THIOL AND DISULFIDE GROUPS IN DOUGH RHEOLOGY[1]

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ABSTRACT

The sensitivity of the rheological properties of dough to oxidation suggests that disulfide bonds play a role in dough rheology. The conversion of thiol groups into additional disulfide cross-links between chain molecules of the protein network has been considered an explanation for the stiffening of dough induced by oxidizing flour improvers. However, thiol blocking reagents, which do not form disulfide bonds, have an effect similar to that of oxidation. The stiffening of dough is apparently not primarily due to the formation of additional disulfide bonds, but rather to the removal of thiol groups. This can be explained by the occurrence of thiol-disulfide interchange reactions in dough. This hypothesis predicts that the elastic deformation of dough is restricted by disulfide cross-links, whereas the viscous deformation increases with increasing thiol content. Experiments with doughs that differed only in their thiol and disulfide contents demonstrated that there is not an unequivocal relation either between the disulfide content and the elastic deformation, or between the thiol content and the viscous deformation. There is evidence that only small fractions of the analytically determined thiol and disulfide groups are rheologically effective. The rheologically effective thiol groups are probably located in small peptides.

Thiol and disulfide groups as well as the rheological properties of dough relate to baking quality of wheat. Therefore, both phenomena have been the object of a variety of basic as well as applied studies.

Rheological properties of dough are important from the technological point of view: they largely determine the volume and crumb structure of the baked loaf of bread. Although the relation to quality has received most attention, in this paper we shall concern ourselves mainly with another aspect of dough rheology. Rheological properties of a variety of materials provide us with information on their structure. We shall apply this principle by drawing from rheological data conclusions on the structure of dough, in particular of its protein constituents.

Most thiol and disulfide groups in dough form part of protein molecules. A chemical approach of baking quality cannot bypass the proteins, and the thiol and disulfide groups of them. Fractionation and reconstitution experiments have demonstrated that baking quality of wheat or flour depends largely on the amount and nature of the proteins, in particular the more insoluble glutenin and gliadin fractions (1–4). During dough development the flour proteins form a network that fills the continuous or gluten phase of dough; this network makes dough an elastoviscous material. Oxidation makes the protein network more rigid, which usually results in a considerable increase in loaf quality. In those countries where the law permits it, this observation is applied in practice by the addition to flour or dough of minute amounts of oxidizing flour improvers such as potassium bromate. The sensitivity of the protein network to oxidation gave cereal chemists a clue to an important factor in baking quality.

The conversion of the amino acid cysteine into cystine, with the formation of additional disulfide cross-links between chain molecules of the protein network, is a logical explanation for the stiffening of dough, induced by oxidizing flour improvers. The reaction can be written as

$$2\ Pr{-}CH_2{-}SH \rightarrow Pr{-}CH_2{-}SS{-}CH_2{-}Pr + 2H$$

[1]Papers with partly the same contents have been published in Proc. 7th Working and Discussion Meetings Intern. Ass. Cereal Chem., Vienna, 1972, 100-7 (publ. 1973), and in Proc. 22nd Ann. Conf., Cereal Chem. Div., Roy. Austral. Chem. Inst., Sydney, 1972, 5-16 (publ. 1973).

in which Pr represents a protein chain molecule. This explanation is supported by the softening action on dough of sulfite and thiol compounds, which split disulfide bonds.

The introduction of the concepts of a protein network and of cross-links between its chain molecules has already made clear that the approach in this paper will be very different from the usual approach in protein chemistry, which starts out by fractionation of the mixture of proteins and characterization of isolated components. The approach in this paper is more inspired by polymer physics. We shall neglect differences between protein chain molecules, except in length and in amount of cross-linking. The model on which the discussion will be based corresponds to the "giant protein molecule" of Meredith (5), that is as large as the macroscopic piece of gluten or dough considered. Both approaches must, in the author's opinion, be taken into account for future progress of understanding of the essence of baking quality in wheat.

Thiol-Disulfide Interchange

The hypothesis that the formation of additional disulfide cross-links explains the stiffening of dough by oxidation, does not explain two observations.

In the first place, thiol blocking reagents, such as *N*-ethylmaleimide (*N*EMI), which do not form disulfide cross-links, have effects on rheological properties similar to effects of oxidizing reagents. The stiffening of dough is apparently not primarily due to the formation of new disulfide bonds, but rather to the removal of thiol groups.

