THE OXIDATION OF WHEAT FLOUR
V. Effect of Lipid Peroxides and Antioxidants[1]

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ABSTRACT

The improver action of peroxidic linoleate and of lipoxidase-linoleate systems was compared with the improver action of hydrogen peroxide which was much greater. The reactivity of a lipoxidase-linoleate system to glutathione was found to be low. Assay of oxidized lipid by the 2-thiobarbituric acid method confirmed the oxidation of lipids in dough during mixing and established, qualitatively, a slight interaction of sulfhydryl and oxidizing lipid. The effect of thioctic acid on the improver action of oxidized lipid was studied and interpreted in terms of the amount of thioctic acid present in flour.

All evidence from these investigations suggests that the role of peroxidic lipid as a sulfhydryl agent is a minor factor of maturing action during mixing.

The lipids represent not more than 1.0 to 2.5% of the weight of flour and, of this amount, only 60 to 70% is soluble in nonpolar sol-

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vents such as petroleum ether and chloroform. In order to obtain complete extraction, solvents with some polar properties (such as alcohol-ether and water-saturated butanol) must be employed or, alternatively, acid hydrolysis.

The present state of our knowledge of the lipids of wheat and their role in dough properties has been the subject of reviews and articles by Fisher (15), Cookson and Coppock (7), Coppock (8), Houston (20), Smith and Andrews (25), Glass (16), and Tsen and Hlynka (31). In a recent series of papers (4,5,6), Carter *et al.* have contributed significantly to our knowledge of the constitution of the wheat flour lipids.

There are several compounds in flour that can function as antioxidants – among others, the tocopherols, thioctic acid, and some pigments. Also, the –SH groups of the flour proteins may be considered as antioxidants of lesser effectiveness.

It seems reasonable to assume that the first stage of natural aging and the action of many maturing agents is the removal of antioxidants. It is known that tocopherols inhibit the start of lipid oxidation and also can destroy preformed hydroperoxides. It has been shown that chlorine dioxide, particularly at high levels, destroys tocopherols (14,22). Thioctic acid and certain other cyclic disulfides also can function as lipid antioxidants (24). Thioctic acid and its probable role will be discussed separately (13).

As flour ages, there is destruction of the natural antioxidants and a gradual increase in the fatty acids, particularly linoleic, caused by enzymatic hydrolysis of the lipids. The longer the storage time and the higher the moisture content and temperature, the greater is the production of fatty acids. The oxygen uptake of doughs during mixing is also increased with storage of the flour (9,18). When present in sufficient amounts, unsaturated fatty acids decrease the extensibility of a dough and give effects similar to overmaturation. These effects are even more marked when the fatty acids are oxidized (28). Flours extracted with petroleum ether show increased strength, greater resistance to extension and lessened extensibility. Flours extracted with petroleum ether and, especially, water-saturated butanol also show less reduction of bromate (10). No definite explanation of this phenomenon is known, but it may be due to the removal of some of the water-soluble sulfhydryl in the butanol extraction and possibly, in the case of the petroleum ether extraction, the removal of the lipoprotein compound, as described by Balls and Hale (1).

Smith and Andrews (25) and Smith, Van Buren, and Andrews (26) have provided valuable data on the uptake of oxygen during mixing

of a dough. They showed that oxidation of linoleic acid, apparently by lipoxidase, was chiefly responsible. Heating the flour reduced the oxygen uptake; the effect of the heat was mainly on the water-soluble fraction and was believed to be due to an enzyme. The changes they observed in the –SH content of dough due to oxidation could not be correlated with changes in physical dough properties as measured by the extensigraph. More recently, Tsen and Hlynka (31) proposed a hypothesis for the role of lipids in the oxidation of dough. They confirmed the oxidation of the lipids during mixing. This oxidation was increased as the concentration of the oxygen was raised. The authors stated that peroxides are formed mainly through peroxidation of the "free" lipids (petroleum ether-extractable) during the mixing process and that lipoxidase catalyzes the oxidation. Tsen and Hlynka further assumed that, since hydroperoxides and peroxides are dough-strengthening agents, they act on the –SH groups. They visualized that, during ing mixing, the unsaturated flour lipids and –SH groups compete with each other for available oxygen, with the fatty acid peroxides oxidizing the –SH groups.

