EFFECT OF BISULFITE AND ACETALDEHYDE ON THE DISULFIDE LINKAGE IN WHEAT PROTEIN[1]

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ABSTRACT

Wheat proteins extracted from flour with $N/100$ acetic acid following a preliminary extraction with water were treated with bisulfite. The increase in sulfhydryl content was followed titrimetrically, using the mercury pool electrode. Only a small increase in –SH was found after 3-minute reaction time. A rapid increase in –SH followed in the next 2 or 3 hours. Urea increased the rate at which equilibrium was attained. With the addition of acetaldehyde a rapid decrease in –SH content was observed at pH 6 and 7 and a slower decrease at pH 4 and 5. N-ethylmaleimide and bromate decreased the titration value prominently. The equilibrium constant for the reaction of bisulfite with wheat proteins was obtained. The above chemical effects are supported by the physical effects of bisulfite and acetaldehyde on the mixing characteristics of dough in the farinograph.

A study of the disulfide-sulfhydryl system of wheat proteins should contribute significantly to a better understanding of the fundamental structure and properties of dough. One approach to this problem has been to focus the attention primarily on the sulfhydryl group as was done by Kong, Mecham, and Pence (9), Bloksma (2), and by Matsumoto and Hlynka (13). Another approach is to place emphasis on the disulfide cross-linkage. This second approach has been adopted in the present investigation. Bisulfite was selected as a reagent that has a prominent effect on the disulfide linkage, and the reaction was followed titrimetrically with the newly developed mercury pool electrode (8).

The effect of bisulfite on the mixing properties of dough was shown by Freilich and Frey (3) in a comparative study of oxidizing and reducing agents. Merritt and Bailey (11) reported that the addi-

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tion of bisulfite resulted in an increased extensibility and a decreased resistance to extension of doughs tested with the extensigraph. Using both the extensigraph and the farinograph, Hlynka (5) studied the effect of bisulfite and acetaldehyde and interpreted his results in terms of the cleavage of a cross-linked network structure of dough.

Additional information was provided by Olcott, Sapirstein, and Blish (14) and Udy (20), who followed the changes in viscosity of gluten dispersions in dilute acetic acid, treated with small amounts of bisulfite. The former authors concluded that the direct reaction of bisulfite with gluten, in decreasing the relative viscosity, was a primary one, and by implication that the activation of proteinase was a minor consideration. Udy suggested a possible fragmentation resulting from the cleavage of the disulfide linkage. He also showed that bisulfite seemed to have some effect on the control of electrostatic forces between protein molecules.

Matsumoto (12) reported a comparative study on the viscosity change and on the sulfhydryl content in gluten determined by amperometric titration (1), using a rotating platinum electrode in ammoniacal buffer and urea solution. He showed that the increased titration value with bisulfite came from the cleavage of the disulfide to sulfhydryl in gluten. The amperometric titration with the rotating mercury pool electrode made possible the titration at a lower pH nearer to that of normal dough. In the present investigation the mercury pool electrode was used, with the modification that the electrolyte was stirred with a magnetic stirrer. The results obtained in the study of the effect of bisulfite and acetaldehyde are reported.

Materials and Methods

The flour used in this study was an unbleached, improver-free, straight-grade flour commercially milled from Manitoba No. 2 Northern wheat. The protein content of the flour was 12.6%. The gluten dispersion was prepared according to the procedure described in our previous paper (13), with details as follows:

The flour was extracted with water at first, with a water : flour ratio of 7:1. The gluten dispersion was obtained by extracting the residue from the water extraction with $N/100$ acetic acid, with the acid solution : original flour ratio of 4:1. This dispersion contained 1.42 to 1.60% protein and had a pH of 5.0. All of the reactions and manipulations reported in this paper were carried on in a nitrogen atmosphere.

Amperometric Titration. The mercury pool electrode, described by Kolthoff, Anastasi, and Tan (8), was used. It was a glass cup about

5 mm. in height and 6 mm. in diameter, filled with mercury. The electrolyte was mixed with a magnetic stirrer at 100 r.p.m. instead of rotating the electrode.