Secondly, a network with permanent cross-links can only be deformed elastically, that is, the deformation disappears when the load is removed. Rheological measurements have demonstrated that, except under special conditions, the deformation of dough is predominantly permanent or viscous. A permanent deformation, however, can be attained only if the cross-links are not permanent. It is, therefore, necessary to introduce in our model a mechanism that makes the disulfide bonds nonpermanent.

Goldstein (6) was the first to suggest that thiol-disulfide interchange reactions are such a mechanism; they may explain the occurrence of permanent deformations of a network with disulfide cross-links. The mechanism is schematically shown in Fig. 1. The first stage shows two protein molecules, cross-linked by two disulfide groups. Reaction (1) with a thiol compound XSH, the nature of which will be discussed later, opens the 1,2-disulfide bond. In the time interval between reactions (1) and (2) the conformations of the protein molecules may change as a result of Brownian motion. In Fig. 1, it is arbitrarily assumed that the upper chain retains its conformation and that the conformation of the lower molecule is changed. Reaction (2) forms a new disulfide cross-link between positions 2 and 3. Reaction (3) is the net result of reactions (1) and (2). The thiol compound XSH is not consumed and becomes available again for another cycle of interchange reactions. A small amount of a thiol compound permits any number of reaction cycles, however large it may be, provided that sufficient time is available. Under stress, Brownian motion has a direction of preference; as a consequence, the combined conformation changes that accompany a large number of reaction cycles then result in measurable deformation.

Direct experimental evidence for the actual occurrence in dough of these interchange reactions has been obtained for the first time by McDermott and

Pace (7). For their experiments they used chemically modified gelatin into which thiol groups had been incorporated. They added this thiolated-gelatin to dough ingredients, isolated gluten from the dough, and found hydroxyproline in its hydrolysate; naturally, this amino acid occurs in gelatin but not in gluten. Similar experiments have been reported by later authors (8–16).

Under certain conditions, the elastic deformation of a network of linear polymer molecules with permanent cross-links, like rubber, is directly proportional to stress, and inversely proportional to the number of cross-links per unit volume (17). By analogy, one could expect that, also with gluten and dough the elastic part of the deformation is at a given stress smaller the more disulfide cross-links are present. One of the purposes of the experiments to be described later in this paper was to verify that at a given stress the elastic part of the deformation of dough is solely determined by the number of disulfide bonds.

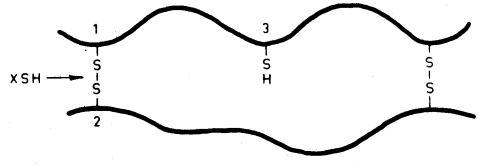
The other point to verify refers to the hypothesis that thiol-disulfide interchange reactions are the mechanism of viscous deformation. The model in Fig. 1 predicts that, other conditions being constant, the viscous part of the deformation increases with increasing thiol content. Therefore, we shall examine the relation between the thiol content of dough and the viscous part of its deformation.

Experimental Data on the Relation Between Thiol and Disulfide Contents and Rheological Properties

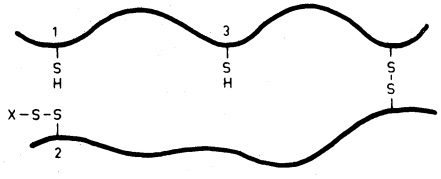
The few experimental data in the literature on the relation between thiol and disulfide contents and rheological properties do not permit a distinction between elastic and viscous deformations. This distinction is, however, essential for testing the hypotheses in the preceding section. To make this distinction possible, the author determined rheological properties of doughs by creep tests, in which shear (deformation) is recorded as a function of time when the test-piece is loaded by a constant shear stress for a certain period. The recovery of the test-piece after removal of the load is a measure of the temporary or elastic part of shear. Its permanent part is considered to be of a viscous nature. Results are expressed as compliances; the compliance is shear divided by shear stress. In the same way as the shear can be divided into an elastic and a viscous part, so can the compliance.

Thiol groups were determined by titration with silver nitrate. In the presence of sulfite the same titration determines the sum of thiol and disulfide groups. Those groups that are determined in the absence of urea are called reactive ones. The increase in titration results upon the addition of urea is considered to measure the nonreactive groups. The distinction merely between reactive and nonreactive groups is a simplification; in reality, there is a distribution of chemical reactivities of thiol and disulfide groups dependent upon the various protein, peptide, and amino acid molecules of which they form part.

