THE ROLE OF LIPIDS IN OXIDATION OF DOUGHS[1]

C. C. Tsen and I. Hlynka

ABSTRACT

Results obtained with the thiobarbituric acid method on lipids extracted from flour provide direct evidence that lipid peroxides are formed in dough during mixing in air or oxygen. Doughs mixed in air or oxygen gave a higher absorbance in the TBA test than did control doughs mixed in nitrogen. Addition of lipoxidase in the form of "Wytase" also increased the rate of peroxidation of flour lipids. On the other hand, addition of NDGA or propyl gallate as antioxidants to flour inhibited the peroxidation essentially to the level of doughs mixed in nitrogen.

Succinic acid peroxide, t-butyl hydroperoxide, hydrogen peroxide, acetyl peroxide, and methyl ethyl ketone peroxide all increased the structural relaxation constant of dough, indicating a similar improving role for flour lipid peroxides.

When sulfhydryl-blocking reagents (PCMB, IAA, $HgCl_2$, and NEMI) and improving agents (iodate and bromate) were incorporated into dough, lipid peroxidation was increased. This suggests that flour lipids compete with sulfhydryl groups for available oxygen in dough.

On the basis of these findings a unified hypothesis for the role of lipids in oxidation of doughs is presented and discussed.

Recent reviews by Cookson and Coppock (5), and by Glass (9) illustrate increasing interest in the chemistry of flour lipids. However, despite the considerable progress that has been made, no general hypothesis has been formulated for the role of lipids in dough chemistry.

A brief review of the pertinent literature follows: Earlier work of Sullivan *et al.* (24,25) showed that unsaturated fatty acids when exposed to oxygen had a marked damaging action on the baking

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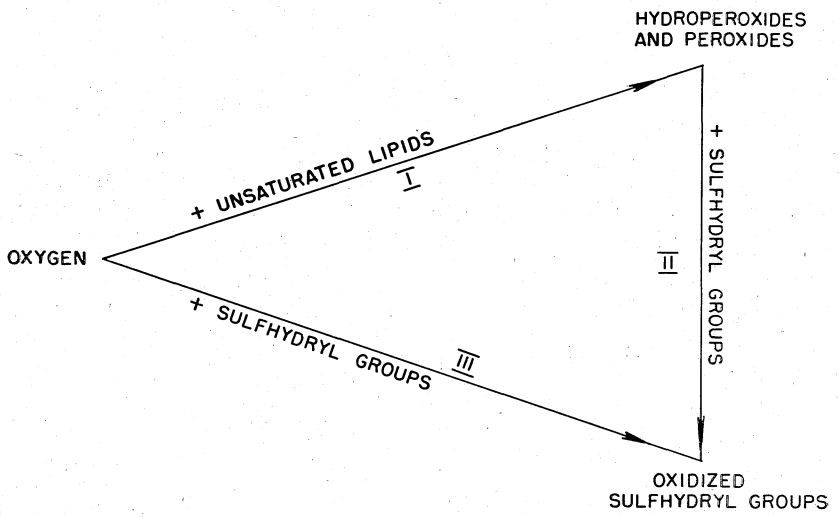
quality of flour; the dough felt dead and broke easily. Hawthorn and Todd (11) found that the bleaching effect in the batter process was due to the action of unsaturated-fat oxidases and, in particular, to that of lipoxidase, but attributed the simultaneous improver effect to direct uptake of oxygen by the flour protein. However, Koch (16) showed that the action of lipoxidase produced physical changes in a dough in addition to its bleaching effect. Using a manometric technique, Cosgrove (6) was able to show that flour suspensions absorbed oxygen. Similarly, Smith and Andrews (22,23) found that the mixing of flour-water doughs was accompanied by an uptake of oxygen. They suggested that utilization of oxygen in dough involved a system comprising lipoxidase and polyunsaturated fatty acid.

The present paper describes experimental data which provide a basis for a unified hypothesis of the role of lipids in dough chemistry. The relationship among the various aspects of lipid chemistry and sulfhydryl groups in dough may be represented in the form of a triangle.



This paper presents the following evidence in support of step I: (a) lipid peroxidation takes place readily during the mixing of flour-water dough under oxygen; (b) the oxygen concentration determines the extent of the peroxidation; (c) "free" lipid is responsible for the peroxidation; and (d) anti-oxidants inhibit the peroxidation. Step II is supported by examining the effect of hydroperoxides and peroxides on rheological properties of dough. Since it is known that sulfhydryl groups are involved in determining rheological properties of dough

(2,8,18), the effect of hydroperoxides and peroxides on the rheological property presumably involves their action on sulfhydryl groups. The relationship between steps I and III is examined by evaluating the effect on lipid peroxidation of blocking the sulfhydryl groups. On the basis of these observations, a mechanism involving competition between flour lipids and sulfhydryl groups for the available oxygen in dough has been formulated.

