-Insoluble
Lawrence	Cysteine-^{35}S	48.8	13.4	10.8	21.8	16.0
Gabo		38.0	17.7	15.2	23.4	20.9
Durral		33.3	20.7	16.5	24.5	21.5
Glenwari		30.1	23.0	18.8	26.7	20.2
Gabo	Cystine-^{35}S	35.5	21.7	16.9	10.8	32.0
Durral		32.1	19.0	15.3	11.5	37.4
Glenwari		26.3	24.3	18.8	9.3	40.1

[a] Calculated indirectly from equilibrium dialysis.
[b] Calculated directly from activities of dough liquor and nondiffusible soluble material after exhaustive dialysis of dough liquor against 0.5*M* sodium chloride.

differed in quality, as judged by their rheological behavior, in the order Lawrence>Gabo>Durral>Glenwari. It can be seen from Table I that the percentage of added cysteine-^{35}S incorporated into the gluten fraction of the doughs decreased in the same order. Conversely, the percentage of added isotope in the soluble protein fraction increased in the same order. The percentage distribution of cysteine was in good general agreement with that observed earlier (12) for doughs mixed under nitrogen.

The diffusible portion of dough liquor was divided into phosphotungstic acid-soluble and -insoluble fractions which correspond, roughly, to cysteine and cystine respectively, and it will be seen that, despite the mixing of the dough under nitrogen, a considerable proportion of added cysteine had been converted to cystine.

The values for soluble protein in columns A and B (Table I) differed consistently, the figures for soluble protein calculated indirectly from equilibrium diffusates (A) being about 20% higher than the values obtained directly (B) by exhaustive dialysis against 0.5*M* sodium chloride. Previously (12) it was shown that the distribution of leucine-^{14}C on equilibrium dialysis of dough liquor was unaffected by a Gibbs-Donnan effect, and that therefore the method of calculation of isotope distribution was valid. However, the indirect calculation makes no allowance for suspended gluten now shown to be present. Thus the values for soluble protein given in column B are almost certainly the more accurate, and the figures for gluten should be corrected upward by the difference between A and B.

The percentage distribution of added cystine-^{35}S in dough fractions followed the same general pattern as was observed with cysteine. The percentage of isotope incorporated into gluten was, however, smaller than that observed with cysteine-^{35}S, whereas the percentage incorporation into soluble proteins was slightly greater than with cysteine. The figures for the diffusible fractions, PTA-soluble and insoluble, representing cysteine and cystine, were, of course, reversed.

Similar percentage distribution figures for the fractions of Gabo dough are shown in Table II from experiments in which NEMI-^{14}C was added; in which the addition of cysteine-^{35}S was followed by swamping with 100 μM of NEMI-^{14}C and in which swamping with 100 μM of NEMI-^{14}C was followed by addition of cystine-^{35}S. For convenience, the figures for straightforward additions of cysteine-^{35}S and cystine to Gabo dough are reproduced from Table I.

Both NEMI and cystine reacted with "available" –SH groups, but, in contrast to cystine, the percentage of NEMI incorporated into soluble proteins was low whereas that into gluten was still fairly high.

The two experiments were not, however, strictly comparable, for while NEMI-^{14}C was added in the presence of 100 μM of carrier NEMI, the cystine-^{35}S was only diluted by endogenous, diffusible disulfide estimated as a maximum of 6 μM.

