Isomeric Ascorbic Acids and Derivatives of L-Ascorbic Acid: Their Effect on the Flow of Dough[1]

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ABSTRACT

Using the spread ratio test, L-ascorbic acid (L-AA) retarded the flow of a nonyeasted dough immediately after mixing and was even more effective after a lay period of 60 min at 30°C. On the other hand, D-ascorbic, L-isoascorbic, and D-isoascorbic acids increased dough flow out of the mixer and gave little or no retardation of flow with increased lay time. Dehydro-L-ascorbic acid and reductic acid gave the same effect as L-AA on the spread of dough. Adding the dehydro form of the other three acids at the dough mixer immediately reduced dough flow but produced no further time-dependent retardation. The 2- and 3-methyl ethers of L-AA did not affect the flow of dough; the 6-bromo- and 5,6-acetonated derivatives gave some retardation. Dehydro-D-isoascorbic acid completely stopped the increase in dough flow caused by addition of cysteine. These results indicate that when L-AA is added to a dough, the immediate reduction in dough flow is due to oxidation of thiol compounds in the water-soluble fraction of wheat flour by dehydro-L-ascorbic acid. Dehydro-L-ascorbic acid is formed by air oxidation of L-AA during dough mixing. The mechanism of the time-dependent retardation of dough flow remains unknown.

Jorgensen (1935) was the first to report that adding L-ascorbic acid (L-AA, Fig. 1) to wheat doughs improves the handling characteristics of the dough. When baked, the dough produces a loaf having good volume and texture. Melville and Shattock (1938) later showed that dehydro-L-ascorbic acid (DE-L-AA, Fig. 2) is the active form of the improver in dough and that DE-L-AA is produced when L-AA is mixed with a wheat flour paste. Furthermore, diketogulonic acid, which forms when the lactone ring of DE-L-AA is hydrolyzed, gives no improving action. Melville and Shattock (1938) proposed that the conversion of L-AA to DE-L-AA was mediated by the enzyme ascorbic acid oxidase. Other investigators (Meredith 1965, Sandstedt and Hites 1945) subsequently demonstrated that oxygen must be present in dough if L-AA is to exert its improving action.

Of the four stereoisomeric, six-carbon ascorbic acids (Fig. 1), only the L-ascorbate isomer improves bread (Feaster and Cathcart 1941, Maltha 1953). Surprisingly, reductic acid is as potent an additive as L-AA in breadmaking, but oxytetronic acid (Fig. 3) has only a modest effect (Maltha 1953).

The selective efficiency of the L-ascorbate isomer as an oxidant in bread dough was first thought to result from the presence in flour of ascorbic acid oxidase, which was thought to be specific for L-AA.

Sandstedt and Hites (1945) originally reported that L-AA disappeared from a boiled extract of flour at half the rate of a nonboiled control. Such a slight difference in rate, however, can be explained either by denaturation of an enzyme or by partial depletion of the oxygen needed in the reaction. Maltha (1953) found that all four of the ascorbic acids (Fig. 1), as well as reductic and oxytetronic acids, are oxidized to approximately the same extent (14–21%) when stirred with a flour extract, pH 5.8, for 0.5 hr. Again, that experiment did not provide unequivocal evidence of the absence of the enzyme, because ascorbic acid oxidase exhibits broad substrate specificity (Johnson and Silva 1937, Malmstrom et al 1975). On the other hand, Grant and Sood (1980) recently reported a 30-fold purification of a factor from wheat flour that catalyzes the oxidation of L-AA.

A generally accepted notion is that the oxidation of ascorbic acid to its dehydro form is not the step that determines whether a given ascorbic acid improves bread. Honald and Stahmann (1968) failed to find ascorbic acid oxidase in a flour extract. Maltha (1953) suggested that peroxidase and polyphenol oxidase may be responsible for enzyme-catalyzed oxidation of ascorbic acid in doughs. Peroxidase has been found in wheat flour, whereas polyphenol oxidase and cytochrome oxidase, other enzymes that oxidize ascorbic acid, are present in small amounts (Honald and Stahmann 1968, Shigeoka et al 1980).