The composition of electrolyte (final concentration) was:

Gluten (dispersed in $N/100$ acetic acid)	0.71–0.77%
Urea	6.7M
KCl	0.08N

The potential of the electrode vs. a saturated calomel electrode was −0.15 volt. The titration was carried out with $M/500$ mercuric chloride at room temperature. For these titrations the pH of the electrolyte was not adjusted in the range of pH 5 to 2; the titration values obtained in this range were within a standard deviation of 2.4%. When the pH was higher than 5, the solution was adjusted to pH 5 with hydrochloric acid. End-points were determined from the current-titration curves. Calculation was made to correspond to the ratio 1 $HgCl_2$ to 2 SH. The recovery of glutathione added to the above electrolyte was 118% by this method.

Farinograph Tests. Dough was mixed from 50 g. flour with 50 p.p.m. bisulfite for 3 minutes in a small farinograph bowl with the normal absorption decreased by 10 ml. Then it was rested for 5 minutes at 30°C. as reaction time. After a rest period, 250 p.p.m. acetaldehyde were incorporated into the dough with 10 ml. of water and the dough was mixed again to obtain a farinogram. In the control experiment 10 ml. of water were added instead of the reagent. pH was adjusted with phosphate buffer.

Results

The results are presented in five sections. Data on the effects of bisulfite and its dependence on reaction time are summarized first. Then follow observations on the effect on the bisulfite reaction of pH, acetaldehyde, oxidizing agents, and specific sulfhydryl reagents. Finally farinogram tests are shown.

1. *Effect of Bisulfite and Its Reaction Time on Sulfhydryl Content of Gluten Dispersion.* Solid bisulfite was added to gluten dispersions. The reaction was carried out under an atmosphere of nitrogen at 30°C. The final concentration of urea in the titration vessel was adjusted to the same level as shown above.

The results are shown in Fig. 1. Both with and without the addition of urea, only a small increase in sulfhydryl was found after 3 minutes' reaction time. An increase in sulfhydryl content followed during the next 2 or 3 hours, but only with solutions containing urea

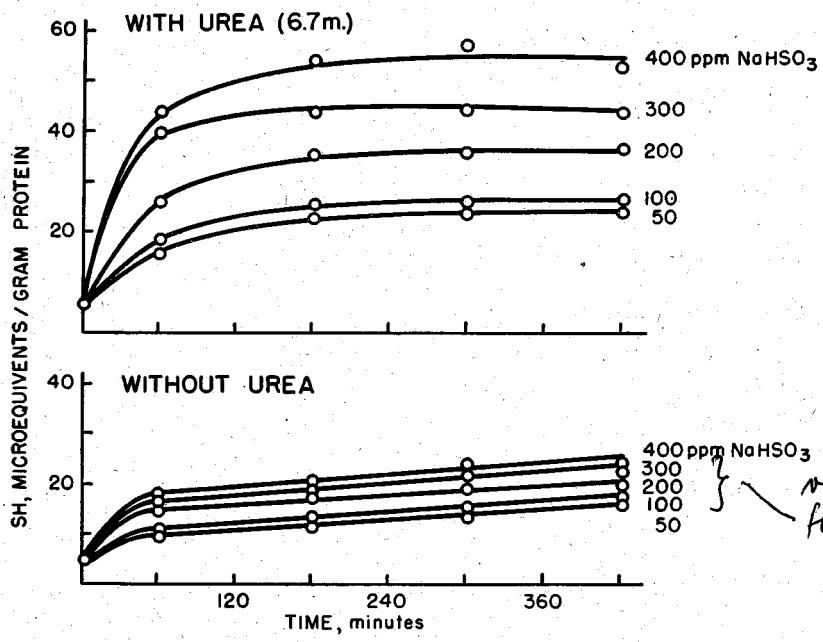

Fig. 1. Sulfhydryl increment versus reaction time on the reaction of gluten dispersion with bisulfite at pH 5.

was equilibrium finally attained in this time. The reaction was faster with urea, as indicated by a higher sulfhydryl content found.

2. *Effect of pH on the Reaction of Bisulfite.* Gluten dispersions without urea cannot be adjusted to the desired pH, since precipitation results. They were therefore treated with 200 p.p.m. bisulfite in 6.7 molar urea at various pH's, adjusted with sodium hydroxide or acetic acid.

The results are shown in Table I. No large difference in the sulf-

TABLE I

EFFECT OF pH ON THE REACTION OF BISULFITE[a]

pH	—SH (Microequiv. per g. Protein)
	After treatment
2.0	10.8
3.0	26.8
4.0	38.0
5.0	37.2
6.0	37.6
	Before treatment
5.0	3.0

[a] 3 hours' treatment with 200 p.p.m. bisulfite.

hydryl increment in the range of pH 4 to 6 was found. The small sulfhydryl increment at pH 2 to 3 seems to show a low reactivity of the disulfide linkage under these conditions.