In the experiment in which the addition of cysteine-^{35}S was followed by swamping with 100 μM of NEMI-^{14}C—which would effectively block all available –SH and halt any further disulfide-sulfhydryl interchange (20)—the percentage of cysteine-^{35}S incorporated into gluten was 80% of the value

TABLE II

DISTRIBUTION OF ADDED ISOTOPES IN VARIOUS FRACTIONS OF DOUGHS MADE FROM GABO FLOUR

Expt. No.	Isotope Added		Percentage of Added Isotope in Fraction							
			Gluten		Soluble Protein[a]		Diffusible Fraction			
							PTA-Soluble		PTA-Insoluble	
	First	Second	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C
1	Cysteine-^{35}S	....	38.0	....	17.7	....	23.4	....	20.9	....
2	Cystine-^{35}S	....	35.5	....	21.7	....	10.8	....	32.0	....
3	NEMI-^{14}C	....	....	44.0	....	5.0	....	24	....	27
4	Cysteine-^{35}S	NEMI-^{14}C	31	42	8	6	33	25	28	27
5	NEMI-^{14}C	Cystine-^{35}S	8	40	4	4	8	26	80	30

[a] Calculated indirectly from equilibrium diffusate.

obtained with cysteine alone. The corresponding figure for soluble protein was less than half that obtained with cysteine alone. This suggests that, normally, the incorporation of cysteine-^{35}S into gluten is rapid and occurs during mixing, whereas that into soluble protein is slower and occurs on standing.

Where the addition of cystine-^{35}S to Gabo dough followed initial swamping with NEMI-^{14}C, little ^{35}S- was incorporated (Table II), for the bulk of the available –SH groups had been blocked by NEMI. Nevertheless, some cystine-^{35}S was incorporated into protein, presumably, by competition between the two –SH reagents for the surviving "available" sulfhydryl.

The specific activities of the various protein fractions in terms of the Folin-biuret reaction are presented in Table III. The figures for cysteine and cystine have been rendered comparable by correction to the same initial addition of isotope. It can be seen that the specific activities of the gluten fraction from all doughs, whether cysteine or cystine was the additive, were higher than the corresponding figures for the soluble proteins.

The specific activities of the gluten fraction from cysteine-treated doughs increased in the order Lawrence<Gabo<Durral<Glenwari; this suggests

TABLE III

SPECIFIC ACTIVITIES (IN TERMS OF FOLIN-BIURET ABSORBANCE) FOR GLUTEN AND SOLUBLE PROTEIN FRACTIONS FROM VARIOUS DOUGHS

Flour	Isotope Added	Specific Activity			
		Gluten Fraction	Salt-Soluble	Albumin Fraction	Globulin[a] Fraction
		counts/min./unit absorbance at 750mμ			
Lawrence	Cysteine-^{35}S	446	288	243	350
Gabo		450	410	429	393
Durral		544	326	375	268
Glenwari		590	503	489	531
Gabo	Cystine-^{35}S	615	437	428	445
Durral		480	315	350	273
Glenwari		530	523	480	597

[a] Globulin fraction calculated by difference from salt-soluble fraction and water-soluble (albumin) fraction.

that the disulfide groups of Glenwari were more susceptible to disulfide-sulfhydryl interchange than those of Lawrence. The same correlation was not observed with the gluten fractions from doughs treated with cystine-^{35}S.