Materials and Methods

An untreated flour milled from Texas and Oklahoma winter wheats and analyzing 0.44% ash and 11.7% protein was used in most tests. In a few tests, where indicated, a spring wheat flour analyzing 0.42% ash and 14.7% protein was employed. Farinograph, extensigraph, and baking tests were conducted as previously described (27). Only the 180-min. reaction time on the extensigraph is reported in most instances. A 45-min. rest period was used for all curves.

Lipoxidase was obtained from the Sigma Chemical Company; it assayed 8,000 units per mg. minimum activity.

Linoleate Peroxides. The peroxides of sodium linoleate were prepared by allowing a 3% solution of sodium linoleate to autoxidize slowly in a 1-liter, cotton-stoppered Erlenmeyer flask in the refrigerator at 5°C. for 6 weeks. This cold temperature was employed to minimize the secondary reactions during the period of oxidation. The peroxide content was assayed by the ferrocyanate procedure (32). At the end of 6 weeks, oxidation of the sodium linoleate had proceeded to a level of 12.6% peroxides.

Thiobarbituric Acid Value. Values for 2-thiobarbituric acid (TBA) were obtained with a method that combined features of the determination outlined by Tarladgis *et al.* (30) and Caldwell and Grogg (2). Fifteen grams of dough was triturated with 35 ml. of glacial acetic

acid for 10 min., using a mortar and pestle. This mixture was allowed to stand 0.5 hr. and then centrifuged. Five milliliters of the centrifugate were combined with 5 ml. of 0.75% TBA and heated in a boiling water bath for 35 min. The solution was chilled in an ice bath and, using gentle suction, filtered through a funnel containing a bed of 2 g. powdered Whatman No. 1 filter paper. The bed was washed twice with 10-ml. portions of water and sucked dry. The red pigment was eluted from the bed with 5- to 10-ml. portions of 75% aqueous pyridine. The amount of red pigment usually was sufficient to be measured in a volume of 10 ml. and the eluate was diluted to this volume with 75% aqueous pyridine and read in a Coleman Junior Spectrophotometer at 520 mμ, using 75% aqueous pyridine as a blank.

Measurement of Reactivity of Linoleic-Lipoxidase System. The maximum amount of lipoxidase was extracted from flour by steeping a slurry of 10 g. of flour in 100 ml. of water at 50°C. for 1.25 hr. The lipoxidase, being stable thermally, was not inactivated by this treatment. After centrifugation, 10 ml. of the centrifugate were combined with 10 ml. of phosphate buffer (pH 6.5). The addition of 36 mg. linoleic acid, dissolved in 0.2 ml. of acetone, formed a lipoxidase-linoleate system that was in excess with respect to the substrate. Incubation of this mixture at 30°C. with shaking developed peroxides at a rate of about 2 micromoles per minute per g. of flour. The reactivity of this system to glutathione (GSH) was studied by adding 26 μmol. of GSH to the incubation mixture. At prescribed time intervals, 2-ml. aliquots were withdrawn and shaken with 2-ml. portions of water-saturated butanol to remove the linoleic acid. One milliliter of the remaining aqueous phase was assayed for GSH by the procedure of Grunert and Phillips (17). Studies were made also with the inclusion of 4 and 20 mg. of thioctic acid in the incubation medium. Thioctic acid was also extracted into the butanol phase.

Results

When added to flour at the high level of 10 mg.% lipoxidase increased by 2 min. the time to maximum on the farinograph curve and produced little or no effect on the extensigraph behavior. The addition of 0.3% of potassium linoleate increased the time to maximum on the farinograph curve from 8 to 15 min., but showed no effect on the extensigram. At a 1% level and in the presence and absence of 10 mg.% lipoxidase, the linoleic acid showed only very slight maturing effect.