Materials and Methods

Reagents. All chemicals used were reagent grade. Tertiary butyl hydroperoxide, acetyl peroxide, methyl ethyl ketone peroxide, succinic acid peroxide were kindly supplied by A. I. Lowell of the Lucidol Division, Wallace & Tiernan, Inc., Buffalo, New York. Linoleic acid was purchased from the Nutritional Biochemicals Company, and 2-thiobarbituric acid from the Distillation Products Industries, Division of Eastman Kodak Company. Ethanol (absolute) was purified by distillation in an all-glass apparatus over potassium permanganate and potassium hydroxide (1 and 2 g., respectively, per liter of ethanol). Diethyl ether was made peroxide-free according to the method of Werner (29). The oxidized linoleic acid was prepared by ultraviolet irradiation of oxygenated linoleic acid.

Flour. The flours used in this study were unbleached, improver-free samples commercially milled from a blend of Canadian hard red spring wheat. The designation and characteristics of these flours are as follows:

Flour	*Moisture*	*Protein Content*	*Ash Content*	*Petroleum Ether Extract*
	%	%	%	%
Straight grade	14.8	13.2	0.43	0.89
Bakers' strong	14.4	15.3	0.60	1.27

Moisture, protein, and ash contents of the flours were determined by the methods of the AACC. Petroleum ether extract was determined by overnight extraction in Goldfisch extractors with petroleum ether (Skellysolve F 95).

Dough. Doughs were mixed in the GRL mixer (12) under nitrogen, air, or oxygen. Flours to be mixed in nitrogen or oxygen were pretreated by purging with nitrogen or oxygen under alternate vacuum and pressure. The solutions were used "as is" for mixing the dough in air, but were saturated with either nitrogen or oxygen when mixing was done in these gases.

Extraction. Lipid was extracted from 25 g. of dough by a rapid method (27) in which total 50 ml. of chloroform was used as the extracting solvent. In order to minimize the oxidation during extraction, the blending was done under nitrogen. After centrifugation and separation, 10 ml. chloroform extract were taken. The solvent was evaporated off in a water bath at 47.5°C., and under a stream of nitrogen. The lipid in 25 g. of dough prepared from each of the two flours was: Straight grade, 0.21 ± 0.01 g. (5 determinations), and Bakers' strong, 0.28 ± 0.02 g. (10 determinations).

Determination of Lipid Peroxidation. A thiobarbituric acid method (1,21) was used to measure lipid peroxidation in dough. Two milliliters of each of 5% trichloroacetic acid and of 0.67% thiobarbituric acid were added to the lipid extract after evaporation. The mixture was placed in a boiling-water bath for 10 minutes, and cooled; water was added to compensate for the loss by evaporation. The contents were then transferred to a separating funnel. Two ml. of ethanol and 4 ml. of diethyl ether were introduced into the funnel. After vigorous shaking of the mixture, the phases were allowed to separate. The bottom phase, containing the colored thiobarbituric acid complex, was separated and its absorbance was measured in a 1-cm. cell with a Beckman Model DU spectrophotometer at 530 mμ against a blank. The blank was prepared in the same manner, except that thiobarbituric acid solution was not added. Since only one-fifth of the chloroform extract was used in each determination, the relative level of lipid peroxides was simply expressed in absorbance units on the basis of the lipids extracted from 5 g. of dough. The TBA method primarily measures malonaldehyde formed in lipid peroxidation. The TBA method has been found comparable with Lundberg and Chipault's method for peroxides, the Kreis test for aldehydes, and with the degree of conjugation when oxidized fatty acid esters were used (15). Because of its sensitivity and simplicity, the TBA method has been widely used to determine the level of lipid peroxidation in tissues (1,26), flour, and cereal products (4,21).

However, the TBA method measures the relative level of lipid peroxidation only. It was therefore standardized against the indophenol method of Glavind and Hartmann (10) which was claimed to be more sensitive than the iodometric method. The standardization was done by measuring the absorbance by these two methods with the same amount of oxidized linoleic acid. By means of the extinction coefficient of Glavind and Hartmann (10), the microequivalents of peroxides in the oxidized linoleic acid were calculated. In turn, the absorbance units obtained from the TBA method

could then be converted into microequivalents of peroxides, as shown in Fig. 1.