The specific activities of gluten from cystine-treated doughs were con-

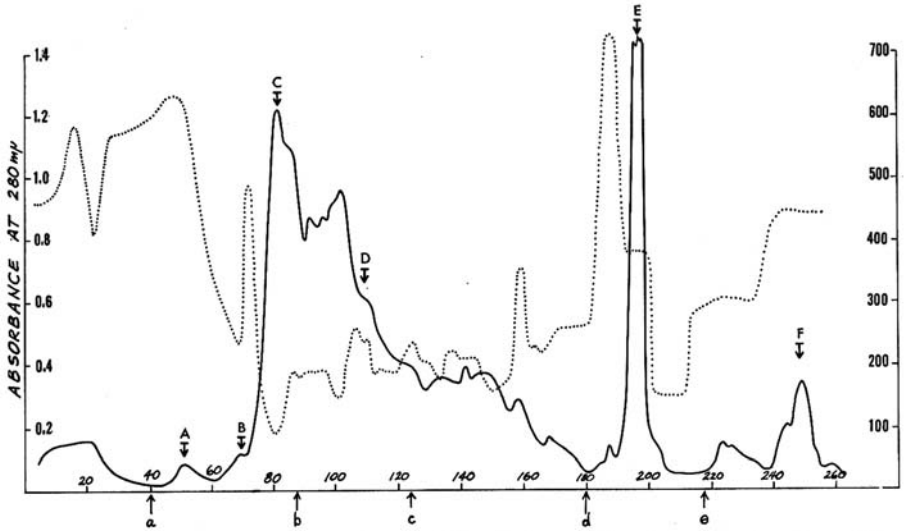

Fig. 1. Chromatographic separation on CM-cellulose of the gluten fraction from Gabo dough mixed with cysteine-^{35}S: solid line, protein concentration in absorbance units at 280 mμ; broken line, specific activity of protein (Folin-biuret reaction) arrows indicate position and width of fractions A to F analyzed for amino acid composition; a to e, start of elution steps. See text for details.

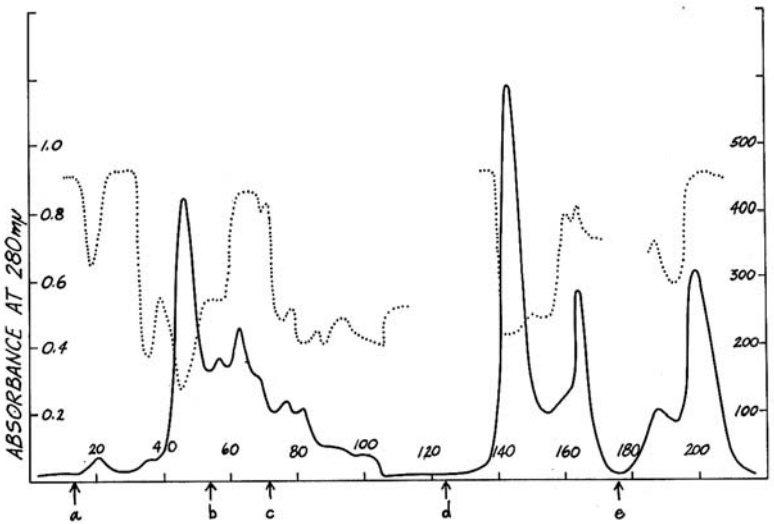

Fig. 2. Chromatographic separation on CM-cellulose of the gluten fraction from Gabo dough labeled with cystine-^{35}S: solid line, protein concentration in absorbance units at 280 mμ; broken line, specific activity of protein (Folin-biuret reaction); a to e, start of elution steps. See text for details.

sistently higher than the corresponding figures from dough treated with cysteine-^{35}S. As there are approximately 25 disulfide bonds for each sulfhydryl group in gluten (17), these figures would suggest that the sulfhydryl groups of gluten are more reactive toward disulfide-sulfhydryl interchange than the disulfide bonds.

The specific activities of the soluble proteins, although interesting, gave no regular correlation either between albumin and globulin fractions or between cystine-treated and cysteine-treated doughs.

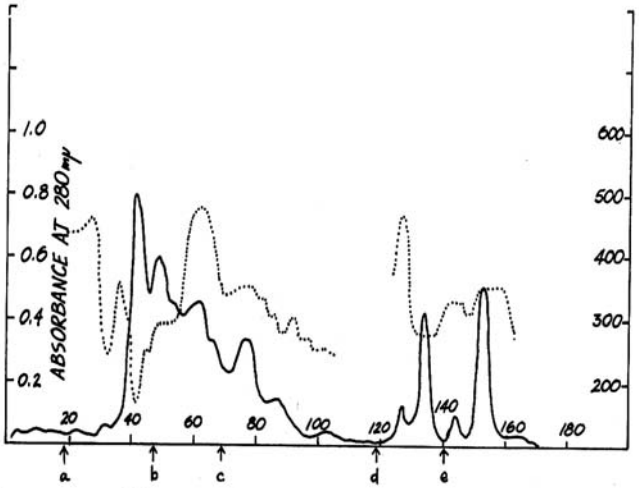

Fig. 3. Chromatographic separation on CM-cellulose of the gluten fraction from Glenwari dough labeled with cystine-^{35}S: solid line, protein concentration in absorbance units at 280 mμ; broken line, specific activity of protein (Folin-biuret reaction); a to e, start of elution steps. See text for details.