The data given in Table I were obtained by using the same absorp-

TABLE I

EFFECT OF LIPOXIDASE AND POTASSIUM LINOLEATE ON FARINOGRAPH CURVE CHARACTERISTICS

	Time to Maximum	Consistency		
		At 10 Minutes	At 20 Minutes	At 30 Minutes
	min.	*B.U.*	*B.U.*	*B.U.*
Control	8	500	480	470
Control + 10 mg.% lipoxidase	10	485	475	470
Control + 0.33% potassium linoleate	15	480	480	475
Control + 0.33% potassium linoleate and 10 mg.% lipoxidase	14	470	470	450

TABLE II

EFFECT OF LIPOXIDASE AND LINOLEIC ACID ON DOUGH CHARACTERISTICS AS MEASURED BY THE EXTENSIGRAPH

Time	No Treatment		10 mg.% Lipoxidase		1% Linoleic Acid		10 mg.% Lipoxidase + 1% Linoleic Acid	
	H[a]	E[a]	H	E	H	E	H	E
min.	*B.U.*	*B.U.*	*B.U.*	*B.U.*	*B.U.*	*B.U.*	*B.U.*	*B.U.*
45	440	240	450	210	515	240	490	240
90	510	210	570	215	590	225	600	230
135	560	215	590	205	630	220	630	230
180	650	190	620	195	680	205	675	225

[a] H = resistance to extension; E = extensibility.

tion. This resulted in small differences in consistency, but this was thought preferable to changing the absorption. Extensigraph data are given in Table II.

A coupled oxidation of glutathione in a wheat lipoxidase-linoleate system could not be demonstrated. Under the ideal condition of excess substrate, the total lipoxidase from 1 g. of flour caused a very small loss of the GSH present in the reaction system, as seen in the table below. There would be much less reactivity to glutathione of the actual lipoxidase-linoleate system existing in dough.

Time	*Glutathione*	*Glutathione Loss*
min.	*μmol.*	*%*
0	26.0	
5	25.2	
10	24.4	
15	25.2	
20	24.4	
25	23.6	9.2

Mapson and Moustafa (21) were able to demonstrate a coupled oxidation of GSH by pea lipoxidase and linoleate in the presence of traces of 2-octanol (8), but were unable to demonstrate this in the absence of 2-octanol. 2-Octanol was not included in our system. Mapson and Moustafa (21) also observed that GSH was not oxidized when incubated in nitrogen with methyl linoleate peroxide.

Among the fatty acids, linoleic occurs in major quantity (60%), with oleic in a lesser amount (13%). Oleic acid is not acted upon by lipoxidase and its rate of oxidation is of the order of one-fortieth of that of linoleic acid (19). Thus, the principal lipid peroxides developed in a flour-dough system must derive from linoleic acid. The investigations of Dahle, Hill, and Holman (12) revealed the inadequacy of the TBA reaction as a means of measuring linoleate oxidation and as a means of quantitatively assessing the oxidation of polyunsaturated mixtures. However, within a given system, TBA values can reveal relative states of oxidation. The data in Fig. 1 show increasing lipid oxidation with increasing time of mixing in air and an increasing accumulation of TBA-reactive material (oxidized lipid) in the absence of free –SH groups, as was demonstrated by Tsen and Hlynka (31). The magnitude of difference in the presence and absence of free –SH groups is small, but does give qualitative evidence of some interaction between sulfhydryl and oxidizing lipid. From this evidence, one cannot interpret that sufficient sulfhydryl is destroyed by oxidized lipid in a normal period of mixing as would be required

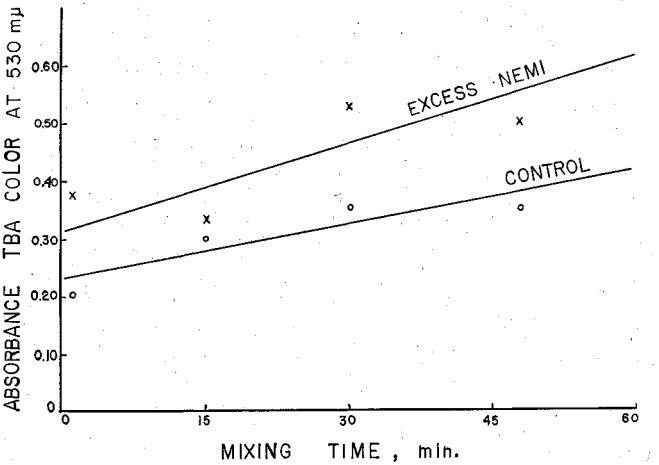

Fig. 1. Effect of mixing on lipid oxidation in the presence and absence of sulfhydryl groups.

for optimum improver action. If mixing time is long or if mixing is accomplished in oxygen, oxidized lipid may have a significant effect (23). One can assume that the increased TBA values in the presence of N-ethylmaleimide are due to the antioxidant effect of the –SH groups on the lipid oxidation.