3. *Effect of Acetaldehyde on Sulfhydryl Content of Gluten Dispersion Treated with Bisulfite.* After 3 hours' reaction time with 200 p.p.m. bisulfite at pH 5, 440 p.p.m. of acetaldehyde were added to the reaction mixture of a gluten dispersion adjusted to the desired pH. The first and the second reactions were carried out in 6.7 molar urea solution dissolved directly in the dispersion.

The results of the determination of free sulfhydryl are shown in Fig. 2. With the addition of acetaldehyde, a rapid decrease in sulfhydryl content was observed at pH 6 and 7 and a slower decrease at pH 5 and 4.

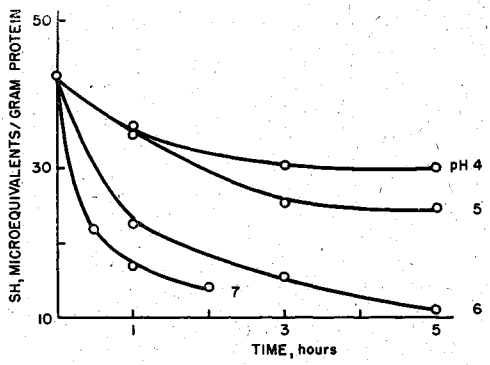

Fig. 2. Effect of acetaldehyde on sulfhydryl groups of gluten dispersion treated th bisulfite at various pH levels.

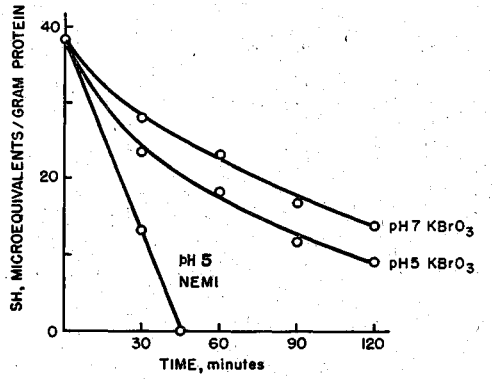

Fig. 3. Effect of bromate or N-ethylmaleimide on sulfhydryl groups of gluten dispersion treated with bisulfite.

4. *Effect of NEMI or Bromate on Sulfhydryl Content of Gluten Dispersion Treated with Bisulfite.* After 3 hours' reaction time with 200 p.p.m. of bisulfite, 400 p.p.m. of N-ethylmaleimide (NEMI), a specific reagent for the sulfhydryl group, or 200 p.p.m. of potassium bromate, were added to the mixture.

The results of the determination of free sulfhydryl groups at various times after the addition of the last reagent are shown in Fig. 3. The dough containing NEMI had a pH of 5 and the doughs with bromate were at pH 5 and pH 7.

5. *Effects of Bisulfite and Acetaldehyde on Farinograms at Different pH.* Bisulfite and acetaldehyde were incorporated into the dough at the same time by Hlynka (5). In this experiment, acetaldehyde was mixed into the dough 8 minutes after the bisulfite had been incorporated initially into the dough at pH 5 or 7. Results are shown in Fig. 4.

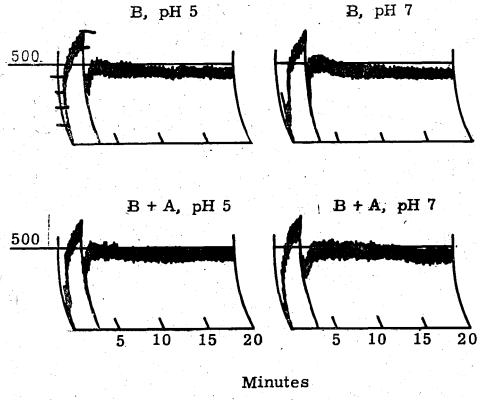

Fig. 4. Effect of bisulfite and acetaldehyde on farinograms at pH 5 and 7. B, Bisulfite, 50 p.p.m.; A, Acetaldehyde, 250 p.p.m. Initial mixing 3 minutes with bisulfite, with absorption decreased by 10 ml. Rest period 5 minutes. Final mixing 17 minutes with or without acetaldehyde in 10 ml. water.

