Disulfide-Sulfhydryl Interchange Studies of Wheat Flour. II. Reaction of Glutathione

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ABSTRACT

Radioactive glutathione ($G^{35}SH$) was used as a tool for investigating disulfide-sulfhydryl interchange reactions in flour suspensions and doughs. Incorporation of $G^{35}SH$, added at a level of 1 μmol./g. flour, was not measurably increased in suspensions by greater alkalinity or by anaerobic conditions. The percent of label incorporated in the soluble and insoluble proteins in suspensions ranged from 10.5 to 25% under all conditions tested. The gluten proteins incorporated only 8.3 to 12.4%. When the same amount of $G^{35}SH$ was added to a dough, 29 and 35.4% of the isotope was incorporated in the protein after 5 and 30 min. of mixing. A dough containing 1 μmol. of $G^{35}SH$/g. flour was lyophilized and remixed with a large excess of glutathione (GSH). The acetic acid-soluble fraction was separated on Sephadex G-100; there was almost complete liberation of the incorporated label. That a GSH-linked hydrogen-transfer system is not involved was proved by adding GSH to synthetic flour made from vital wheat gluten and wheat starch. The same weakening effect was observed as in normal flour. Also, a heated flour used in a suspension gave the same results on counting as the unheated flour. These findings are discussed in relation to disulfide-sulfhydryl interchange.

The evidence indicates that the rheological properties of dough and its three-dimensional network are dependent on the arrangement and number of SS bridges and SH groups of the proteins. The hydration and configuration of the higher-molecular-weight proteins of gluten are such that some SS linkages probably are inaccessible to attack by SH reagents. Also, several investigators have shown that some of the SH groups of flour proteins are much more labile than others.

Flour contains only about 1 μmol. of sulfhydryl per g. compared to 15 to 20 μmol. disulfide. Since less than half of the SH groups are affected by oxidation, new SS cross-linkages, if formed, would be increased only slightly compared to those already present. Goldstein (1) and then numerous other investigators postulated a disulfide-sulfhydryl interchange which involves the cleavage and reformation of the SS bonds mediated by SH groups of flour or by relatively small amounts of added SH compounds.

McDermott and Pace (2) demonstrated disulfide-sulfhydryl interchange by use of thiolated gelatin. Hydroxy proline originating in the gelatin was detected in the gluten. Frater and Hird (3) interpreted the effects on flour dough which they obtained with glutathione (GSH), oxidized glutathione (GSSG), and reduced gluten as evidence of disulfide-sulfhydryl interchange. Mauritzen and Stewart (4,5,6) and Stewart and Mauritzen (7) developed a technique for the fractionation of dough and employed both ^{35}S-cysteine and ^{35}S-cystine for a more direct means of demonstrating disulfide-sulfhydryl interchange. Villegas *et al.* (8) mixed doughs with gelatins which had been thiolated to various stages and discussed the rheological properties of the doughs, comparing the extent of thiolation with the effect of GSH. Sullivan and Dahle (9) showed that formamidine disulfide acts as a blocking agent

for the SH groups of flour and obtained no significant evidence that the formamidine disulfide residue participated in disulfide-sulfhydryl interchange at the normal pH range of dough.

It is well documented that the addition to flour of GSH and other SH reagents at a level of 1 μmol./g. drastically weakens the rheological properties of the flour. Since flour contains 15 to 20 SS groups per g., an exchange reaction of these groups with GSH at a level of 1 μmol./g. could be expected, on the basis of stoichiometry, to leave most, if not all, the reacted GSH attached to protein molecules in an SS linkage, as shown in equation 1.

$$PrSSPr + GSH \rightleftarrows PrSSG + PrSH \quad (1)$$

Also, since GSH is not present in stoichiometric excess, reaction 2 would not be expected to occur to any significant degree.

$$PrSSG + GSH \rightarrow PrSH + GSSG \quad (2)$$

On the other hand, the addition of excess GSH would result in reaction 2.