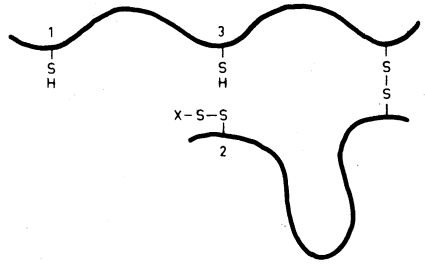
Experiments were made with doughs that varied only in their thiol and disulfide contents. Variations in these contents were obtained by making all doughs from one untreated and unbleached flour, and adding to the dough ingredients various amounts of the oxidizing flour improvers bromate or iodate, the thiol blocking reagent *N*-ethylmaleimide, or the thiol compound glutathione, or by mixing in air or oxygen instead of nitrogen. These experiments have been described fully in reference (18).



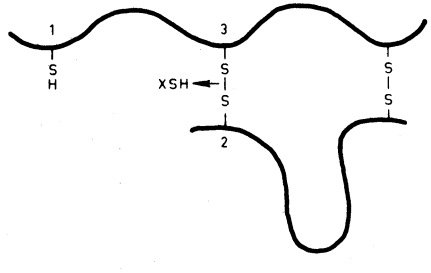
$$R_1SSR_2 + XSH \rightarrow R_1SH + R_2SSX \quad (1)$$



Brownian motion



$$R_2SSX + R_3SH \rightarrow R_2SSR_3 + XSH \quad (2)$$



$$R_1SSR_2 + R_3SH \rightarrow R_1SH + R_2SSR_3 \quad (3)$$

Fig. 1. Viscous flow as a result of thiol-disulfide interchange reactions. Explanation in text.

Figures 2 to 5 show how the total and reactive thiol and disulfide contents change upon the addition of reagents. Glutathione increases both the total and reactive thiol content, whereas iodate decreases them. The effect of bromate is small in respect to that of an equivalent amount of iodate. Changes in disulfide contents upon the addition of reagents are relatively small. In the most interesting range, the ratio of reactive to total thiol (SH) is 80%, with only small variations. Reactive disulfide bonds are a much smaller fraction of total disulfide bonds, namely 12–15%.

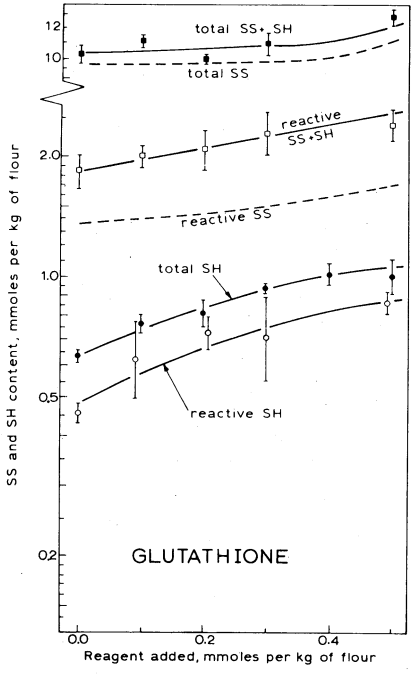
Figures 6 to 9 depict the changes in rheological properties. All compliances increase by the addition of glutathione; that is, the dough becomes softer. Bromate, iodate, and *N*-ethylmaleimide stiffen the dough. As with the thiol content, the effect of bromate is smaller than that of an equivalent amount of iodate; the difference is, however, smaller than with the thiol contents.

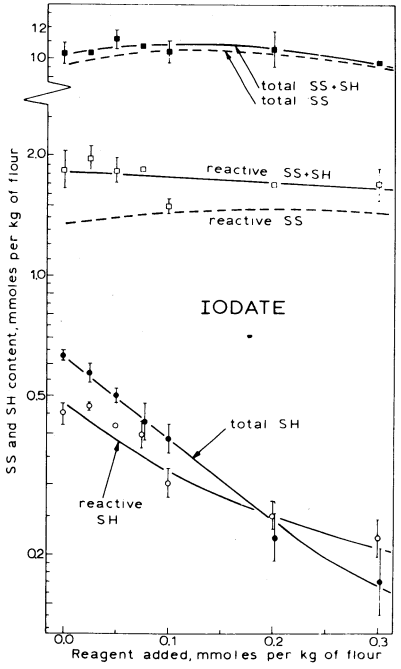
Having established the relations between the amounts of reagents added on one hand, and the total and reactive thiol and disulfide contents on the other, and between the amounts of reagents and the rheological properties, one can derive graphs showing the relation between the thiol and disulfide contents and the rheological properties. Figures 10 to 12 show these graphs from which the amounts of reagents are eliminated.