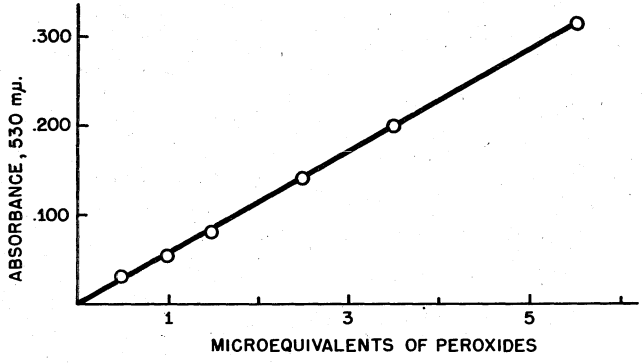


Fig. 1. Calibration of the thiobarbituric acid method.

In addition to the TBA method, the iodometric method of Lundberg and Chipault (17) was used to supplement the determination of the extent of lipid peroxidation during the mixing.

Results and Discussion

Lipid Peroxidation during the Mixing of Doughs. Figure 2 shows that the lipid peroxidation as indicated by absorbance takes place readily during the mixing of flour-water doughs under oxygen. But under nitrogen, the level of lipid peroxides remains the same throughout the mixing period; the apparent low peroxide level of the control is probably due to the small amount of lipid peroxides originally present in the flours or to some oxidation that occurred during the extraction and analysis.

Further results were obtained by the iodometric method to confirm the validity of the TBA method for the study of peroxidation. The peroxide values were found to be 1.8, 13.0, 16.8, and 20.9 μeq. of peroxides in the lipids extracted from 25 g. of dough (Bakers' strong flour) mixed under oxygen for 2.5, 5, 10, and 20 minutes, respectively. While under nitrogen, the method could not detect any peroxides in the lipids from doughs mixed for the same periods as were done under oxygen.

The results agree well with the observation of Smith and Andrews (22) that lipids are involved in oxygen uptake during the mixing of flour-water doughs. The present study, however, offers direct evidence that flour lipids undergo the peroxidation during the mixing.

Further evidence of peroxidation was obtained by preparing the

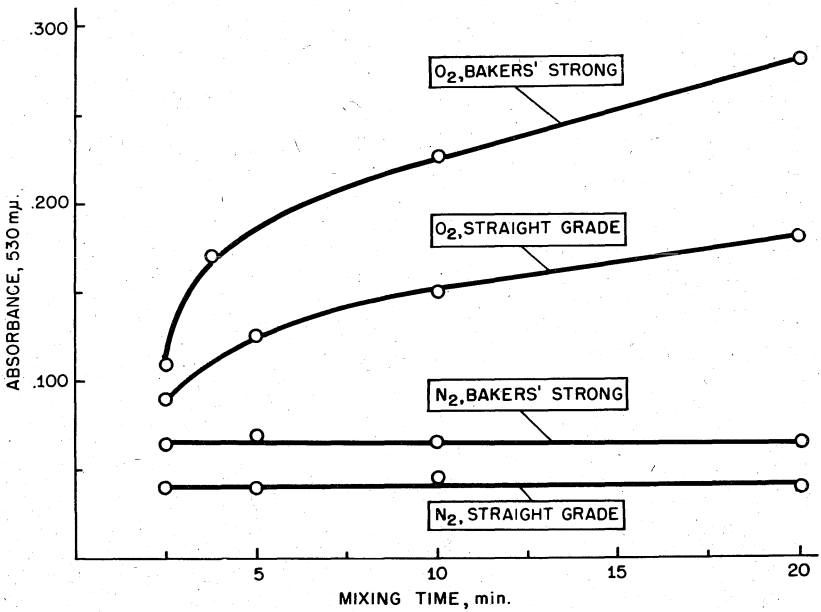


Fig. 2. Lipid peroxidation during the mixing of flour-water dough. Flour, 100 g., was mixed with 34 ml. of water and 25 ml. of 4% sodium chloride.

dough under various concentrations of oxygen. The results (Table I) show that the peroxidation increases as the concentration of oxygen is raised. It seems that the available oxygen in dough determines the extent of the peroxidation.

TABLE I

EFFECT OF OXYGEN CONCENTRATION ON LIPID PEROXIDATION[a]

ATMOSPHERE	ABSORBANCE (530 mμ)
Nitrogen	0.064
Air	0.097
Oxygen, 40%	0.129
Oxygen, 100%	0.169

[a] 100 g. of flour (Bakers' strong) were mixed with 34 ml. of water and 25 ml. of 4% NaCl for 5 minutes.