The above rationale led to the experiments with GSH reported in this paper.

MATERIALS AND METHODS

The flour was an untreated spring-winter blend analyzing 0.47% ash and 14.2% protein (14% m. b.). The vital wheat gluten was purchased from Midwest Solvents Co., Atchison, Kansas; GSH and $G^{35}SH$ were purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. Measurement of radioactivity was made at the University of Minnesota, with a Tricarb Liquid Scintillation Counter, Model 314F, Packard Instrument Co., Downers Grove, Ill.

Counting Radioactivity

For counting radioactivity the supporting gel was prepared by mixing 25 g. of thixotropic gel powder (Cab-O-Sil, Packard) and 1.5 g. of ppo-2,5-diphenyloxazole (Packard) in 500 ml. of 70% toluol-30% absolute alcohol (v./v.) solution. Maximum amounts are 0.4 ml. for solution and 100 mg. for powdered material, and counting was done between 1,000 and 10 000. A control vessel which contains the sample and a known standard solution must be prepared for colored material, as color may reduce the count.

Synthetic Flour

Enzyme-free synthetic flour was prepared by mixing starch and wheat gluten 19:81 (w./w.). The synthetic flour analyzed 9.0% moisture, 0.26% ash, and 14.2% protein.

Heat-Treatment of Flour

Flour (20 g.) was placed in a lightly stoppered, 125-ml. flask and heated at 100°–110° C. for 1 hr.

Flour Slurries at Various pH Levels

For the anaerobic experiments, 20 g. of flour in a 125-ml. flask was evacuated under vacuum for 30 min. and the flask was refilled with nitrogen gas.

The $G^{35}SH$ was dissolved in water (1 μmol. per 3 ml.) that had been

deionized, boiled for 10 min., and cooled. Flour suspensions of various pH levels were prepared by slurrying 20 g. in 60 ml. of $G^{35}SH$ solution, followed by the addition of the appropriate amount of acid or alkali: pH 5.0, 0.6 ml. of 1*M* acetic acid; pH 5.9 required no addition; pH 8.5, 1.1 ml. of 0.5*N* sodium hydroxide; and pH 9.6, 1.6 ml. of 0.5*N* sodium hydroxide. Immediately afterward nitrogen gas was passed into the suspension for 10 min. and all slurries were incubated for 60 min. at 30°C.; then the pH of each was adjusted to 5.9 by addition of 0.5*N* sodium hydroxide or 1*M* acetic acid.

Fractionation of Flour Slurries

The separation of the freeze-dried precipitate, supernatant liquids 1 and 2, and trichloroacetic acid (TCA) filtrate is shown in the diagram.

To recover unreacted $G^{35}SH$ and $G^{35}S^{35}SG$ completely, a settling procedure was employed, following Mattern and Sandstedt (10), who found that settling and decanting a flour suspension prevented formation of the gluten complex, allowing a more complete separation of water-soluble substances. The study of flour-water systems which contain the cohesive gluten complex is complicated by the fact that gluten and starch may occlude unreactive compounds which ordinarily would remain in solution. GSH has the advantage that its oxidized form, GSSG, is very soluble in water.

In all the experiments on dough described in this paper, GSH was added to the total absorption water prior to the addition of flour.

Because of the difficulty in extracting water-soluble compounds such as GSH and GSSG from raw dough, the dough was lyophilized. The ground dough was employed for remixing with excess GSH.

Farinograph Experiments

Absorptions were determined for a maximum consistency of 500 B.U., and the total weight of the dough for each experiment was 80 g.

GSH or $G^{35}SH$ was added as a solution, the concentration of which was 1 μmol./g. of flour. GSH was used for the farinograms shown in Fig. 1.