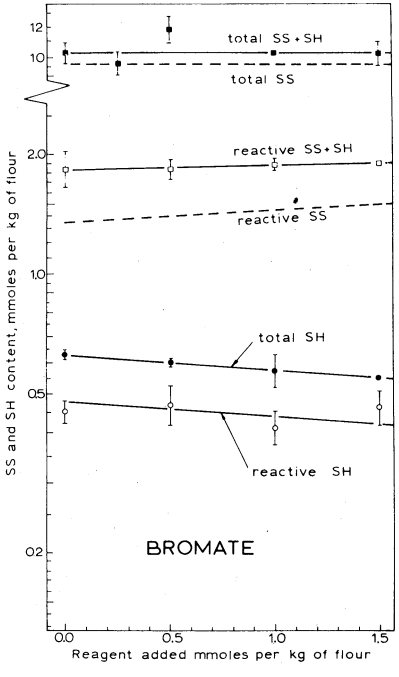
Figure 10 shows that in spite of very small changes in disulfide content the elastic compliance can change markedly. Glutathione increases the elastic compliance but also the total and reactive disulfide content; this is inconsistent with the hypothesis under discussion. Decreases in elastic compliance occur combined with increases as well as decreases in reactive disulfide content. A remarkable feature is that there is no unequivocal relation between the disulfide content and the rheological properties: this relation depends upon the reagent used.

Corresponding graphs for the viscous compliance as a function of total or reactive thiol content in Figs. 11 and 12 are, to some extent, in agreement with the hypothesis that thiol groups are required for the viscous deformation of dough. For all reagents an increase in thiol content corresponds to an increase in compliance, and conversely. However, the lines for the various reagents do not coincide; as for the disulfide content and the elastic compliance, there is no unequivocal relation between the total or reactive thiol content and the viscous compliance. Bromate has a considerable rheological effect in spite of the small decrease in thiol content. The position of oxygen is intermediate between those of bromate on the one hand and iodate and *N*-ethylmaleimide on the other. Apart from a shift parallel to the SH-axis, the graph for the reactive thiol content is very similar to that for the total thiol content; this reflects the constant ratio between reactive and total thiol groups, which has been noted earlier in this paper.

Figs. 2–5. Thiol and disulfide contents of doughs in the presence of various reagents. The lengths of the vertical lines at the experimental points correspond to the 95% confidence interval, that is, four times the standard error of estimate; if there is no vertical line, this length is smaller than the size of the sign. Full lines correspond to SS + SH or to SH as obtained by titration. Dashed lines show the disulfide contents obtained by subtraction of the thiol contents from the observed sums of disulfide and thiol contents. Fig. 2. glutathione, Fig. 3. potassium iodate, Fig. 4. potassium bromate, Fig. 5. *N*-ethylmaleimide.


total SS+SH
total SS
reactive SS+SH
reactive SS
total SH
reactive SH
GLUTATHIONE
SS and SH content, mmoles per kg of flour
Reagent added, mmoles per kg of flour


total SS+SH
total SS
reactive SS+SH
reactive SS
IODATE
total SH
reactive SH
SS and SH content, mmoles per kg of flour
Reagent added, mmoles per kg of flour


total SS+SH
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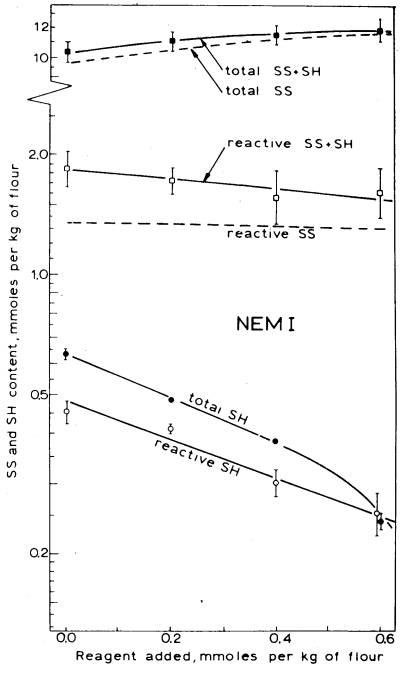
reactive SS
total SH
reactive SH
BROMATE
SS and SH content, mmoles per kg of flour
Reagent added mmoles per kg of flour


