

THE SULFHYDRYL CONTENT OF DOUGHS MIXED UNDER NITROGEN¹

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ABSTRACT

The sulfhydryl content of flour-water doughs mixed under nitrogen in a farinograph was determined by amperometric titration with silver nitrate. During mixing periods of 15 to 50 min., sulfhydryl content increased in seven hard red winter and four hard red spring wheat flours. The increases ranged from 0.14 to 0.40 $\mu\text{eq.}$ per g. The mechanical action of dough mixing was required for the increase. The pattern of changes up to 15 min. differed among six flours; in two, no changes were found, but in the others sulfhydryl rapidly decreased and then recovered to near the flour value. With the latter four flours, removal of free lipids with carbon tetrachloride eliminated the initial rapid decrease. Both the increase over the original sulfhydryl content and the initial loss and recovery can be significant factors in relating sulfhydryl losses to amounts of oxidant consumed in doughs.

The sulfhydryl groups of flour proteins appear to be more important to the physical properties and baking behavior of doughs than their low concentration would lead us to expect. For example, sulfhydryl (-SH) contents decrease when flours or doughs are matured by oxidation, apparently irrespective of the improving agent. Also, if the dough is to have normal elasticity and extensibility, some -SH groups must be present during dough mixing, perhaps to promote sulfhydryl-disulfide interchange. Most of the publications pertinent to these generalizations were cited by Sullivan, Dahle, and Schipke (1); other more recent publications are cited below.

Doughs mixed in air, so that some -SH groups are oxidized (2), differ in physical properties from doughs mixed in nitrogen. In load-extension tests, the latter doughs are more extensible and less resistant to deformation. Mixing curves obtained in air and in nitrogen also have been observed to differ (3,4), but not as consistently as load-extension curves. When attempts have been made to relate such differences to the -SH content of doughs, the -SH level during mixing under nitrogen has been assumed not to change significantly. However, in two of the more recent papers, Tsen and Bushuk (5) and Bloksma (6) noted that in doughs mixed under nitrogen the -SH content may increase slightly. They give data for three flours, although Bloksma also states that the "slow increase is occasionally found with both normal and defatted flour."

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When similar experiments were made in this Laboratory, substantial increases were sometimes found after extended mixing. More information was collected, primarily to learn the extent of differences among flours. The results, presented below, show considerable differences among flours and suggest that the increases may be significant in relation to several questions about -SH groups and dough behavior.

Materials and Methods

Analytical data and farinograph curve characteristics for the flours used are given in Table I. Farinograph curve characteristics for four

TABLE I
SOURCE, ANALYTICAL DATA, AND FARINOGRAPH CURVE CHARACTERISTICS OF FLOURS

CLASS	WHEAT		FLOUR			DOUGH ^a		
	Area	Variety	Mill	Protein ^b	Ash ^b	Absorption ^c	Peak Time	Stability
				%	%	%	min.	min.
HRW	Nebr.	(Mixed)	QJ ^d	15.3	0.67	60.4	7.5	22.5
	Nebr.	Pawnee	QJ	12.4	0.55	58.2	4.5	9.5
	Kans.	Comanche	Multimat	15.0	0.47	60.4	4.5	19.5
	Kans.	Pawnee	Multimat	10.7	0.47	55.8	2.0	4.0
	Kans.	Bison	Multimat	12.6	0.49	56.2	2.5	18.5
	Kans.	Triumph	Multimat	12.3	0.44	57.4	4.5	8.0
	Kans.	Wichita	Multimat	12.8	0.45	55.8	5.0	10.0
HRS	N.D.	Conley	Multimat	15.2	0.45	64.0	6.5	15.5
	N.D.	Lee	Multimat	16.0	0.50	64.0	7.5	14.5
	N.D.	Selkirk	Multimat	14.8	0.48	63.4	4.5	9.0
	N.D.	Thatcher	Multimat	13.6	0.51	62.8	5.0	15.5
	(Mixed)	Commercial	15.6	0.45	65.8	5.0	9.5
Carbon Tetrachloride-Washed Flours								
HRW	Nebr.	(Mixed)	QJ	63.2	9.0	13.0
	Nebr.	Pawnee	QJ	61.0	5.5	8.0
	Kans.	Comanche	Multimat	62.4	8.5	12.5
	Kans.	Pawnee	Multimat	57.4	3.0	6.5

^a Doughs mixed under nitrogen.