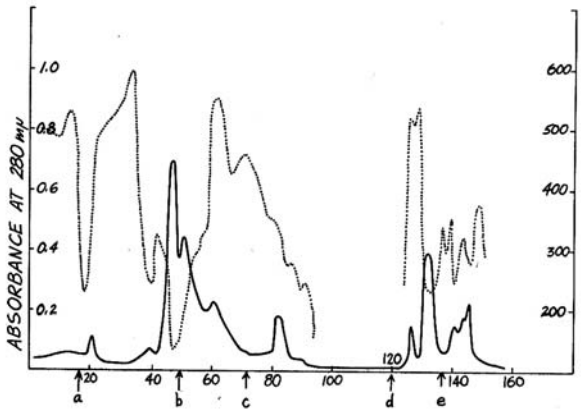

Fig. 4. Chromatographic separation on CM-cellulose of the gluten fraction from Glenwari dough labeled with cystine-^{35}S: solid line, protein concentration in absorbance units at 280 mμ; broken line, specific activity of protein (Folin-biuret reaction); a to e, start of elution steps. See text for details.

The gluten fractions from the doughs were further fractionated by ion-exchange chromatography to determine whether cystine-^{35}S and cysteine-^{35}S were incorporated uniformly into gluten proteins.

The elution patterns of gluten from Gabo and Glenwari doughs treated with cysteine-^{35}S, cystine-^{35}S, and NEMI-^{14}C, are shown in Figs. 1 to 5.

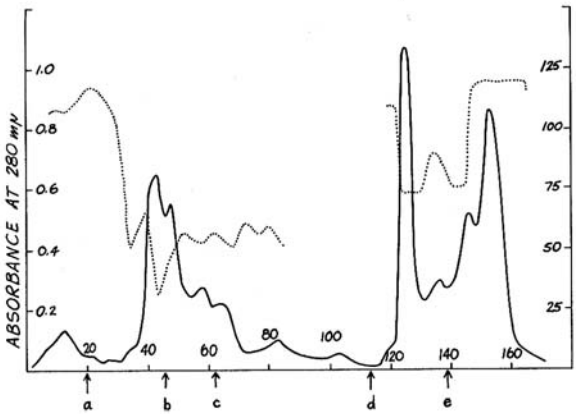

Fig. 5. Chromatographic separation on CM-cellulose of the gluten fraction from Gabo dough labeled with NEMI-^{14}C: solid line, protein concentration in absorbance units at 280 mμ; broken line, specific activity of protein (Folin-biuret reaction); a to e, start of elution steps. See text for details.

TABLE IV

AMINO ACID COMPOSITION OF GLUTEN FRACTIONS OBTAINED BY ION-EXCHANGE CHROMATOGRAPHY OF THE GLUTEN FROM A DOUGH MIXED WITH GABO FLOUR