total SS+SH
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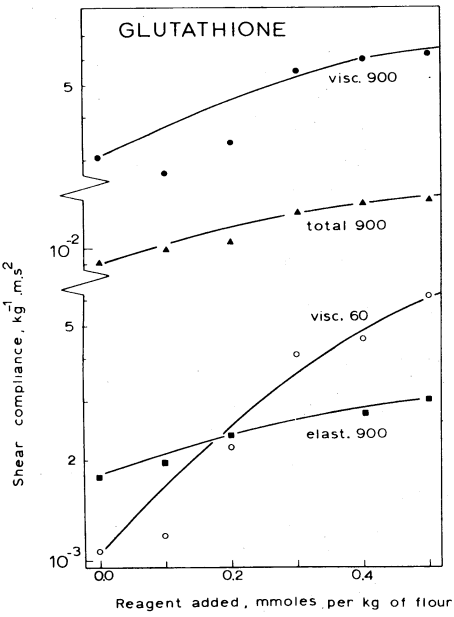
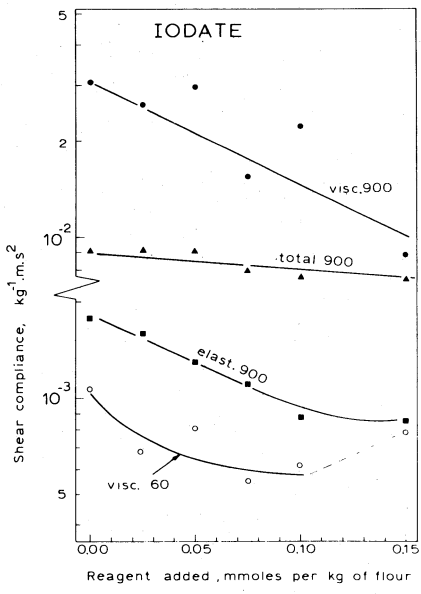
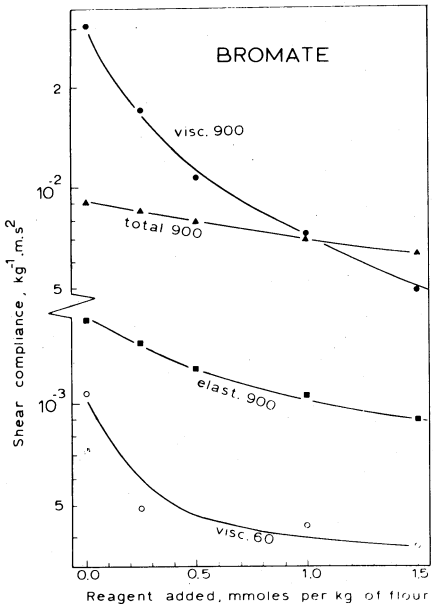
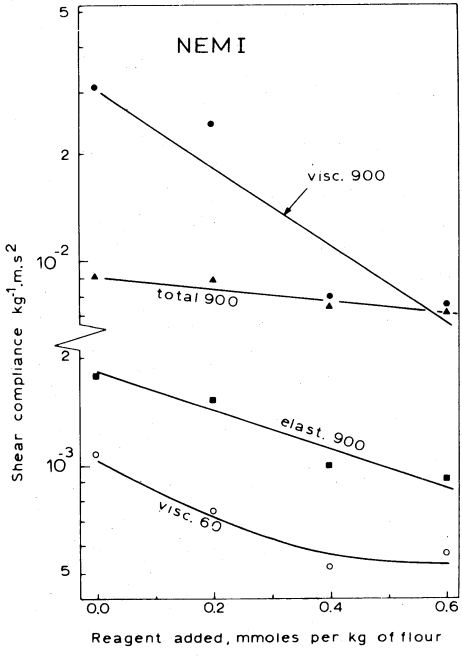
SS and SH content, mmoles per kg of flour
Reagent added, mmoles per kg of flour









Figs. 6–9. Compliances of doughs after 60 or 900 sec in the presence of various reagents. Viscous and elastic compliances are measured at a shear stress of 90 N/m^2, the total compliance at such shear stresses that after 900 sec a shear of 0.60 is attained. Fig. 6. glutathione, Fig. 7. potassium iodate, Fig. 8. potassium bromate, Fig. 9. *N*-ethylmaleimide.