^b Dry basis.

^c 14% Moisture basis.

^d Quadrumat Junior.

flours after extraction of lipids with carbon tetrachloride are included. Those flours milled on a Miag Multimat² mill were provided by the Northern Utilization Research and Development Division.

Doughs were mixed in a 50-g. farinograph bowl. Oxygen was excluded from flour, water, and dough as follows: flour samples were weighed out and placed in a desiccator; the desiccator was evacuated, filled with nitrogen, and then held overnight at 35°C. A "cleaner"

²Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

dough was mixed to check and adjust the farinograph and then discarded. One of the flour samples was placed in the farinograph bowl, and the bowl was covered with a fitted cover of clear plastic. The farinograph was run for 2- or 3 min. with a stream of nitrogen gas passing through the bowl and over the dry flour. Water (freshly boiled and flushed with nitrogen before being placed in the buret) then was added, and the dough mixed as usual. The flow of nitrogen continued throughout mixing. (The nitrogen was passed through two gas-washing bottles containing water to humidify the gas and prevent drying of the dough.) When mixing was stopped, doughs were immediately pressed into thin sheets between two blocks of dry ice, and the frozen doughs lyophilized. The dried doughs were ground through a 60-mesh screen (Wiley mill), allowed to absorb moisture from room air for a day to facilitate weighing, and stored at 35°C.

Less flushing with nitrogen did not result in lower -SH contents. Thus, doughs mixed 5 min. from one flour, gave the following: regular procedure, 1.00 μ eq. sulfhydryl per g. (dry basis); held in air overnight, but given 10 min. of stirring under nitrogen in farinograph bowl before water was added, 1.01 μ eq.; in air overnight, 1-min. stirring under nitrogen, 1.04 μ eq. With a second flour, the 1-min. stirring under nitrogen gave the following for doughs mixed 5 min. in duplicate: after storage in air, 0.96, 1.01 μ eq.; after storage under nitrogen, 0.98, 0.96 μ eq. per g. The precautions for exclusion of air therefore must have been more than adequate.

Sulfhydryl determinations (7) were made by amperometric titration with silver nitrate in Tris buffer-urea suspensions (8). Each value reported is based on at least three titrations, with sample size varied. For flour and most dough samples, 200, 400, and 600 mg. were titrated; but smaller samples were titrated when -SH contents were unusually high. From the titers, the slope of the line representing ml. titrant per g. sample was calculated by a least-squares method.

Results and Discussion

Changes in Sulfhydryl Content with Mixing. Initial observations were made on a few doughs mixed 20 min. under nitrogen. In most instances the -SH content was higher than that of the flour, but differences were small. To obtain a more detailed picture of the time course of changes, series of doughs differing in mixing time by 5- or 10-min. increments up to 50 min. were prepared. Increases in -SH content were definite, usually easily detectable with less than 50 min. of mixing. Results with four flours giving about the smallest and largest increases are presented in Fig. 1. As shown, the largest increases

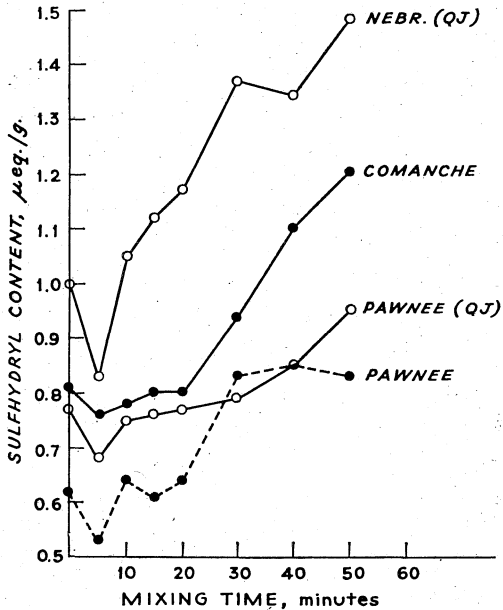


Fig. 1. Sulphydryl content of flour-water doughs mixed under nitrogen in a 50-g. farinograph bowl. The Pawnee curve (dashed lined) has been displaced downward by 0.1 $\mu\text{eq. per g.}$ QJ designates flours milled on a Quadrumat Junior mill; the other two were milled on a Multimat.

were about 0.4 $\mu\text{eq. per g.}$ flour. This increase approaches 50% over the flour value in the Comanche set.