Because ascorbic acid is oxidized more rapidly during mixing in a dough than in a flour-water slurry, the oxidation of ascorbic acid in dough is probably due to oxygen and metallic catalysts such as copper and iron ions (Khan and Martell 1967). Furthermore, work in at least three laboratories has shown that oxidation of ascorbic

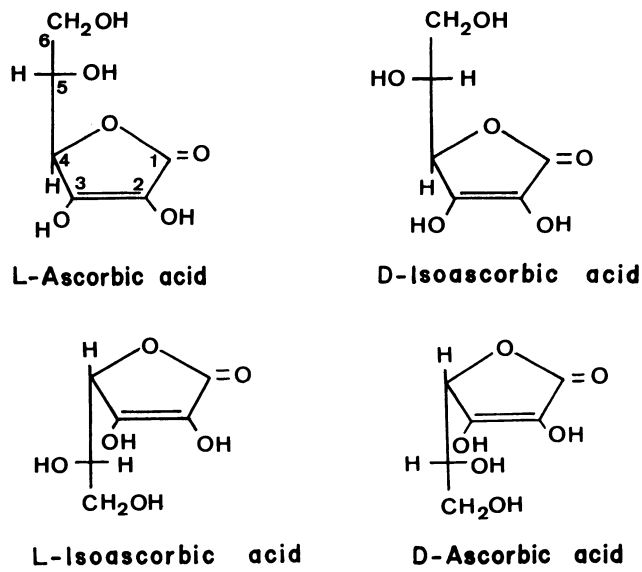

Fig. 1. Stereoisomeric six-carbon ascorbic acids.

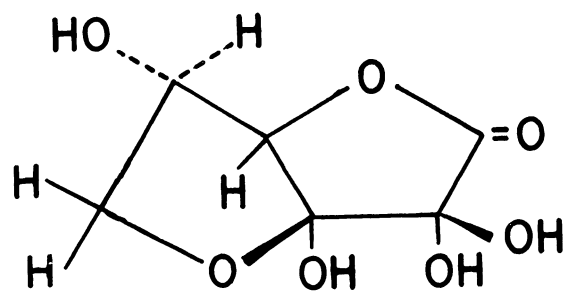

Fig. 2. Dehydro-L-ascorbic acid (Hvoslef and Pederson 1979).

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acid is not the rate-limiting step in the compound's bread-improving action. After 5 min of mixing of flour-water doughs containing 100 ppm of L-AA based on flour (14% mb), 54–78% of L-AA was oxidized (Carter and Pace 1965, Kuninori and Matsumoto 1963). Those results were recently confirmed in yeast-leavened doughs mixed to optimum consistency. ElKassabany et al (1980) found that 60–80% of L-AA and D-isoascorbic acid (D-IAA) at 50 ppm was converted to oxidized forms during dough mixing. Because doughs are fully oxidized by ~15 ppm of added dehydro-L-ascorbic acid (ElKassabany and Hoseney 1980), only ~15% oxidation of L-AA (100 ppm) is needed during mixing to fully oxidize a dough.

The stereospecific action of L-AA in improving bread has been explained most recently by the removal of sulfhydryl (SH) groups in a dough because of the action of glutathione dehydrogenase (GSH-DH) (Carter and Pace 1965, Kuninori and Matsumoto 1964, Mair and Grosch 1979, Sandstedt and Hites 1945, Tsen 1965). The enzyme uses DE-L-AA as a cofactor to accept the electrons removed from glutathione in the following reaction:

$$2\ GSH + DE\text{-}L\text{-}AA \xrightarrow{\text{dehydrogenase}} GSSG + L\text{-}AA.$$

GSH-DH occurs widely in plants and in wheat flour from which it has been purified 500-fold to an essentially homogeneous protein (Boeck and Grosch 1976). Wheat GSH-DH was completely active on GSH but was incapable of oxidizing L-cysteine, $NADH_2$, and

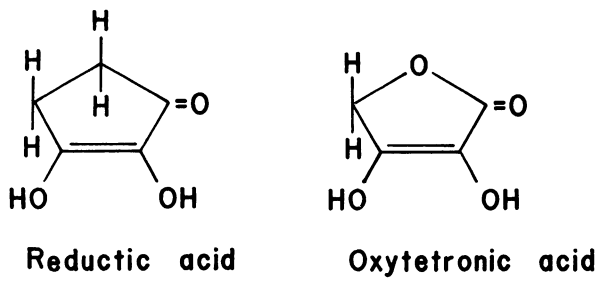

Fig. 3. Reductic acid (**left**) and oxytetronic acid (**right**).