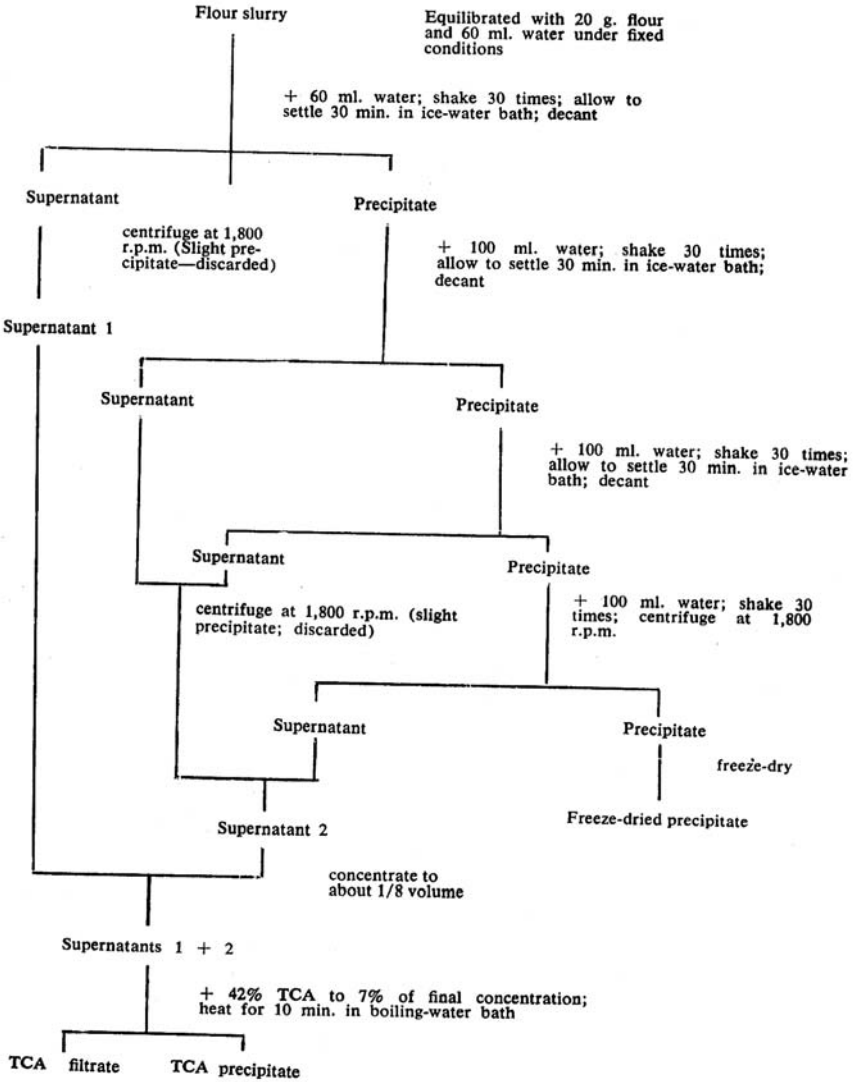
Measurement of ^{35}S Added as $G^{35}SH$ in Dough

Fifty grams of flour and 31.15 ml. of water containing 50 μmol. of $G^{35}SH$ were mixed on the farinograph for 5 min. Twenty grams of the dough was weighed and dispersed in 50 ml. of 7% TCA in a Waring Blendor, and the dispersion was centrifuged. The precipitate was washed with 50 ml. of 7% TCA in a centrifuge bottle, centrifuged out, and freeze-dried (TCA precipitate). The TCA supernatant liquid was heated in a boiling-water bath for 10 min., and the TCA filtrate was separated from the slight amount of precipitate. Radioactivity was counted in the original $G^{35}SH$ solution, the TCA precipitate and the TCA filtrate. The procedure was repeated on a dough mixed for 30 min.