These farinograms show that the addition of acetaldehyde, after the reaction of dough with bisulfite, had little effect at pH 5. At pH 7 a definite increase in consistency is shown as a result of treatment with acetaldehyde.

Acetaldehyde without bisulfite was confirmed to have no effect on the farinogram at both pH 5 and 7. However, it is not shown in Fig. 4, as the result is the same as reported by Hlynka (5).

Discussion

Some studies on the effect of bisulfite on the disulfide linkage of

cystine, oxidized glutathione, and protein (16,17) bovine serum albumin (8), and wool (4,18) have been published recently. In these studies the following mechanism of the reaction is generally accepted.

$$P\begin{matrix}S\\|\\S\end{matrix} + SO_3^{=} + H^+ \rightleftharpoons P\begin{matrix}SH\\|\\S.SO_3^-\end{matrix} \qquad (1)$$

where the protein disulfide is denoted by $P\begin{matrix}S\\|\\S\end{matrix}$.

It is reasonable to assume the same type of reaction for gluten and bisulfite. This reaction of bisulfite with gluten, breaking the disulfide linkages of protein into sulfhydryl and sulfonate groups, has been confirmed experimentally in the present study through amperometric titration of the sulfhydryl group produced.

However, some comment should be made on the effect of bisulfite on this titration itself. The condition of this titration is quite the same as Kolthoff's (8), except that urea is used as a denaturing agent instead of guanidine hydrochloride, and the sample is gluten dispersion in acetic acid. The urea and acetic acid were found to have no inhibiting effect on this titration in blank tests with them. On the basis of this observation and the fact that N-ethylmaleimide (which is a specific reagent for the sulfhydryl group) can cancel the titration value, the increased titration value is presumed to come from sulfhydryl groups of gluten.

The effect of bromate in markedly decreasing the titration value should be considered from two aspects. One is a reaction of bromate with sulfite which causes shifting the equation (1) to the left, and the other is the direct oxidation of sulfhydryl groups of gluten. The effect of pH on the oxidation seems to support the latter possibility. The question arises whether the sulfhydryl is produced during the reaction, or results from the treatment of the titration electrolyte with urea. The results obtained for the 3 minutes' reaction time in Fig. 1 indicate very little increase of titration value. Thus the increased titration value may be considered as a result of reaction time and not as that of treatment during the titration. This is also known from titration curves which show no decrease in current with each addition of mercuric chloride solution. These curves, which served mainly as primary data, are not shown in this paper.

The data shown in Fig. 1 indicate that urea increases the velocity of attaining equilibrium and also produces more sulfhydryl groups

than is produced in the reaction without urea. It is reasonable to assume that the breaking of hydrogen bonds by urea may increase the susceptibility of protein toward bisulfite.

An equilibrium constant for equation (1) can be calculated from titration values with the assumption that all disulfide groups in gluten are reactive and have the same equilibrium constant, and the reactions are consecutive.

The constant is shown by the equation (2) at a given pH.

$$K = \frac{[-SH]\ [-S.SO_3^-]}{[P(S-S)]\ [SO_3^=]} \qquad (2)$$

The values for each bisulfite concentration in Fig. 1 are summarized in Table II.

TABLE II

EQUILIBRIUM CONSTANT IN EQUATION (2) AT 30°C. WITH OR WITHOUT 6.7*M* UREA AT PH 5.0

BISULFITE CONCENTRATION	MOL. CONCENTRATION IN SOLUTION [a]				K
	PSH	$PSSO_3^-$	P(S–S)	$SO_3^=$	
ppm	$\times 10^{-4}$	$\times 10^{-4}$	$\times 10^{-4}$	$\times 10^{-4}$	
With urea					
50	3.7	3.0	10.0	0.59	1.88
100	4.2	3.5	9.5	2.01	0.77
200	5.8	5.1	7.9	4.65	0.81
300	7.1	6.4	6.6	7.39	0.93
400	9.1	8.4	4.6	10.00	1.66
Blank	0.7	0	13.0	....	...
Without urea					
300	3.2	2.5	9.3	8.80	0.09

[a] After equilibrium was attained.