Optimum improvement, as judged by the extensigraph, was obtained by 1.4 μmol. sodium linoleate peroxide per g. of flour, or 420 μmol. for 300 g. flour. This was much less maturing than shown by

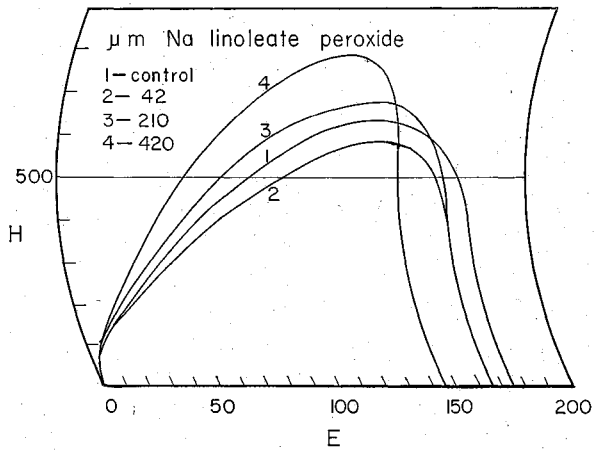

Fig. 2a. Extensigrams at 180 minutes' reaction time with varying levels of sodium linoleate peroxide.

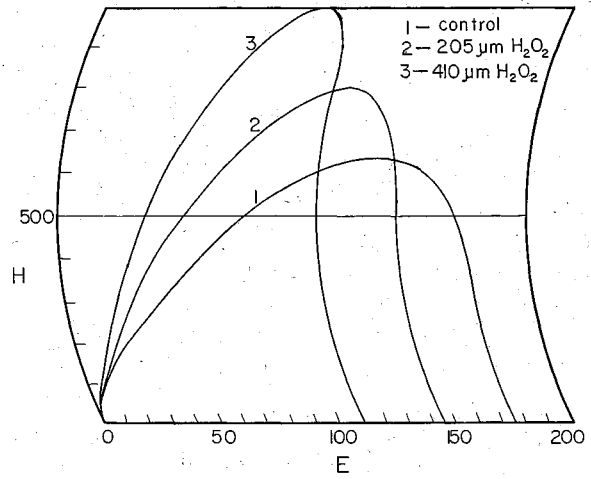

Fig. 2b. Extensigrams at 180 minutes' reaction time with different levels of hydrogen peroxide.

the equivalent amount of hydrogen peroxide, as illustrated in Figs. 2a and 2b. In fact, half the amount of hydrogen peroxide was as effective. This does not take into account any decomposition of hydrogen peroxide by catalase which, if significant, would make even less hydrogen peroxide, on an equivalence basis, as effective.

Baking data on hydrogen peroxide and acetone peroxides are given in Table III. Optimum improvement was shown at 200 μmol. hydrogen peroxide per 300 g. of flour, as also shown for the extensigraph. Acetone peroxides gave excellent improvement at a level of 300 μmol. with the particular flour used, and the dough was drier than the doughs treated with hydrogen peroxide.

TABLE III

EFFECT ON BAKING PROPERTIES OF VARYING LEVELS OF HYDROGEN PEROXIDE AND OF ACETONE PEROXIDES

	Mixing Time	Volume	Grain
	min.	*% of standard*	
Control	6	98	98
	8	95	98
Control + 100 μmol. hydrogen peroxide	6	98	98
	8	100	99
Control + 200 μmol. hydrogen peroxide	6	106	100
	8	102	100
Control + 400 μmol. hydrogen peroxide	6	103	99
	8	97	98
Control + 300 μmol. acetone peroxides	6	103	100
	8	98	101