Measurement of Radioactivity in Fractions Separated on a Sephadex G-100 Column

To prepare the dough, 60 g. of flour, 1 μmol. of $G^{35}SH$/g. of flour (total activity 23.6 μc) and 37.5 ml. of water were mixed for 5 min. on the farinograph. The dough was freeze-dried on a glass plate and then ground in the Stein mill for 3 min. Fifty grams of this dough was mixed with 31.15 ml. of water and 2,500 μmol. of GSH for 25 min. on the farinograph.

SEPARATION OF FLOUR FRACTIONS



The remixed dough was freeze-dried and then ground, as above. As a control, the procedure was repeated without GSH. To extract acetic acid solubles, 5 g. of the remixed, freeze-dried and ground dough was dispersed in 25 ml. of 0.05*M* acetic acid for 1½ min. in a Waring Blendor. The extract was centrifuged at 1,800 r.p.m. Acid solubles were fractionated on a Sephadex G-100 column which was 17 mm. in diameter and 750 mm. long and had been equilibrated with 0.05*M* acetic acid. The eluant was 0.05*M* acetic acid. Tubes containing the fractions were observed for absorbance at 280 mμ, for nitroprusside reaction, and for radioactivity.

RESULTS AND DISCUSSION

Effects of pH and Atmosphere on the Incorporation of G^{35}SH in a Flour Slurry

Table I gives the percent of radioactivity in the separations made of flour slurries at various pH levels, outlined in the diagram. Around 10% of the radioactivity was detected in the precipitate (largely gluten and starch). About 80 to 90% of the radioactivity was found in the supernatant liquid, which consisted of water and salt-soluble proteins of lower molecular weight as well as other soluble material. Interchange reactions should take place more easily under alkaline and anaerobic conditions than in aerobic and acid media, but these data show no evidence of increased rates of reaction with increasing pH and/or removal of oxygen. It is probable that part of the small amount of radioactivity found in the precipitate might have come from adsorption of smaller-molecular-weight proteins. There was no evidence of significant interchange reaction in the flour slurry even under anaerobic and high pH conditions.

TABLE I

EFFECT OF PH AND ATMOSPHERE ON THE INCORPORATION OF G^{35}SH IN SLURRIES

pH	Condition	Distribution of Radioactive Isotope			
		Flour Slurry	Freeze-Dried Precipitate	Supernatant Liquids 1 and 2	TCA Filtrate
		%	%	%	%
5.0	Aerobic	100.0	10.3	92.0	83.9
5.9	Aerobic	100.0	8.3	81.9	75.0
8.5	Aerobic	100.0	12.4	84.7	80.1
8.5	Anaerobic	100.0	8.3	85.7	77.9
9.6	Anaerobic	100.0	9.0	93.1	89.5

Effect of Heat-Treatment on Incorporation of G^{35}SH in a Flour Slurry

It was thought there might be a possibility that some enzyme systems in flour would influence the reaction of SH groups. Therefore, experiments were made with heat-treated flour. The distribution of radioactivity in the flour slurry is shown in Table II. The data show that the precipitate from both the untreated and heat-treated flours retained only 8% of radioactivity. The data also indicate that enzyme systems are not involved in the reaction of GSH in a flour slurry.

Reaction of GSH in Enzyme-Inactivated Flour Dough Systems

Farinograph tests were made with a blend of vital wheat gluten and purified wheat starch. This synthetic flour was, presumably, enzyme-free.

TABLE II

EFFECT OF HEAT-TREATMENT OF FLOUR ON INCORPORATION OF G^{35}SH IN SLURRIES

FLOUR	DISTRIBUTION OF RADIOACTIVE ISOTOPE			
	Slurry	Freeze-Dried Precipitate[a]	Supernatant Liquids 1 and 2	TCA Filtrate[b]
	%	%	%	%
Untreated	100.0	8.8	82.3	77.5
Heat-treated	100.0	7.8	87.5	82.7

[a]N contents: 332.3 mg. for untreated and 333.1 mg. for heat-treated flour.
[b]N contents: 24.5 mg. for untreated and 19.1 mg. for heat-treated flour.