In these calculations, $SO_3^=$ was obtained from bisulfite added to the solution through the following equations:

$$HSO_3^- \rightleftharpoons H^+ + SO_3^= \qquad (3)$$

$$H_2SO_3 \rightleftharpoons H^+ + HSO_3^- \qquad (4)$$

Denoting by α the expression,

$$\frac{SO_3^=}{H_2SO_3 + HSO_3^- + SO_3^=}$$

$$\text{then } \frac{1}{\alpha} = 1 + \frac{1}{K_2}\ [H^+] + \frac{1}{K_1K_2}\ [H^+]^2 \qquad (5)$$

where K_1 and K_2 are the equilibrium constants of equations (4) and (3), and were taken as 1.7×10^{-2} and 5×10^{-6} respectively (10). The calculated α was 0.33 at pH 5. P (S–S) in the table was obtained from the total cystine content and the reacted disulfide. For the calibration the cystine content was assumed to be 2% of the total gluten. This

value was based on published literature (13,15) and was also checked experimentally.

Some of the equilibrium constants at each bisulfite concentration were closely reproducible, while some indicated higher values. These higher values may be explained with the assumption that the sulfhydryl groups that come into solution at lowest and highest bisulfite concentrations have a different reactivity. The K value in the reaction without urea is about one-eighth of that with urea. This reactive disulfide, calculated from the data obtained in the reaction with bisulfite at 400 p.p.m. in the reaction mixture which produced maximum sulfhydryl content in Fig. 1, was 63% of total disulfide calculated from the cystine content.

Udy (20) showed that the intrinsic viscosity of gluten dispersions from different flours which originally had different values reached the same value after the reaction with bisulfite. The differences in intrinsic viscosity of gluten dispersions may be related to the reactive disulfide, because both the differences between samples in intrinsic viscosity and in reactive disulfide linkages disappear after the reaction with bisulfite.

The results in Fig. 2 show very good agreement with those reported by Hlynka (5) in studies of the physical properties of dough and gluten. The reaction of acetaldehyde which decreased the sulfhydryl groups produced by bisulfite shows strong evidence of its counteraction to bisulfite, although this is very slow except at pH 6 and 7.

This reverse reaction may be explained by the following possible reactions:

(a) *Reverse Reaction of Equation (1).* In the addition of acetaldehyde to the reaction mixture, bisulfite is removed from the reacting system by forming an addition compound with acetaldehyde which shows very little dissociation at pH lower than 8 (19). The equation (1) is shifted to the left, assuming it to be reversible. As a result, the titratable sulfhydryl groups are decreased.

(b) *Mercaptal Formation.* Acetaldehyde may react with sulfhydryl groups of gluten produced by the reaction with sulfite, to form a mercaptal at high pH level which prevents the sulfhydryl groups from titration with mercuric ions.

(c) *Oxidation of Sulfhydryl Group.* A small amount of oxygen may be left in the reaction mixture, and may oxidize sulfhydryl groups at a higher pH level in the medium where excess bisulfite was eliminated from the reaction mixture.

This third possibility can be neglected, because in this study acetaldehyde was added to the mixture which was freed from oxygen

by twice-repeated evacuations and filling with nitrogen alternately.

The first possibility depends on the reversibility of equation (1). The authors checked this reversibility by breaking the equilibrium of the equation through vacuum evaporation of sulfur dioxide at a low pH level. At pH 2.8 the titration value of the reaction mixture of Section 1 was approximately the same before and after evaporation of sulfur dioxide. However, the mixture raised to pH 6 after the evaporation at pH 2.8 decreased in sulfhydryl content to about one-half. Stricks and Kolthoff (16) described the reversibility of the cysteine-cystine system in the presence of sulfite in the pH range from 8 to 13. Thus the first mechanism appears to be more probable.

However, the alternate possibility of mercaptal formation cannot be neglected.

The farinograms shown in Fig. 4 also strongly support these results. That is, a definite increase of consistency is observed on the addition of acetaldehyde at pH 7 but not at pH 5, where little decrease of sulfhydryl groups is detected in Section 3. The free sulfhydryl groups in water-soluble and acid-soluble proteins were estimated before and after oxidation by Matsumoto and Hlynka (13). The value of free sulfhydryl was very small compared with disulfide linkage. Thus the sulfhydryl-disulfide exchange reaction is suggested as part of this system too (6,7). The reactive disulfide in gluten studied in this paper also seems to play an important part in the sulfhydryl-disulfide exchange reaction. Further work on this disulfide linkage may eventually build up a bridge between rheological behavior and basic structure of dough.

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