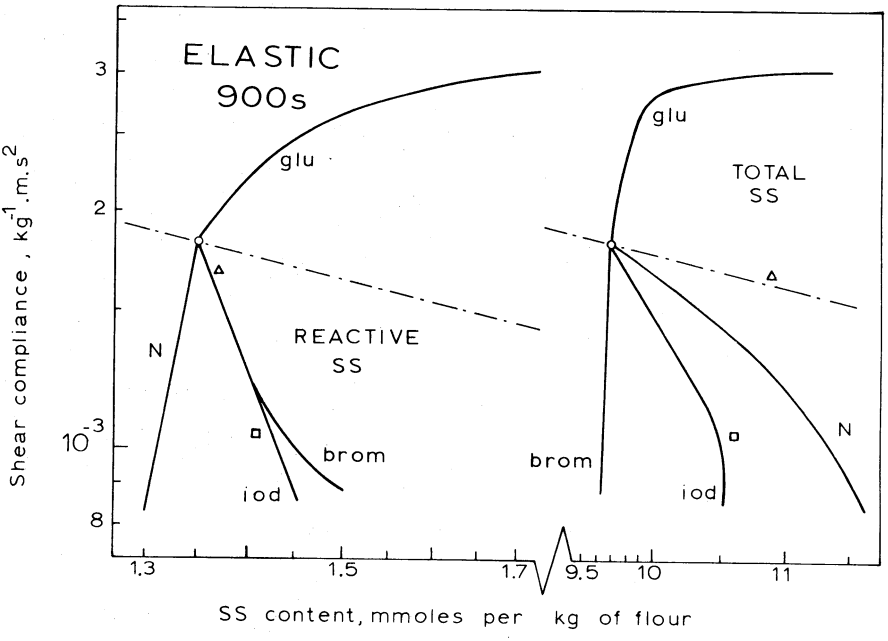


Fig. 10. Elastic compliance after 900 sec at a shear stress of 90 N/m^2 as a function of total or reactive disulfide content. Circle = control dough (mixed in nitrogen); triangle = dough mixed in air; square = dough mixed in oxygen. Abbreviations: glu = glutathione; brom = bromate; iod = iodate; N = *N*-ethylmaleimide. Dash-dot lines correspond to inverse proportionality between disulfide content and compliance.

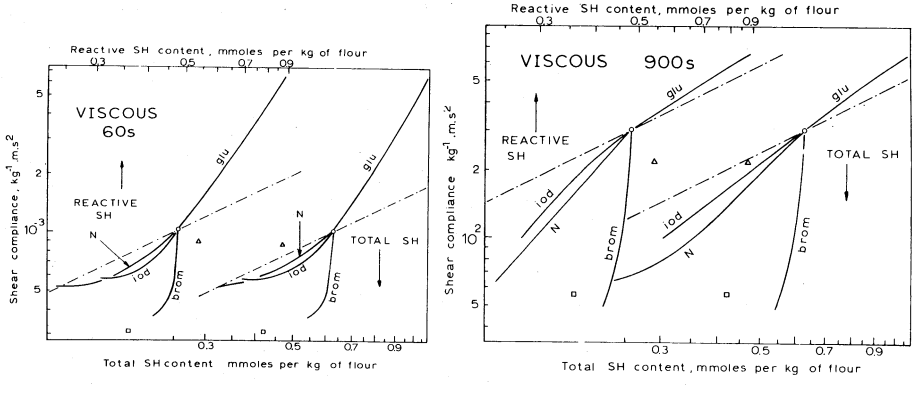


Fig. 11–12. Viscous compliances after 60 or 900 sec at a shear stress of 90 N/m^2 as a function of total or reactive thiol content. Symbols and abbreviations as in Fig. 10. Dash-dot lines correspond to direct proportionality between thiol content and compliance.

The results in Figs. 10 to 12 do not confirm the hypotheses that disulfide bonds restrict the elastic deformation of dough, and that thiol groups permit its viscous deformation. There is not an unequivocal relation either between the total or reactive disulfide content and the elastic deformation or between the total or reactive thiol content and the viscous deformation. Nevertheless, the evidence that thiol and disulfide groups play a role in dough rheology, and that thiol groups are involved in the improver reaction, is too specific to be disregarded. Therefore, we have to modify the above hypotheses, or to look for an alternative that explains the specific effects of oxidation, thiol blocking, and disulfide bond splitting.