There appears to be no reason to doubt the over-all upward trends with long mixing. However, the precision of the analyses is not sufficient to establish small differences in rates or patterns of increase among flours. The irregularities in the plotted lines in Fig. 1 show this imprecision; the standard deviation of the value for a single point (calculated from the three individual titrations) averaged $\pm 0.026 \mu\text{eq. per g.}$, or, expressed in relation to the $-\text{SH}$ content of each sample, $\pm 2.9\%$.

The graphs of most flours, including those shown in Fig. 1, have a dip which shows a decrease in $-\text{SH}$ content during the initial 5 min. of mixing, followed by an about equally rapid recovery to near the original $-\text{SH}$ content of the dough. This dip has been observed to different degrees with several flours run at different times. It also appears in data of Bloksma (6) and Tsen and Bushuk (5), although in the latter paper the recovery did not reach the original flour value in 40 min. of mixing under nitrogen. Although this rapid drop and recovery is not commented upon by the authors cited above (5,6), its

occurrence in those investigations, as well as the present work, makes it appear to be a common pattern.

Free lipids were removed from the four flours of Fig. 1 with carbon tetrachloride, and doughs were again prepared. As shown in Fig. 2,

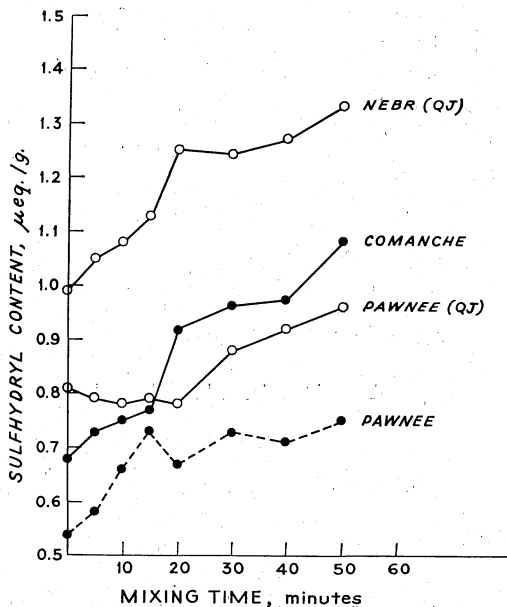


Fig. 2. Changes in $-SH$ content of carbon tetrachloride-extracted flour-water doughs mixed under nitrogen. The Pawnee curve (dashed line) has been displaced downward by $0.1 \mu\text{eq. per g.}$ Other details as for Fig. 1.

removal of lipid eliminated the dip with all four flours. Lipids were not washed from other flours, but Bloksma (6) gives results showing that removal of lipids with petroleum ether eliminated the dip. The ability of fatty acid peroxides to lower sulfhydryl content of doughs is well established (9,10). Perhaps the lipids in the flours that show loss and recovery of sulfhydryl groups had been oxidized to a degree capable in turn of oxidizing some $-SH$ groups very quickly.

A dip was not observed with all flours, however; the commercial hard red spring (HRS) wheat flour did not give one. Attempts to produce a dip by storage of the flour at room temperature in air or by addition of Cu(II) ion to doughs were unsuccessful. Additions of oxidized linoleic acid, introducing from 0.9 to $2.9 \mu\text{eq. peroxide per g. flour}$, decreased the $-SH$ content of doughs mixed for 5 min., as expected; but an increase between 5 and 10 min. of mixing was not obtained consistently. The original flour value was never regained even

in those samples giving an increase. No explanation for the inconsistency of these observations, nor for the wide variations in size of the dip among flours, can be given at present.

Comparison of Various Flours. Several other flours were tested. In most cases, -SH contents were determined only for flour and for 15- and 50-min. doughs. These determinations were sufficient to demonstrate an upward trend with long mixing when present (Table II).