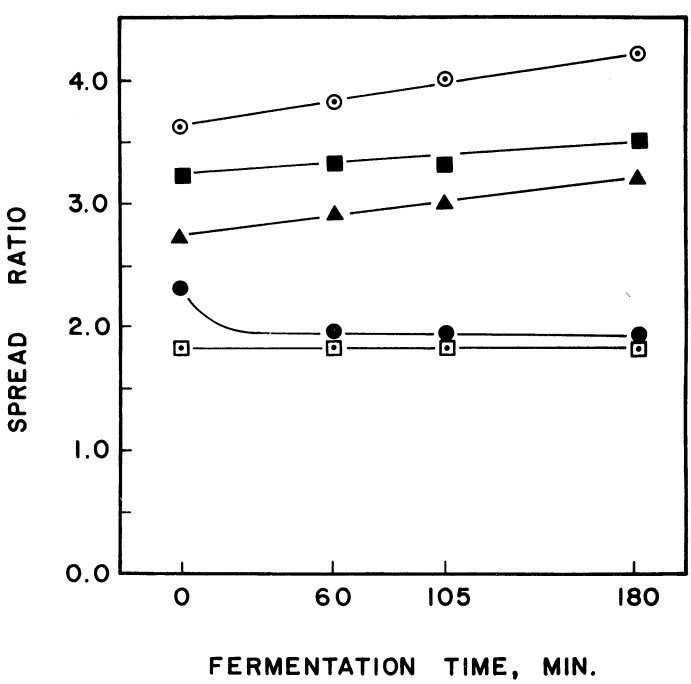

Fig. 4. Spread ratio of several additives on flour-water doughs. ▲ = control, ● = L-ascorbic acid (100 ppm based on flour), ■ = D-isoascorbic acid (100 ppm based on flour), ⊙ = L-cysteine (30 ppm based on flour), and ⊡ = iodate (30 ppm based on flour).

thioglycolic acid (Kuninori and Matsumoto 1964). In a model system at pH 7 and at the physiological concentrations of GSH and DE-L-AA normally used in dough (Grosch 1975), the electrons were transferred by GSH-DH from GSH to DE-L-AA five to 10 times faster than to dehydro-L- or D-isoascorbic acids. However, the data on dough suggesting preferential oxidation by DE-L-AA of glutathione (Mair and Grosch 1979) or of all combined sulfhydryl compounds (Tsen 1965) are not convincing. For example, when 20 ppm of dehydro-L-ascorbic acid or dehydro-D-isoascorbic acid was added to two flours (Mair and Grosch 1979), the two compounds in both flours gave practically the same percentage of oxidation of GSH to its disulfide during 1 min of mixing. In addition, when no dehydroascorbic was added, glutathione still disappeared and could not be detected in a dough after 5 min of mixing.

ElKassabany and Hoseney (1980) fractionated wheat flour into starch, gluten, and water-soluble fractions. Using reconstitution techniques and a spread ratio test on dough, they concluded that the water-soluble fraction contained a deleterious substance responsible for the flow of underoxidized dough. They postulated that oxidants such as dehydro-L-ascorbic acid deactivate an enzyme in the water-solubles that causes dough to flow. L-Ascorbic acid has been shown to both inhibit enzymes (Melville and Shattock 1938) and to stimulate them (Ettlinger et al 1961, Levene et al 1974). Thus, DE-L-AA may activate an enzyme in the water-solubles that somehow counteracts deformation of dough.

Meredith (1965) was the first to divide the dough-improving action of L-AA on dough flow into two time periods, immediate and time-dependent. The main objective of our investigation was to use the dough-spread test to examine the two phases of the dough-improving action of the four isomeric six-carbon ascorbic acids, their dehydro forms, several derivatives of L-AA, and reductic acid.