Rheologically-Effective and -Ineffective Thiol and Disulfide Groups

A possible modification is the introduction of the concept of rheological effectiveness of thiol and disulfide groups. This concept can more easily be explained with reference to the thiol groups.

So far, a distinction between thiol groups forming part of different compounds has only been made in respect of their behavior in the analytical determination. It is to be expected, however, that thiol groups in different protein or peptide molecules also play different roles in the rheological properties. Thiol groups can only contribute to the viscous compliance by entering the chemical reaction (1) of Fig. 1. As a consequence, one expects a correlation between chemical reactivity and rheological effectiveness. Small compounds, like cysteine and glutathione, diffuse much more rapidly in dough than do proteins. Therefore, they are expected to be chemically more reactive as well as rheologically more effective. With extensigraph measurements, added glutathione (mol wt 307) was, indeed, found to be more effective than thiolated-gelatin (mol wt 10,000 or more) with the same amount of thiol groups (19). Although there is probably a distribution of rheological effectivenesses, in the same way as the chemical reactivities are distributed over a certain range, we shall, for simplicity, only make a distinction between two classes: rheologically-effective and -ineffective groups. The rheologically-effective thiol groups are defined as those that, by way of interchange reactions, contribute to the viscous compliance.

The distinction between rheologically-effective and -ineffective thiol groups does not coincide with that between chemically-reactive and -nonreactive groups, if the latter is defined on the basis of the titration procedure described. This is shown by Figs. 11 and 12, in which the graphs of viscous compliance *vs.* reactive thiol content are as complicated as those *vs.* the total thiol content. This suggests that the rheologically-effective thiol groups form only a small fraction as compared with the chemically-reactive groups. Further evidence for this suggestion comes from the observation that iodoacetic acid is a highly-efficient flour improver with an optimum addition in baking of only 30 μmol per kg of flour (20). If so small an amount of a thiol blocking reagent is effective, the number of rheologically-effective groups cannot be much greater. In mixing experiments in the Brabender Farinograph, the first 400–560 μmol *N*-ethylmaleimide per kg of flour (25–35% of the total thiol content) affected the mixing tolerance much more than did further additions (21).

Comparison of the contents of rheologically-effective thiol groups with the contents of various classes of thiol compounds in Table I suggests that glutathione and other thiol peptides are the rheologically-effective compounds,

which is indicated by XSH in Fig. 1. It has been demonstrated that some thiol peptides, in fact, react with iodate and iodoacetic acid (28,29).

Observations by the author on the differences between the various oxidizing or thiol-blocking reagents can be explained by the assumption that only a small fraction of the thiol groups is rheologically-effective, if it is further assumed that bromate reacts more specifically with these effective thiol groups than does iodate or *N*-ethylmaleimide. This means that, at the same decrease in total thiol content, bromate has removed more rheologically-effective thiol groups.

For the disulfide bonds, a relation between the chemical reactivity and the rheological effectiveness is not as obvious as for the thiol groups. If the rubber-like model, explained earlier in this paper, is applicable, only those interchain disulfide cross-links that are present in excess of one per polypeptide chain are effective in restricting the elastic deformation. They were called "branching cross-links" by Ewart (30). By application of the theory of rubber elasticity, the number of rheologically-effective disulfide cross-links can be calculated from the modulus of gluten. Results are shown in Table II, which, for comparison also shows results for total and chemically-reactive disulfide contents. An estimate of the rheologically-effective disulfide bonds that is independent of a molecular model can be made on the basis of the effect of dithiothreitol (a compound with two thiol groups) on the farinogram. The first 0.3 mmol dithiothreitol per kg of flour affected dough development time more than did further additions; using the resistance to mixing as a criterion, the rheologically-effective disulfide content is 1.1–1.7 mmol/kg (21).

TABLE I
Contents of Some Classes of Thiol Compounds in Flour and Dough

Class	Contents μmol/kg flour basis	Reference
Total	600–1,800	22
	650	18
Chemically-reactive	500	18
Compounds with low molecular-weight, not further specified	200–440	23
	110–160	24
Sum of glutathione, L-cysteinyl glycine, and an unidentified thiol peptide	11	25
Glutathione	8	26
	42–65	24
	4	25
	34–132	27
Rheologically-effective thiol groups		
From baking experiments	≤30	20
From mixing experiments	≤400–560[a]	21

[a]Total thiol contents 1,600–1,900 μmol/kg.