TABLE II
SULFHYDRYL CONTENT OF FLOURS AND DOUGHS MIXED UNDER NITROGEN^a

	SULFHYDRYL CONTENT PER G. FLOUR			INCREASE PER G. PROTEIN	
	Flour	15-min. Dough	50-min. Dough	Flour-to- 50-min.	15-to- 50-min.
	$\mu\text{eq.}$	$\mu\text{eq.}$	$\mu\text{eq.}$	$\mu\text{eq. -SH}$	$\mu\text{eq. -SH}$
Nebraska, QJ	1.00	1.12	1.48	3.14	2.35
Pawnee, QJ	0.77	0.76	0.95	1.45	1.53
Comanche	0.81	0.80	1.20	2.60	2.93
Pawnee	0.72	0.71	0.93	1.96	2.05
Bison	0.68	0.81	0.95	2.14	1.31
Triumph	0.72	0.75	0.95	1.87	1.63
Wichita	0.66	0.75	0.94	2.18	1.48
Conley	1.02	1.07	1.23	1.38	1.05
Lee	1.03	1.09	1.30	1.69	1.31
Selkirk	0.82	0.88	1.12	2.03	1.62
Thatcher	0.88	0.92	1.11	1.69	1.40
Carbon tetrachloride — washed					
Nebraska, QJ	0.95	1.13	1.33	2.48	1.31
Pawnee, QJ	0.80	0.79	0.96	1.29	1.59
Comanche	0.68	0.77	1.08	2.67	2.06
Pawnee	0.64	0.83	0.85	1.77	1.96

^a All values, dry basis. Flour characteristics in Table I. Doughs mixed in farinograph, 50-g. bowl.

The change from flour to dough mixed for 50 min. measures total increase, but the change from 15 to 50 min. may reflect better the maximum rate of change after the irregularities of the first minutes are past. The increases in -SH content, referred to the flours (50-min. dough value minus the flour value), ranged from 0.18 to 0.48 $\mu\text{eq.}$ per g. flour; for the 15- to 50-min. doughs, they ranged from 0.14 to 0.40 $\mu\text{eq.}$ per g. flour. Values for the increases expressed per g. of protein are included in Table II. The ratio of maximum to minimum values among the various flours remains nearly as large on a protein basis as on a flour basis.

Scatter diagrams of these -SH values plotted against protein content and farinograph curve characteristics indicated that no high correlations were present. However, the scatter diagram of farinograph stability under nitrogen *vs.* the -SH increase for flour to 50-min. dough appeared to have the least randomness; and the correlation coefficient was +0.68*.

The -SH increases per g. of protein do not vary widely among most of the flours that had different protein contents. The Nebraska-QJ and Comanche samples are exceptions, with large increases per g. of protein accompanying a high protein level.

Accessibility of Sulfhydryl Groups. The results presented above, plus those of Bloksma (6) and Tsen and Bushuk (5), show that the titratable -SH content of doughs increases upon mixing. The results, however, do not differentiate between formation of new -SH groups and exposure of -SH groups already present in flour but not titratable under the particular conditions employed in the separate investigations. New -SH groups presumably could arise by mechanical scission of disulfide bonds (11), by enzymatic reduction of disulfide bonds (12), or by formation from labile grouping such as thiol esters (13). Alternatively, the exposure of -SH groups in the 6M urea in which the titration is conducted might be incomplete in the flour samples, particularly if large aggregates of protein or protein complexed with carbohydrate or lipid are present. Mixing then could break up the aggregates and make more -SH groups accessible to the silver ion for titration.

Bushuk (14) and Bloksma (15) measured the accessibility of -SH groups in flours and doughs by their consumption of iodate. Titrations of -SH groups in the absence of urea were made by Tsen and Dempster (16) for the same purpose. The latter method was preferred in the present work, because the oxidation of -SH groups was not the principal concern. The carbon tetrachloride-washed Comanche and Pawnee flour sets were chosen because of their contrast with respect to total increase, and accessible -SH contents were determined.