To ascertain whether "free" or "bound" lipid is responsible for the peroxidation, the following study was made. Defatted flours were prepared by extracting Bakers' strong flour with petroleum ether (Skellysolve F 95) in a large Soxhlet extractor for 16 hours. The flour was then removed and spread out in shallow dishes under a stream of nitrogen to facilitate the evaporation of the solvent. The flour, after removal of "free" lipids by extraction (19), was used for mixing under nitrogen or oxygen. The results, given in Table II, clearly

demonstrate that the peroxides are formed mainly through the peroxidation of "free" lipid during the mixing process.

TABLE II
PEROXIDATION OF "BOUND" LIPID IN DOUGH[a]

MIXING TIME	ABSORBANCE (530 mμ)	
	Nitrogen	Oxygen
minutes		
2.5	0.057	0.061
10.0	0.071	0.073

[a] Experimental conditions the same as in Table I.

Effect of Lipoxidase on the Peroxidation. The oxidation appeared to be catalyzed by lipoxidase in flour. When Wytase (a commercial lipoxidase preparation) was introduced before the mixing, the amount of peroxides formed in the dough was higher than that of the control sample (Table III). The presence of lipoxidase in various North American wheats was reported by Irvine and Anderson (14) and in different flour fractions by Miller and Kummerow (20). Recently Smith and Andrews (22) found that the mixing of flour-water dough was accompanied by an uptake of oxygen. The rate of oxygen absorption depended on the water-soluble component, lipids, and gluten of flour. Removal of any one component greatly reduced the rate. The amount of oxygen uptake could be increased by adding linoleic or linolenic acid, or Wytase. Their results, obtained by manometric technique, demonstrate clearly that lipoxidase is involved in this peroxidation. By measuring the level of lipid peroxidation in dough, the same conclusion is obtained from the present study: flour lipoxidase catalyzes the oxidation of unsaturated lipids during the mixing process.

TABLE III
LIPID PEROXIDATION CATALYZED BY LIPOXIDASE[a]

LIPOXIDASE (WYTASE)	ABSORBANCE (530 mμ)
% flour	
0	0.120
1	0.204
5	0.334

[a] Flour, 100 g. (Bakers' strong), was mixed with Wytase, 34 ml. of water, and 25 ml. of 4% NaCl under oxygen for 2.5 minutes.

Effect of Antioxidant on the Peroxidation. Additional information on lipid peroxidation was obtained by using antioxidants to inhibit peroxide formation. The results in Table IV show that the peroxida-

tion is kept to the control level by use of nordihydroguaiaretic acid or propyl gallate. This provides further evidence that the peroxidation takes place during the mixing of dough.

TABLE IV

INHIBITION OF THE PEROXIDATION BY ANTIOXIDANTS[a]

ANTIOXIDANT	ABSORBANCE (530 mμ)
Control (nitrogen)	0.061
NDGA (nordihydroguaiaretic acid) ($1 \times 10^{-2}M$)	0.061
PG (n-propyl gallate) ($1 \times 10^{-2}M$)	0.062

[a] Bakers' strong flour (200 g.) was mixed under nitrogen with 18 ml. of water, 50 ml. of antioxidant solution, and 50 ml. of 4% NaCl for 5 minutes, and rested for 10 minutes. The atmosphere of the mixer was then changed from nitrogen to oxygen. The dough was remixed under oxygen for 5 minutes.

Effect of Hydroperoxides and Peroxides on Rheological Properties of Dough. The products of lipid peroxidation are, of course, hydroperoxides, peroxides, and others. An attempt was therefore made to study the effect of representative, readily available hydroperoxides and peroxides on rheological properties of dough. The compounds tested include hydrogen peroxide, t-butyl hydroperoxide, succinic acid peroxide, acetyl peroxide, and methyl ethyl ketone peroxide. A quantity of 201.8 g. flour (straight grade) was mixed with 70.4 ml. of $2.84 \times 10^{-3}M$ hydroperoxide or peroxide (except that $7.10 \times 10^{-4}M$ was used for methyl ethyl ketone peroxide) and 50 ml. of 4% sodium chloride under nitrogen for 2.5 minutes. Because of insolubilities of acetyl peroxide and methyl ethyl ketone peroxide in water, these two reagents were first dissolved in 2 ml. of ethanol and then dispersed in 68.4 ml. of water. The results are shown in Table V. On the basis of the relaxation constants (13), it can be concluded that all the hydroperoxides and peroxides tested are good dough-strengthening agents. It was also found that the addition of oxidized linoleic acid increased the constant as compared with that of linoleic acid. Since sulfhydryl groups are known to be involved in determining rheological properties of dough (2,8,18), it indicates that these hydroperoxides