MATERIALS AND METHODS

General

Solutions were evaporated under diminished pressure and below 40°C. Melting points, determined on a Fisher-Johns melting-point (MP) apparatus, were not corrected. Thin-layer chromatography (TLC) was performed on plates coated with silica gel G (Brinkman Instruments, Inc., Westbury, NY). After the plates were developed, components were located by spraying with 50% aqueous sulfuric acid and charring on a hot plate. A Beckman DB-G spectrophotometer was used to record ultraviolet (UV) spectra. The nitrogen used to purge solvents and flour was a prepurified grade (Matheson Co., Inc., East Rutherford, NJ).

Ascorbic Acids

L-Ascorbic acid was purchased from Fisher Scientific Co., Pittsburgh, PA, and D-isoascorbic acid from Aldrich Chemical Co., Inc., Milwaukee, WI. L-Isoascorbic acid and D-ascorbic acids were synthesized by recemization of L-AA and D-IAA, respectively (Brenner et al 1964). Reductic acid (mp 208–9°C) was a gift of M. S. Feather of the University of Missouri, Columbia.

3-*O*-Methyl-L-ascorbic acid (mp 121–2°C) was prepared by methylation of L-AA with diazomethane in methanol solution. The 3-methyl ether was purified by column chromatography on silica gel as described by Shrihatti and Nair (1977). Ultraviolet spectrophotometry of the 3-methyl ether in water showed a λ_{max} of 245 nm at pH 2 and 7.

2-*O*-Methyl-L-ascorbic acid was prepared by methylation of 5,6-*O*-isopropylidene-L-ascorbic acid with dimethylsulfate in alkali, followed by hydrolytic removal of the 5,6-blocking group (Lillard 1980). The 2-methyl ether was a syrup characterized by comparing its UV and ^{13}C nmr spectra with those of L-ascorbic acid and its 3-methyl ether (Lillard 1980). Results of UV analysis of the 2-methyl ether were as follows: pH 2, λ_{max} = 240 nm; and pH 7, λ_{max} = 260 nm; water, λ_{max} = 245 nm (Haworth et al 1937); and alkali, λ_{max} = 260 nm.

The dehydro derivatives were prepared by reaction of a methanolic solution of ascorbic acid with molecular oxygen in the presence of charcoal (Ohmori and Takagi 1978). After removal of

methanol, the dehydro forms were dissolved in water and their purity checked by TLC immediately before use. In chloroform-methanol, 4:1 (v/v), the starting ascorbic acid had relative mobility (R_f) of 0.2, whereas a dehydro form had R_f of 0.6.

6-Bromo-6-deoxy-L-ascorbic acid (mp 170°C) was prepared by treatment of L-AA with hydrogen bromide in acetic acid (Bock et al 1979). 5,6-*O*-Isopropylidene-L-ascorbic acid (mp 217–223°C) was synthesized by a reaction among L-AA, acetone, and hydrogen chloride (Jackson and Jones 1969).

Spread Ratios

The flour used was a composite of many hard winter wheat varieties grown throughout the Great Plains of the United States. It contained 12.2% protein (N × 5.7) and 0.42% ash.

Doughs were mixed with a pin mixer from 100-g flour to optimum consistency and development. Doughs were fermented or left standing at 30°C and 90% rh. After 0, 60, 105, and 180 min, doughs were molded and permitted to rest 60 min. Their spread ratios then were determined by dividing the width of a molded dough piece by its height (Hoseney et al 1979). The standard deviation of a spread ratio of 3.1 was ± 0.05.

When doughs were mixed under nitrogen, the gas in the flour was first exchanged five times by alternately subjecting the flour in a desiccator to a vacuum (~0.1 mm) and replenishing the desiccator's atmosphere with nitrogen. The desiccator and a pin mixer were placed in a large plastic bag that was inflated by a constant stream of nitrogen. Flour, water, and yeast were mixed 2 min in the nitrogen atmosphere, and the dough was allowed to rest 4 min, during which time the yeast rapidly depleted the oxygen in the dough.[4] Then a solution (1.0 ml) containing ascorbic acid or a blank solution (1.0 ml) was added and the dough mixed to optimum. The ascorbic acid solution (1 g/100 ml) also contained compressed yeast (1 g/100 ml) to prevent oxidation of ascorbic acid in the stock solution.[4] The blank solution contained 1 g of compressed yeast per 100 ml. After mixing, the doughs were fermented and spread ratios measured as described previously.