Table III shows appreciable differences between flours. All the -SH

TABLE III
PERCENT ACCESSIBLE SULFHYDRYL GROUPS DURING DOUGH MIXING

DOUGH MIXING TIME	ACCESSIBILITY		
	CCl ₄ -Washed Flours		Unwashed Pawnee
	Comanche	Pawnee	
<i>min.</i>	<i>% of total^a</i>	<i>% of total^a</i>	<i>% of total^a</i>
0 (flour)	82	92	90
5	92	100	89
10	93	100	100
15	88	95	100
20	67	95	100
30	72	83	100
40	79	99	100
50	68	86	100

^a (-SH groups titratable in absence of urea/-SH groups titratable in 6M urea) × 100. Dough mixed under nitrogen in 50-g. farinograph bowl.

groups of the washed Pawnee flour quickly became accessible to titration in the absence of urea as dough mixing proceeded. Even -SH groups above those originally present in the flour that became titratable in urea after longer mixing were for the most part also titratable in the absence of urea. The Comanche flour at first showed similar behavior in that the proportion of accessible sulfhydryl increased, but with longer mixing and with a larger increase in the total -SH groups (see Fig. 2), the proportion of accessible groups diminished. Because the high level of accessibility in the carbon tetrachloride-washed Pawnee flour was unexpected, titrations without urea were also made on unwashed Pawnee flour. The increase in accessible groups in unwashed Pawnee flour was consistent, and at the end of 10 min. of mixing all -SH groups were accessible without urea (Table III).

A ratio of two titrations is involved in the "percent accessible" figure, and an "inaccessible" figure would require use of a difference between two larger values. The precision of the determinations did not seem to be sufficient to justify determinations of accessible -SH groups in additional sets at present.

Rested Doughs. If enzymatic reduction of disulfide to -SH groups caused the increase in -SH content, it seemed possible that merely holding a dough would increase -SH content. Accordingly, pairs of doughs from the Bison, Triumph, and Wichita flours were mixed 15 min. in the farinograph. One dough of the pair then was allowed to rest under nitrogen in the bowl for 35 min. before freezing and drying; the other dough of the pair was mixed continuously under nitrogen for the additional 35 min. before freezing and drying. The results consistently showed little change in the resting doughs (as expected from other reports) and an increase in the 15- to 50-min. mixing (as given in Table II). Actual values for the change from 15 to 50 min. were as follows: resting doughs, -0.06, 0.00, and +0.04 $\mu\text{eq. per g.}$ for Bison, Triumph, and Wichita flours; continual mixing, +0.14, 0.20, and 0.19 $\mu\text{eq. per g.}$, in the same order.

Effects of Salt and N-Ethylmaleimide. Sodium chloride definitely modifies the physical properties of doughs and affects their response to iodate added in excess of -SH content of the flour (17). Therefore, the effect of salt at levels normal to dough was examined in some limited comparisons with the Nebraska-QJ and commercial HRS wheat flours. As shown in Table IV, increases in -SH content appeared to occur as readily in the presence as in the absence of salt.

Sulfhydryl content also increased when N-ethylmaleimide was added to block groups initially present (Table IV). In this way, sulfhydryl could be reduced to very low levels where increases were easy to

TABLE IV
EFFECT OF SALT AND N-ETHYLMALEIMIDE ON SULFHYDRYL INCREASES^a

MIXING TIME	ADDITIVE		
	None	2% NaCl ^b	NEMI ^c
	$\mu\text{eq./g.}$	$\mu\text{eq./g.}$	$\mu\text{eq./g.}$
HRS wheat flour			
Flour	0.92
2-min. dough	0.75	0.75	...
5-min. dough	0.76	0.73	...
10-min. dough	0.88	0.88	...
Nebraska, QJ flour			
Flour	1.00
10-min. dough	1.05	...	0.22
20-min. dough	1.17	...	0.37
25-min. dough	... ^d	1.18	...
50-min. dough	1.48	1.54	0.55

^aDoughs mixed under nitrogen in 50-g. farinograph bowl.

^b2% NaCl on flour weight basis.

^c1 $\mu\text{eq.}$ per g. flour.