TABLE V

EFFECT OF HYDROPEROXIDES AND PEROXIDES ON THE RELAXATION CONSTANT OF DOUGH

REAGENT	RELAXATION CONSTANT
Control	3290
t-Butyl hydroperoxide	4000
Succinic acid peroxide	4650
Hydrogen peroxide	5150
Control with 2 ml. ethanol	3320
Acetyl peroxide	7000
Methyl ethyl ketone peroxide	7170

and peroxides strengthen dough by oxidizing sulfhydryl groups.

The Competitive Mechanism of Lipid Peroxidation and Sulfhydryl Oxidation. The data in Table VI strongly suggest that flour lipids compete with sulfhydryl groups for the available oxygen in dough. *p*-Chloromercuric benzoate (PCMB), iodoacetic acid (IAA), N-ethylmaleimide (NEMI) and mercuric chloride are known sulfhydryl reagents. The improvers, potassium bromate and iodate, have been found to react with sulfhydryl groups of flour and gluten. Once sulfhydryl groups are blocked or reacted by these reagents, the lipid peroxidation increases. This oxygen-competitive mechanism probably plays an important role in determining dough properties.

TABLE VI

EFFECT OF VARIOUS REAGENTS ON LIPID PEROXIDATION[a]

TYPE OF REAGENTS	REAGENT (CONC.)		ABSORBANCE (530 mμ)
Control	.....		0.156
Sulfhydryl reagents	PCMB	($2 \times 10^{-3}M$)	0.270
	IAA	($4 \times 10^{-3}M$)	0.221
	$HgCl_2$	($2 \times 10^{-3}M$)	0.228
	NEMI	($4 \times 10^{-3}M$)	0.218
	NEMI	($1.6 \times 10^{-2}M$)	0.235
Improvers	Iodate	($3 \times 10^{-4}M$)	0.177
	Iodate	($6 \times 10^{-4}M$)	0.184
	Iodate	($1.2 \times 10^{-3}M$)	0.193
	Bromate	($6 \times 10^{-4}M$)	0.165
	Bromate	($2.4 \times 10^{-3}M$)	0.179

[a] Experimental conditions the same as in Table IV.

General Discussion

The study of the oxidative change of lipids is usually hampered by the long extracting process and complicated analysis. As unsaturated lipids are readily oxidizable, the change will likely take place during the process of extraction and analysis. The present study employs the rapid method to extract the lipids and the thiobarbituric acid methed to measure the level of lipid peroxidation. By the rapid method, the extraction and evaporation are done under nitrogen and the centrifugation is carried out at 0°C. Thus, the oxidative change of lipids is minimized. The TBA method provides a sensitive procedure for detecting lipid peroxidation. With these two methods, together with the iodometric method, it is thus possible to demonstrate that the lipid peroxides are formed during the mixing of flour-water doughs.

The peroxidation is an enzyme-catalyzed reaction. The presence of molecular oxygen is necessary for the action of lipoxidase. Antioxi-

dants are effective inhibitors of lipoxidase. Wytase (a commercial lipoxidase preparation) can increase the level of the peroxidation. All these facts have been demonstrated in the present study, and point to the involvement of the lipoxidase-polyunsaturated fatty acid system. This conclusion is in accord with that of Smith and Andrews obtained through a different approach.

Sulfhydryl groups are oxidized readily by molecular oxygen in the presence of a trace of a metallic catalyst (28). During the mixing of doughs, flour lipids and sulfhydryl groups, both oxygen-labile, can be visualized as competing with each other for the available oxygen. This competition is demonstrable; for, when sulfhydryl groups are blocked or reacted, the level of the lipid peroxidation increases significantly. The relationship has been presented in the form of a triangle and is useful in explaining the changes among lipids, sulfhydryl groups, oxygen, and other improving agents during the mixing of dough. For example, Cunningham and Hlynka (7) have found that removal of flour lipids increases the inhibitory action of oxygen on the bromate uptake. Bushuk and Hlynka (3) have also demonstrated that cumene hydroperoxide inhibits the bromate reaction. Cookson and Coppock (5) have observed that defatted flour gives a dough of greater resistance and lower extensibility, similar to the effect of the oxidizing agents. Sullivan (24) has shown that oxidizing agents act more effectively on defatted flour than normal flour. The reaction mechanism responsible for these observations can be readily understood according to the scheme outlined in the present paper.

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