The effect on the farinograms of GSH added at a level of 1 μmol./g. flour is illustrated in Fig. 1. Comparison of the control and GSH dough of

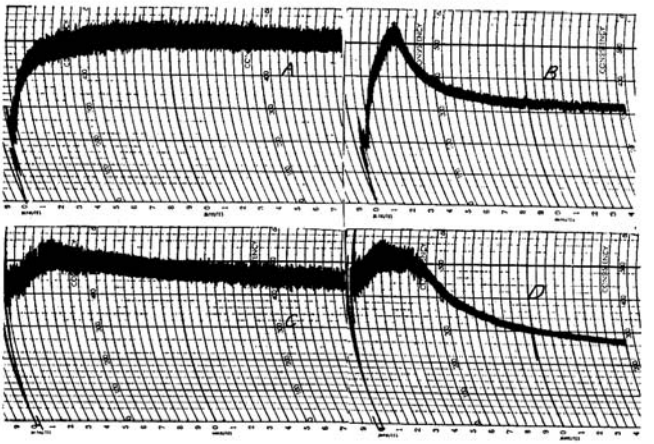


Fig. 1. Effect of GSH on natural and "synthetic" flours: A, natural flour; B, natural flour with GSH (1 μmol./g.); C, "synthetic" flour; D, "synthetic" flour with GSH (1 μmol./g.).

normal flour and synthetic flour showed that GSH reduced consistency rapidly after 3 min. and narrowed the band width on both flours. Thus, it appears that the dough-weakening action of GSH is not enzyme-dependent.

Distribution of ^{35}S Added as G^{35}SH in Dough

Significant interchange did not seem to take place in flour suspensions even at a pH higher than 7.0, as has already been shown. Therefore, it was of interest to study the effect of dough mixing on interchange reactions.

G^{35}SH was mixed with flour and water in a farinograph at the same level used in the slurries (1 μmol./g. flour). In the previous experiment, the weakening effect of GSH appeared at 3 min. of mixing and, after 6 min., the decrease in consistency was very slow. The radioactivity of ^{35}S was checked in doughs mixed 5 and 30 min., with the results shown in Table III.

All of the gluten protein and some occluded soluble protein were contained in the TCA precipitate (N content 295 mg.). The TCA filtrate con-

TABLE III
DISTRIBUTION OF ^{35}S ADDED AS $G^{35}SH$ IN DOUGHS

Mixing Time	Distribution of Radioactive Isotope		
	Original Dough (20 g.)	TCA Precipitate[a]	TCA Filtrate[a]
min.	*%*	*% of total*	*% of total*
5	100.0[b]	28.8	70.7
30	100.0[c]	33.9	64.6

[a]Details are given in "Materials and Methods."
[b]1,344,474 counts/min.
[c]1,363,435 counts/min.

tained nonprotein nitrogen (12 mg.) which constituted about 4% of the total nitrogen of the original dough (12.3 g. of flour; N content 307 mg.). Incorporation of radioactivity increased very little between 5 and 30 min. of reaction. About 30% of the original radioactivity was found in the precipitate of both doughs. This percentage is considerably higher than that found in the precipitate of the flour slurry, where only 10% radioactivity was found. From these data, it appears that mixing promotes the reaction of protein SS groups and GSH, although extended mixing increased only slightly the rate of reaction.

Distribution of ^{35}S Added as $G^{35}SH$ in Dough—Sephadex Separation of Acetic Acid Solubles

The following reactions proceed between $G^{35}SH$ and protein disulfide, although the whole reaction is more complicated as oxygen relates to the exchange.

$$PrSSPr + G^{35}SH \rightarrow PrS^{35}SG + PrSH \quad (1)$$
$$PrS^{35}SG + GSH \rightarrow GS^{35}SG + PrSH \quad (2)$$
$$GS^{35}SG + GSH \rightarrow GSSG + G^{35}SH \quad (3)$$

Reaction 2 and/or 3 occurs when GSH is present in excess.