RESULTS AND DISCUSSION

The use of the spread ratio test to measure the effects of several flour additives on dough is illustrated in Fig. 4. When a control flour-water dough was mixed to optimum, the width divided by the height of the dough (spread ratio) increased from 2.7 to 3.2 as the lay time of the dough increased from 0 to 180 min. Potassium iodate reduced spread immediately to give a width-to-height ratio of 1.8, which remained constant as lay time increased up to 180 min. L-Ascorbic acid gave an immediate reduction in dough flow, followed by more resistance to flow with increasing lay time. Thus, iodate exerted its strengthening effect rapidly and completely after mixing, as expected, whereas L-AA displayed an immediate and time-dependent effect as described by Meredith (1965) and confirmed by ElKassabany and Hoseney (1980). Cysteine and D-IAA produced an immediate increase in dough flow (Fig. 4), but they did not affect the rate of dough flow with increasing lay time. In a dough oxidized to optimum with potassium bromate and yeast fermentation, not shown in Fig. 4, the spread ratio began at ~2.5 and declined to ~1.5 in 180 min.

Figure 5 shows the effects of adding the four isomeric ascorbic acids to a flour-water dough. Only the L-ascorbic isomer strengthened dough against flow. In fact, compared with a control, the other three isomers increased the flow of dough, a fact observed by ElKassabany and Hoseney (1980) but not by Maltha (1953) or Sandstedt and Hites (1945). We believe that the additional flow could be caused by competition in the dough for available oxygen. Oxygen may react with either the SH groups or the ascorbic acid. If fewer SH groups are oxidized during mixing, the residual SH groups will cause an immediate increase in the flow of dough that will be sustained as lay time increases.

Most researchers (Bloksma 1972, Mair and Grosch 1979, Sokol et al 1960, Tsen and Bushuk 1963) have found that SH groups disappear during dough mixing. In contrast to our explanation of the reducing effects of three of the ascorbic acids, Tsen (1965) did not find an increase in SH groups in doughs mixed with added D-IAA. Zentner (1968) also failed to find any free SH in wheat gluten after adding 750 ppm of L-AA. However, Graveland et al (1978) suggested that the analytical method of determining SH groups in dough (Bloksma 1972, Sokol et al 1960) needs to be reinvestigated.

The spread ratios of yeasted dough containing three of the isomeric ascorbic acids are shown in Table I. The improving effect of the combined L-AA and yeast is somewhat greater after 180 min of fermentation time (spread ratio = 1.7) than with L-AA alone (Fig. 5, spread ratio = 1.8–1.9). The data show that the other isomers do not effectively improve dough.

When a yeasted dough containing 100 ppm of L-ascorbic acid

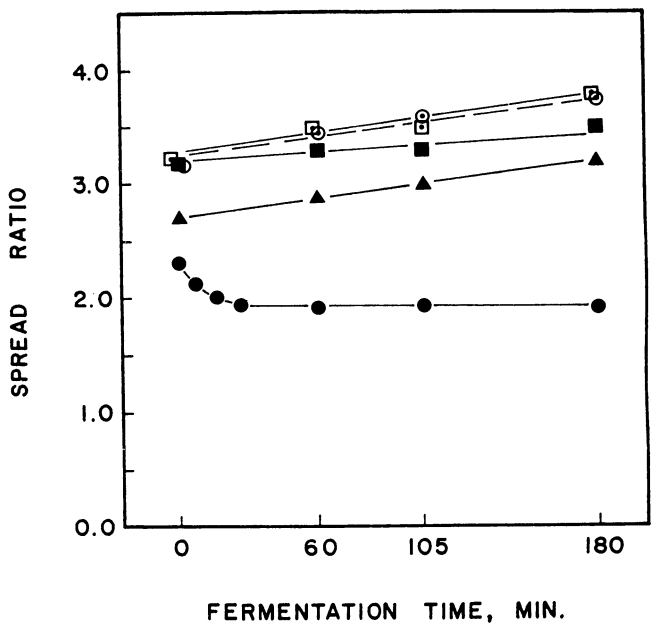

Fig. 5. Effect of reduced forms of ascorbic acids on the spread of flour-water doughs. ▲ = control, ● = L-ascorbic acid, ■ = D-isoascorbic acid, ⊙ = L-ascorbic acid, and ⊡ = L-isoascorbic acid.