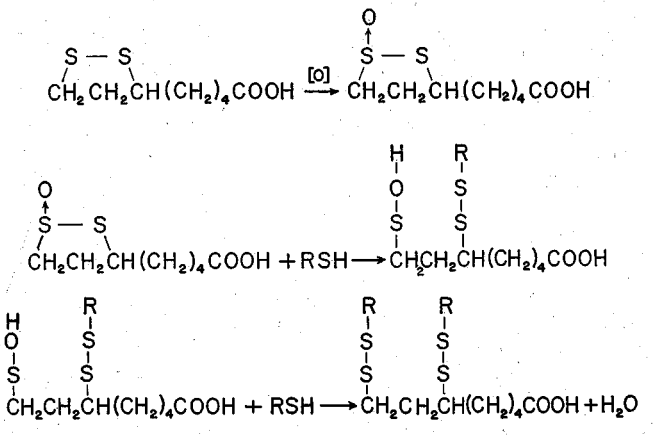

Fig. 3. Some reactions of thioctic acid.

Role of Thioctic Acid. Some of the possible reactions of thioctic acid are shown in Fig. 3.

With our present procedures, less than 6 mg. (30 μmol.) of thioctic acid per kilo of flour have been found, the amount varying with the flour. This corresponds stoichiometrically to about one-tenth of the –SH value of flour. The particular manner in which thioctic acid is combined in flour is not known.

The sequence of reactions in natural aging may be visualized as follows:

Tocopherols —(air, O_2)→ oxidized tocopherols

Unsaturated lipids —(air, O_2; lipoxidase)→ lipid peroxides

Lipid peroxides + tocopherols → oxidized tocopherols

Lipid peroxides + thioctic acid → thioctic monoxide

Thioctic monoxide + 2RSH → $CH(R)S_2CH_2CH(CH_2)_4COOH$

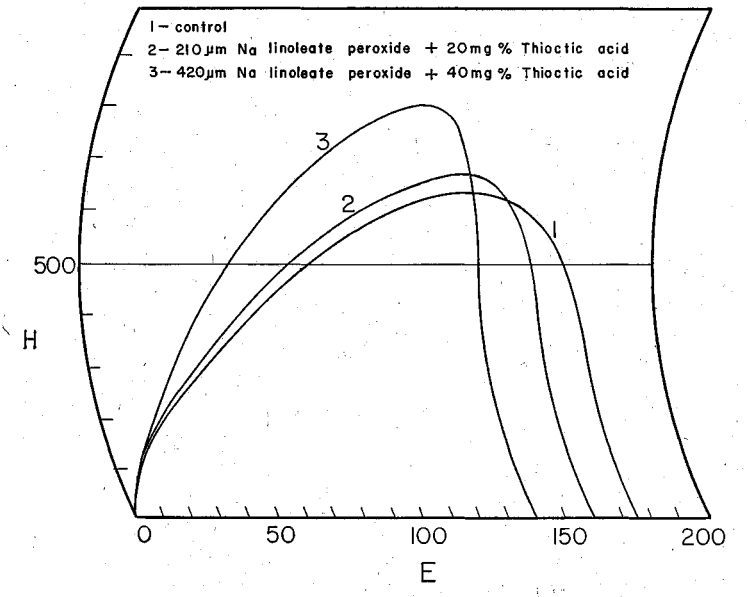

Fig. 4. Extensigrams at 180 minutes' reaction time with varying levels of sodium linoleate peroxide and thioctic acid.

When tocopherols and other antioxidants, including thioctic acid, are oxidized, the lipoxidase-catalyzed oxidation of unsaturated lipids occurs (29). Thioctic acid monoxide formed in this process can oxidize the –SH groups of flour.

Thioctic acid is a patented, edible antioxidant (24) and reacts with hydrogen peroxide and lipid peroxides to form the monoxide (3,11). The improver action, if any, offered by thioctic acid in a dough system must come about through its oxidation by lipid peroxides to the monoxide which, in turn, reacts with –SH groups, as indicated in the above reactions.

High levels of thioctic acid were added to flour prior to the addition of peroxidic sodium linoleate. Figure 4 shows the difference of improver action offered by the thioctic acid when compared to Fig. 2a.

Extensigrams with thioctic acid showed slightly increased maturing at earlier stretches, but no significant change at 180 min., indicating that the reactivity of thioctic acid to sulfhydryl in this system is comparable to that of the original lipid peroxides.