	AMINO ACID (As percentage of total μmoles [a])					
FRACTION	A	B	C	D	E	F
	μmoles	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Lysine	0.8	0.7	0.5	0.7	1.0	1.0
Histidine	1.0	0.7	1.2	1.5	2.1	2.4
Arginine	1.0	0.9	1.2	2.1	2.5	3.2
Ammonia	35.0	36.7	35.8	29.2	28.4	29.0
Aspartic acid	2.9	1.5	2.0	1.9	3.0	3.5
Threonine	2.7	2.3	2.0	2.6	3.4	2.9
Serine	5.1	4.4	4.0	5.3	5.4	5.9
Glutamic acid	43.1	44.1	40.2	36.6	32.7	33.2
Proline	20.3	21.7	18.3	15.4	14.1	13.0
Glycine	7.5	4.4	3.3	5.2	7.1	7.6
Alanine	2.4	2.0	2.9	2.8	4.0	4.5
Cystine	0.5	0.6	3.0	3.2	1.8	0.7
Valine	2.5	1.3	3.8	4.2	4.6	4.4
Methionine	....	0.7	1.3	1.6	1.5	6.7
Isoleucine	1.6	2.1	3.8	3.6	3.4	3.3
Leucine	4.5	3.8	6.4	7.3	7.2	7.3
Tyrosine	1.4	2.4	1.6	2.4	2.5	2.5
Phenylalanine	4.0	6.5	4.7	3.6	3.5	3.9

[a] Total exclusive ammonia; no allowance has been made for hydrolytic destruction of serine and threonine; tryptophan was not estimated.

The patterns for Gabo and Glenwari are very similar. The percentage of the total protein eluted from carboxymethyl-cellulose by pH 12 phosphate or by dilute alkali was greater with Gabo than with Glenwari dough, but otherwise the elution patterns of gluten from the two doughs appeared very similar. The most interesting feature of the elution patterns was the fluctuation of specific activity, which would suggest a range of gluten species which have undergone disulfide-sulfhydryl interchange to different extents. It should be noted that the fluctuation of specific activity of the gluten fractions followed the same general pattern irrespective of which isotope was used.

The fractions marked A to E in Fig. 1 were analyzed for their amino acid content, and the results are shown in Table IV. Note that the content of cystine and cysteine, estimated as cystine, varied considerably from 0.5 to 0.6 μM per cent in fractions A and B to 3.0 and 3.2 μM per cent in fractions C and D.

Discussion

Disulfide-sulfhydryl interchange can take place by the following reactions:

1) $\text{Gluten–SS–Gluten} + \text{Cy–}\overset{*}{\text{S}}\text{H} \rightleftarrows \text{Gluten–}\overset{*}{\text{S}}\text{S–Cy} + \text{Gluten–SH}$

2) $\text{Gluten–SH} + \text{Cy–}\overset{**}{\text{S}}\text{S–Cy} \rightleftarrows \text{Gluten–}\overset{*}{\text{S}}\text{S–Cy} + \text{Cy–}\overset{*}{\text{S}}\text{H}$

3) $\text{Gluten–SS–Gluten} + \text{Protein–SH} \rightleftarrows \text{Protein–SS–Gluten} + \text{Gluten–SH}$

4) $\text{Cy–}\overset{**}{\text{S}}\text{S–Cy} + \text{Cy–SH} \rightleftarrows \text{Cy–}\overset{*}{\text{S}}\text{S–Cy} + \text{Cy–}\overset{*}{\text{S}}\text{H}$

5) $\text{Cy–}\overset{*}{\text{S}}\text{H} + \text{Cy–}\overset{*}{\text{S}}\text{H} + [\text{O}] \rightarrow \text{Cy–}\overset{**}{\text{S}}\text{S–Cy} + \text{H}_2\text{O}$

Gluten-SS-Gluten represents an inter- or intramolecular disulfide bond in gluten; Protein–SH, the sulfhydryl group of soluble protein; and Cy–SS–Cy and Cy–SH, cystine and cysteine (or small peptides containing disulfide or sulfhydryl groups).

It can be seen that interchange reactions with proteins using ^{35}S-labeled cysteine or cystine can occur directly by reactions 1 and 2 but can also take place indirectly by reactions 4, 5, and 3.