TABLE I
Spread Ratio of 2%-Yeasted Doughs Containing Various Ascorbic Acids (100 ppm)

	Fermentation Time (min)	
Treatment	**0**	**180**
Control	2.9	1.9
L-Ascorbic acid	2.3	1.7
L-Isoascorbic acid	3.1	2.3
D-Ascorbic acid	3.4	2.6

TABLE II
Spread Ratios of 2%-Yeasted Doughs Containing L-Ascorbic Acid (100 ppm) Mixed In a Nitrogen Atmosphere

	Fermentation Time (min)	
Treatment	**0**	**180**
Control[a]	3.1	2.6
Control mixed in air	2.9	1.9
L-Ascorbic acid	3.2	1.8
Dehydro-L-ascorbic acid	2.4	1.7

[a] Air in flour exchanged five times by nitrogen and dough mixed in an N_2 atmosphere.

[4] Liang and Seib. Personal communication.

was mixed with nitrogen, the immediate strengthening effect of L-AA was largely eliminated (spread ratio = 3.2 versus control spread ratio of 3.1 in Table II). On the other hand, when dehydro-L-ascorbic acid was added during mixing, the dough was immediately strengthened. Both additives, however, produced the same reduction in the flow of dough at fermentation times of 180 min. The oxidation effect of L-AA at 180 min for doughs mixed with nitrogen indicates either that we did not completely exclude oxygen from the dough or that some other oxidant in dough converted at least 15% of L-AA to DE-L-AA. Nevertheless, the spread ratios at zero fermentation time led us to postulate that L-ascorbic acid behaves like the three other isomers, competing with native SH groups for molecular oxygen during dough mixing. In doughs containing added L-AA, and when the oxygen tension is reduced or short mixing times are used, the extra SH groups in doughs reduce mixing time and power requirements. The softening effect of L-AA (Mauseth and Johnston 1968, Zentner 1968) can be explained by this postulate.

Figure 6 shows the effects of adding the dehydro forms of each of the four isomeric ascorbic acids on the spread of flour-water doughs. The curve for dehydro-L-ascorbic acid in Fig. 6 can be superimposed on the curve for L-AA in Fig. 5; this confirms the findings of ElKassabany et al (1980), who showed that L-AA at 50 ppm in yeasted dough is ~80% oxidized at the optimum mixing time. The spread ratio data in Figs. 5 and 6 do not support the suggestion that DE-L-AA is a more effective dough improver than L-AA (Maltha 1953, Sandstedt and Hites 1945). The dehydro forms of L-IAA, D-AA, and D-IAA produced almost no effect on the initial flow of dough, but they did slightly negate the time-dependent increase in flow (Fig. 6).

The 2-methyl and 3-methyl ethers of L-AA were ineffective in preventing dough flow (Fig. 7) because they are difficult to oxidize to DE-L-AA. This demonstrates again that the dehydro form of L-AA is the active dough improver. 5,6-*O*-Isopropylidene-L-ascorbic acid, 6-bromo-6-deoxy-L-ascorbic acids, and dehydro-6-deoxy-6-bromo-L-ascorbic acid (Fig. 7) were partly effective in decreasing dough flow, compared with that of L-AA and reductic acid (Maltha 1953).