The assumption that there are thiol and disulfide groups of various rheological effectivenesses may explain the discrepancies between the experimental results in Figs. 10 to 12, and the predictions based on the earlier hypotheses. If this assumption is true, the rheologically-effective groups must be located in particular protein and peptide fractions. This hypothesis might be verified by experiments similar to those described in this paper, but with determinations of thiol and disulfide groups in specific protein and peptide fractions. A technique that may serve for the determination of thiol groups in protein and peptide fractions has been developed by Lee *et al.* (35–38) and by Tkachuk and Hlynka (39). In this technique, one blocks the thiol groups by adding an excess of *N*-ethylmaleimide, and then hydrolyzes the protein. After that, the amino acids are separated by chromatography; each cysteine in the original material produces S-succinyl-L-cysteine, which can be identified because of its position in the chromatogram. Its amount is a measure of the thiol content. This technique can readily be combined with the separation of the proteins into a number of fractions between blocking of the thiol groups and hydrolysis.

Alternative Explanations

There are reasons to question the existence in dough of a continuous protein network with disulfide cross-links. Ewart (30) did so because of the sharp drop of viscosity of glutenin solutions upon reduction. Jones and Carnegie (40) drew attention to the following facts that are contradictory to this model. In the first place, it is possible to extract the major portion of the wheat proteins from dough by solvents that do not split disulfide bonds. Secondly, the electrophoresis patterns of gliadins and soluble proteins do not change when a flour is mixed to a dough, and hence the network would be restricted to the glutenin fraction or even part of it.

TABLE II
Contents of Some Classes of Disulfide Compounds in Flour, Dough, and Gluten

Class	Contents mmol/kg		Reference
	Protein basis	Flour basis	
Total	80–130	8–16	22
	110	12	18
	90–100[a]		31
	90–100	9–13	21
Chemically-reactive	18	2	18
	70–85[a]		31
Rheologically-effective disulfide groups			
From modulus of gluten	2.5		32
	0.05		33, 34
From mixing experiments			
Dough development time	≤3	≤0.3	21
Resistance to mixing	≤10–13	≤1.1–1.7	

[a]Glutenin fraction only.

There is still another argument against the explanation of elasticity in dough on the basis of a cross-linked network. The modulus of rubber-like materials is proportional to the absolute temperature (17). Contrary to this, the modulus of dough (41–43) and gluten (33) decreases with increasing temperature up to 45° and 50°C, respectively[2] (45). Therefore, it is unlikely that the elasticity of dough and gluten is due to a network of chain molecules with permanent cross-links.

Ewart proposed a model in which glutenin molecules are linked up to linear concatenations by one (30) or two (44) disulfide bonds between each molecule and the next. In his model, the concatenations are cross-linked by chain entanglements and secondary forces. There is no proof, however, that these cross-links are strong enough; if they are not, the network will show only viscous deformations and no elasticity at all. In Ewart's model thiol-disulfide interchange is a mechanism for stress relaxation; viscous flow is accounted for by unraveling of chain entanglements, and breaking and reformation of secondary interchain bonds. This seems illogical, since, rheologically, viscous flow and stress relaxation are the same.

The explanation proposed by Jones and Carnegie (40) is quite different. It is based upon the assumption that in the endosperm and in flour the protein conformation is such as to expose the nonpolar regions. During mixing with water the proteins will tend to adjust their conformation, leading to burying of nonpolar and exposure of polar groups. The ability of a protein to adapt its conformation may be hindered by intramolecular disulfide bonds. Then thiol-disulfide interchange may facilitate the adjustment of the protein conformation. As a consequence, oxidation will retard the change in conformation, resulting in more hydrophobic and less polar bonding between proteins. The suggestion of Jones and Carnegie explains some observations that are not accounted for by the original or modified hypotheses. Therefore, it is worthwhile to be explored further, but two objections can readily be raised. In the first place, their model does not explain the occurrence of elasticity in dough. Secondly, it predicts that oxidation may affect the viscous compliance of dough, but it fails to predict the direction of the effect, let alone its magnitude. A definite assessment of the validity of the suggestion of Jones and Carnegie cannot be given until it has been as severely tested as Goldstein's hypothesis of thiol-disulfide interchange.

[2]Note added in proof: Contrary to these observations, Funt and Lerchenthal recently reported a decrease in compliance of gluten when the temperature increased from 293 to 313 K.

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