^dInterpolation on Fig. 1 gives a value of 1.27.

detect. The increase of 0.33 $\mu\text{eq.}$ per g. from 10 to 50 min. compares with 0.43 $\mu\text{eq.}$ per g. in the control doughs. The precision of results is only sufficient to state that the increase was of the same order of magnitude as in the control. However, this result suggests that the presence of NEMI does not facilitate scission of disulfide (17). On the other hand, the increase was not markedly inhibited, as might occur if the -SH groups were formed by an enzyme requiring free -SH groups for activity.

General Discussion

It seems certain that the titratable -SH content of doughs increases as they are mixed in a nitrogen atmosphere. Flours vary considerably in this regard, but values reported from two other laboratories show increases also. The published and present observations also agree that the dough loses -SH groups early in mixing and later returns to nearly the original -SH content; but the exact course of these latter changes is difficult to follow with present analytical methods.

If these changes are accepted as established, some previous results should be reconsidered. Specifically, reported variations in the ratio of sulfhydryl lost to iodate consumed appear to arise from unrecognized formation of additional -SH groups. To illustrate, Tsen and Bushuk (5) mixed doughs 5 and 15 min. under nitrogen with 1.4 $\mu\text{eq.}$ iodate added per g. flour. In the 5-min. dough, 1.15 $\mu\text{eq.}$ iodate was consumed and 0.43 $\mu\text{eq.}$ sulfhydryl per g. remained; in the 15-min. dough, 1.39 $\mu\text{eq.}$ iodate per g. was consumed, but 0.42 $\mu\text{eq.}$ sulfhydryl per g. still remained. The production of enough -SH groups to maintain the -SH

content, replacing those oxidized by the iodate consumed, seems the simplest explanation. Similarly, a marked increase in "accessible" -SH groups with increase in mixing time was observed by Bushuk (14); e.g., 5.7 $\mu\text{eq.}$ per g. protein with 5 min. of mixing under nitrogen, and 10.8 $\mu\text{eq.}$ per g. protein with 15 min. of mixing. Such a marked increase could be accounted for in part by an increase in total -SH content during mixing.

Instances of the reversal of an effect of sulfhydryl-blocking or oxidizing agents also could be explained by the production, release, or "unmasking" of additional -SH groups. For example, Meredith and Bushuk (4) added various concentrations of NEMI to doughs mixed under nitrogen. When added NEMI exceeded a critical level, gluten could not be washed from doughs after short or moderate mixing times. After extended mixing, however, gluten could again be recovered. Perhaps longer mixing produced sufficient -SH groups to restore cohesiveness of the gluten components by redistribution of disulfide bonds through sulfhydryl-disulfide interchange reactions.

The rapid initial loss of -SH groups, giving the dip in the curves for -SH content *vs.* mixing time, also appears to be strongly involved in the variations in reported ratios of sulfhydryl lost to oxidant consumed. Tsen and Bushuk (5) reported high molar ratios, beginning at 7.0 with low iodate additions, decreasing to 3.5 with higher iodate additions. We recalculated the ratio, using as a base the -SH value for their 5-min. dough mixed under nitrogen with no added iodate, rather than the flour value. This calculation would seem to give a truer estimate of the effects of iodate, as the 5-min. dough with no iodate shows a loss of sulfhydryl. Ratios in the range from 2.5 to 3.1 then were obtained for all levels of iodate (their Table II). Bloksma (15) also gives results that show that the critical iodate addition is larger after lipids are extracted from flour with light petroleum; this again appears to reflect the removal of oxidized lipids which, in our observations as well as Bloksma's, eliminates the initial rapid loss of -SH groups.

Other examples could be given, but the above should be sufficient to indicate that in relating sulfhydryl loss to oxidant consumed, both the production or release of titratable or reactive -SH groups and the rapid initial loss frequently encountered even when mixing under nitrogen must be taken into account.

Our studies thus far have used a nitrogen atmosphere and very long mixing times, but relatively gentle mixing treatment. This procedure has been useful to trace out various features of behavior of the -SH groups. The significance of the observations to actual baking con-

ditions largely remains to be evaluated. Both the more vigorous mixing and the reducing environment of a fermenting dough, however, suggest that the observations will be relevant. The differing increases in sulfhydryl content of doughs from various flours suggest a basis for differing requirements for oxidants, even though the -SH content of flours involved may be equal.

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