In Fig. 2 results are given on the measurement of radioactivity, absorbance, and nitroprusside reaction of doughs which had been treated with $G^{35}SH$ (1 μmol./g. flour) and remixed with excess GSH (50 μmol./g. flour) after separation of the acetic-acid-soluble material on Sephadex G-100.

In comparing absorbance at 280 mμ for the control (solid line, closed circle) and the GSH extract (solid line, open circle), both have two significant peaks, the first peak resulting from the higher-molecular-weight proteins and the second from more soluble proteins. It would appear that some of the acetic acid-insoluble protein was solubilized by the GSH in that extract; the open-circle peak appeared earlier and is higher and sharper than that of the control (solid-circle peak) at 50 ml. of effluent volume. Some of the gluten appeared to be cleaved or otherwise disaggregated to lower-molecular-weight protein and appeared in later effluents after mixing with excess GSH. The second E_{280} peak of the open-circle line (GSH) is higher than the solid-circle line (control).

Since the extract of the dough with excess GSH might contain noticeable unreacted and/or oxidized GSH, nitroprusside tests were made on the effluents. The peak of color intensity was at 165 ml. and occurred 10 ml.

later than the second E_{280} peak of the same sample. The peak corresponds to that of free GSH or GSSG.

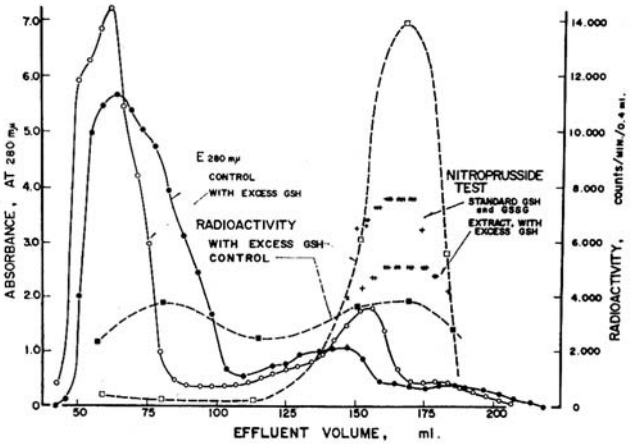


Fig. 2. Measurement of absorbance, radioactivity, and nitroprusside test on fractionation on Sephadex G-100 of acetic acid solubles of dough which had been mixed with 1 μmol. of $G^{35}SH$/g. flour and were remixed with and without excess GSH (50 μmol./g. flour). Absorbance at 280 mμ is shown by solid lines; closed circle, control; open circle, excess GSH; radioactivity is shown by broken lines; solid squares, control; open squares, excess GSH.

The distribution of the radioactivity showed that reactions 2 and 3, above, took place. In the control, the distribution of radioactivity was spread to all fractions at nearly the same position as the two E_{280} peaks (broken line, solid squares). The last part of the radioactivity of the second peak could be unreacted $G^{35}SH$ and/or $G^{35}S^{35}SG$. The sample from the remixed dough with excess GSH showed one significant peak at the same position as the nitroprusside peak of GSSG, and no peak was detected at the positions of the E_{280} peaks of the control.

The data obtained from these investigations with $G^{35}SH$ are in general agreement with the findings of Stewart and Mauritzen (7), who used ^{35}S-cysteine in dough-mixing experiments. They found 24 to 38% ^{35}S-cysteine incorporated into gluten proteins with dough-mixing times ranging from 3½ min. to 1 hr. The findings reported here show the incorporation of 29 to 35.4% $G^{35}SH$ into gluten protein with mixing times from 5 to 30 min. Assuming that SS cleavage occurred with this incorporation of $G^{35}SH$, it can be estimated that approximately 2%, or less, of gluten SS bonds are broken during normal dough mixing, employing the relatively high GSH level of 1 μmol./g. of flour. Mauritzen estimated that 1 to 2% of gluten SS bonds were broken by cysteine unless a large excess of cysteine was employed.