Although efforts were made to minimize oxidative conversion of cysteine to cystine (reaction 5) by mixing the doughs under nitrogen, there is evidence from the results of phosphotungstic acid precipitation of dough liquor that both cystine-^{35}S and cysteine-^{35}S were formed during mixing; the former by oxidation of added cysteine-^{35}S (reaction 5) and the latter by interchange reaction of added cystine-^{35}S with protein sulfhydryl. Both the cystine-^{35}S and cysteine-^{35}S formed could then react further with either protein –SH or protein disulfide. This constitutes a serious difficulty in distinguishing between the primary reaction of these two compounds with proteins of dough. A more important area of uncertainty concerns the level of endogenous, diffusible disulfide and sulfhydryl compounds in flour and therefore the extent to which the added, labeled compound is diluted by endogenous carrier.

Comparisons of the distribution and specific incorporation of added cysteine-^{35}S and cystine-^{35}S into the fractions of various doughs should, therefore, be made with the above reservations in mind.

At first sight, the increased percentage incorporation of added cysteine into gluten in the order Lawrence>Gabo>Durral>Glenwari (Table I) would

suggest a greater reactivity of gluten disulfide bonds toward disulfide-sulfhydryl interchange reactions in the same order.

There are probably two reasons for the observed figures in Table I: (a) the gluten content of the flours decreased in the above order, Lawrence having nearly twice the gluten content of Glenwari; (b) it had been shown earlier (17) that, in contrast to the others, the dough liquors from both Gabo and Lawrence contained no detectable diffusible –SH material and that, if any were present in the flour, it had been consumed during mixing. There would be less endogenous dilution of the added label and thus, while the percentage of added cysteine-^{35}S incorporated into gluten was greater with Lawrence than Glenwari, the extent of incorporation could have been less. This is borne out by the higher specific activity of gluten from Glenwari compared with gluten from Lawrence (Table III).

The ratio of disulfide bonds to sulfhydryl groups in soluble proteins is roughly 6:1; the corresponding figure for gluten is approximately 25:1 (17). Despite the preponderance of S–S to –SH, added cystine is incorporated equally and in some cases more effectively than added cysteine, suggesting that the sulfhydryl groups of proteins are more accessible to disulfide-sulfhydryl interchange reaction than protein disulfide bonds.

Similarly, the greater proportion of –SH groups in soluble protein as against –SH groups in gluten is not reflected in higher specific activities of soluble proteins, when cystine-^{35}S is added, suggesting that the –SH groups of gluten are more reactive than those of the soluble proteins.

The experiment in which incorporation of cysteine-^{35}S was followed by swamping with NEMI-^{14}C confirms the earlier observation (12) that most disulfide-sulfhydryl interchange in gluten takes place in the first few minutes of mixing, whereas interchange with soluble proteins takes place more slowly (Table II). Moreover, when swamping with NEMI-^{14}C was followed by the addition of cystine-^{35}S, very little ^{35}S- was incorporated into soluble proteins, whereas considerable incorporation into the gluten fraction was still observed, which again suggests that –SH-blocking and disulfide-sulfhydryl interchange in gluten occurs mainly during mixing.

The specific activities of gluten fractions obtained by chromatography varied considerably, although this variation was not simply related to the cystine (cysteine) content of the gluten fractions. It is likely that the gluten fractions with high specific activities are those which possess a more open three-dimensional structure permitting easier access of small molecules for interchange reaction.

The amino acid analyses of gluten fractions are in good agreement with those of Woychik *et al.* (18). The omega and alpha glutens of these authors have almost identical composition with chromatographic fractions B and F (Fig. 1) respectively of the present study.

In general, the results of this and other work (9,12,17) support the belief that, in untreated doughs, rheologically important disulfide-sulfhydryl interchange occurs mainly between diffusible sulfhydryl or disulfide compounds on the one hand and gluten on the other, and further, that such interchange reactions occur most rapidly during actual mixing of the dough.

Interpretation of the results of the above experiments was hampered by lack of knowledge of the endogenous flour content of low-molecular-weight disulfide and sulfhydryl compounds. It is clear that adequate interpretation of future studies of the incorporation of labeled disulfide and sulfhydryl compounds into dough will require a precise knowledge of the disulfide and sulfhydryl contents of all fractions both in flour and dough.

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