Thioctic acid manifests an antioxidant effect in a lipoxidase-linoleate system and is compared with the lesser antioxidant effect of glutathione, as shown in the table below.

Antioxidant	*Concentration*	*Decrease of Peroxide (Inhibition)*
	in $10^{-3}M$	*%*
Thioctic acid	1.17	67.7
	2.27	99.4
	3.31	100.0
Glutathione	0.93	. . .
	1.86	7.0
	2.79	12.8

TABLE IV

REACTIVITY OF LIPOXIDASE-LINOLEATE-THIOCTIC ACID SYSTEM TO GLUTATHIONE

THIOCTIC ACID	TIME	GLUTATHIONE	GLUTATHIONE LOSS
μmol.	*min.*	*μmol.*	*%*
20.0	0	26.0	
20.0	5	25.6	
20.0	10	25.2	
20.0	15	24.8	
20.0	20	24.3	
20.0	25	24.8	4.6
100.0	0	26.0	
100.0	5	25.5	
100.0	10	25.0	
100.0	15	23.4	
100.0	20	23.4	
100.0	25	22.8	12.0

The inclusion of thioctic acid in a lipoxidase-linoleate system does not appear to greatly change its reactivity to glutathione, as illustrated by the data in Table IV.

The small amount of thioctic acid found, in addition to the other evidence given above, indicates that the improver role of thioctic monoxide is probably not a major factor in mixing. In natural aging of flour where longer reaction times are involved, its improver role may be significant.

Discussion

The oxygen uptake of dough during mixing has been shown to be due to the oxidation of unsaturated lipids, particularly those containing linoleic acid. It is also known that the –SH groups oxidize during mixing in air, especially in the presence of traces of copper and other metals. The evidence obtained thus far does not indicate that, under normal mixing procedures, the fatty acid peroxides oxidize the –SH groups to any significant degree. The amount of hydroperoxides actually formed during mixing at present is not readily measured, but it would appear to be small and the reactivity between the peroxides formed and the hydrophilic –SH groups is poor in an aqueous medium. It has been found, however, that the reactivity between these compounds is enhanced in solvent mixtures more compatible to both, such as 50% ethanol (11). Moreover, even glutathione is only very slowly oxidized in a lipoxidase-linoleate system at the pH of dough. On the other hand, water-soluble peroxides, such as hydrogen peroxide, t-butyl peroxide, or acetone peroxide, are excellent improvers. Fat-soluble peroxides such as benzoyl peroxide, while good bleaching agents, are poor improvers.

The relative contribution of the lipoxidase-linoleate system to improver action in a dough is further reflected in the data of Smith, Van Buren, and Andrews (26). The oxygen uptake mainly attributable to this system was compared on a patent and second clear flour. These flours showed a comparable improvement on a 10-min. mixing time, during which the second clear absorbed over five times the quantity of oxygen absorbed by the patent. This amount of oxygen corresponded to 8.6 μmol. per g. of second clear flour. These authors found no relationship between either the change in sulfhydryl or the final sulfhydryl value and changes occurring in dough properties as measured by the extensigraph.

As has already been mentioned, Mapson and Moustafa (21) observed that 2-octanol couples the oxidation of GSH in a lipoxidase-linoleate

system. If a substance having similar properties is present in dough, perhaps more sulfhydryl would be oxidized by this system.

There are undoubtedly other minor oxidation systems functioning in the natural aging and chemical maturing of flour and in the mixing of dough. Present work has verified our original supposition that the main reactions responsible for flour maturing involve the –SH and S–S groups of the proteins. Removal of most of the water-soluble –SH groups seems to be the dominant means of effecting improvement. There is some oxidation of these –SH groups by air and by oxidized lipid, but the latter effect is relatively slight compared to the effect of chemical improvers, except with long aging of the flour or the use of long mixing periods. The concentration of linoleate peroxides required to effect improvement is undoubtedly manyfold greater than the amount produced during normal mixing and is at least double the concentration of water-soluble peroxides required to obtain optimum rheological properties of dough.

Although much has been learned in recent years about the various mechanisms responsible for flour improvement, further work is needed to evaluate the relative importance of lipid and other oxidizing systems and their effect on the –SH–S–S interchange and subsequent dough properties.

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