The high sensitivity of mixing doughs to free thiols, even at low levels, and the speed of this change suggest some kind of rapid mechanism. The rate or extent of disulfide-sulfhydryl interchange, demonstrated by the experiments reported in this paper and by those of Mauritzen and Stewart

(4,5,6), is not as much as might be expected. It may be that a certain few SS bonds are crucial to dough integrity and are more vulnerable to SH attack. In a more dilute slurry, stirring does not provide sufficient energy to affect either hydrogen-bond breakage or strained SS linkages. The experiment in which Sephadex was employed to separate flour proteins demonstrated a disproportionation of proteins toward lower molecular weight, as a consequence of reaction with a large excess of GSH. It would appear that excess GSH fragmented some gluten proteins by inter-SS cleavage, or otherwise disaggregated large protein complexes.

The loss of conformational stability of flour proteins due to scission of intra-SS bonds is a possible effect of thiols on mixing and must be considered in the interpretation of these experimental findings.

It seems most likely that the major part of the energy employed in mixing breaks the hydrogen bonds of gluten and starch; then certain of the SS bonds are stretched and strained, making them more vulnerable to attack by SH groups by changing the configuration and arrangement of the protein structure.

There is the possibility that, in dough systems, the free radical of GSH (GS°), which is formed by oxygen, causes scission of protein disulfide into $2PrS^\circ$. Gilbert *et al.* (11) detected GS° in the solutions of deoxyribonucleic acid or sodium alginate, which caused a decrease in the viscosity of the solutions. Although Axford *et al.* (12) obtained no indication of the presence of free radicals in dough during mixing, the formation of GS° could lead to SS interchange in dough.

$$2GS^\circ + PrSSPr \rightarrow GSSG + 2Pr^\circ \quad (1)$$

$$GS^\circ + PrS^\circ \rightarrow GSSPr \quad (2)$$

The reaction rate may be proportional to the concentration of reactants, which might account, in some measure, for more interchange in dough than in a suspension.

In addition to the equations for exchange of PrSSPr with GSH, given in the introductory section, there are other possibilities. Cecil and Weitzman (13) and Weitzman (14) demonstrated that the redox potential, E_o', for disulfide-sulfhydryl systems of insulin and some other proteins, is fairly comparable to that of the cysteine-cystine system. Isles and Jocelyn (15) showed that the incubation of cysteine and some proteins induced formation of mixed disulfides and that GSH regenerated the SH groups of bovine serum albumin lost in the reaction with cystine. This behavior might be applied to the reaction of GSH and protein SS bonds in dough, as follows:

$$PrSSPr + GSH \rightarrow PrSH + PrSSG \quad (1)$$

$$2PrSH \xrightarrow{\frac{1}{2}\,O_2} PrSSPr + H_2O \quad (2)$$

$$2GSH \xrightarrow{\frac{1}{2}\,O_2} GSSG + H_2O \quad (3)$$

$$PrSH + GSSG \rightarrow PrSSPr + GSH \quad (4)$$

It must be kept in mind that the experiments reported here, and those conducted by Mauritzen and Stewart (4,5,6), tested the exchange of flour protein disulfides with molecular thiols, glutathione, and/or cysteine. This

does not answer the question of the amount of exchange that would exist in a normal dough, since endogenous protein thiol is present in very small amounts in flour and is probably much less reactive, owing to the large steric requirement, as compared with smaller thiol compounds. Interchange reactions between protein disulfide groups and thiols may be governed largely by the reactivity of the latter. More experimental data are needed before the rheological behavior of dough, as affected by oxidizing and reducing substances, can be satisfactorily explained.

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