We believe that L-AA and the other isomeric ascorbic acids are

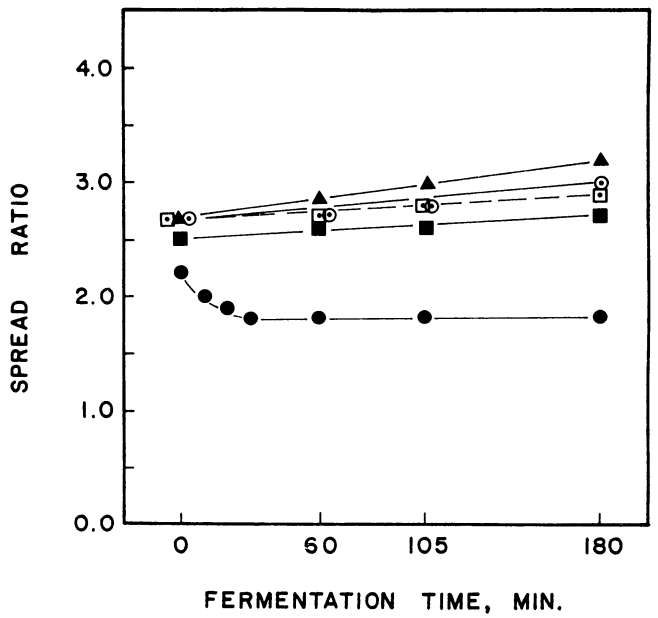

Fig. 6. Spread ratio of oxidized forms of ascorbic acid, flour-water doughs. ▲ = control, ● = dehydro-L-ascorbic acid, ■ = dehydro-D-isoascorbic acid, ⊙ = dehydro-D-ascorbic acid, and ⊡ = dehydro-L-isoascorbic acid.

TABLE III
Spread Ratios of Flour-Water Doughs Containing L-Ascorbic Acid, Hydrogen Peroxide, and/or Catalase

Treatment	Fermentation Time (min)			
	0	60	105	180
Control	2.7	2.9	3.2	3.4
Control + catalase (4,500 units)[a]	2.8	3.0	3.2	3.4
Hydrogen peroxide (1,200 ppm)	1.6	1.6	1.6	1.6
+ catalase (4,500 units)	2.8	3.0	3.2	3.4
L-Ascorbic acid (100 ppm)	2.3	1.9	1.9	1.9
L-Ascorbic acid (100 ppm) + catalase (4,500 units)	2.2	1.9	1.9	1.9

[a] One unit of catalase decomposes 1 μmol of hydrogen peroxide per min at 25°C and pH 7.

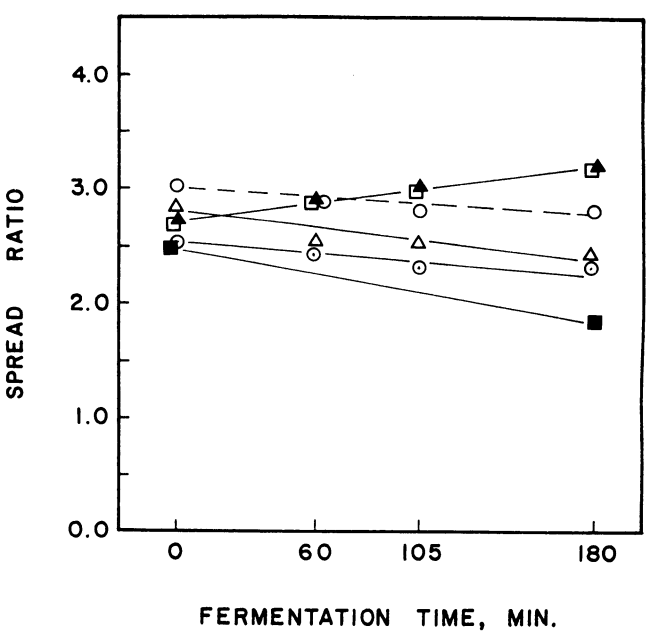

Fig. 7. Spread ratios of reductic acid, L-ascorbic acid, and selected derivatives. ▲ = control, □ = 2- or 3-*O*-methyl-L-ascorbic acid, O = 6-bromo-6-deoxy-L-ascorbic acid, ⊙ = dehydro-6-bromo-6-deoxy-L-ascorbic acid, ■ = reductic acid and L-ascorbic acid, and △ = 5,6-*O*-isopropylidene-L-ascorbic acid.

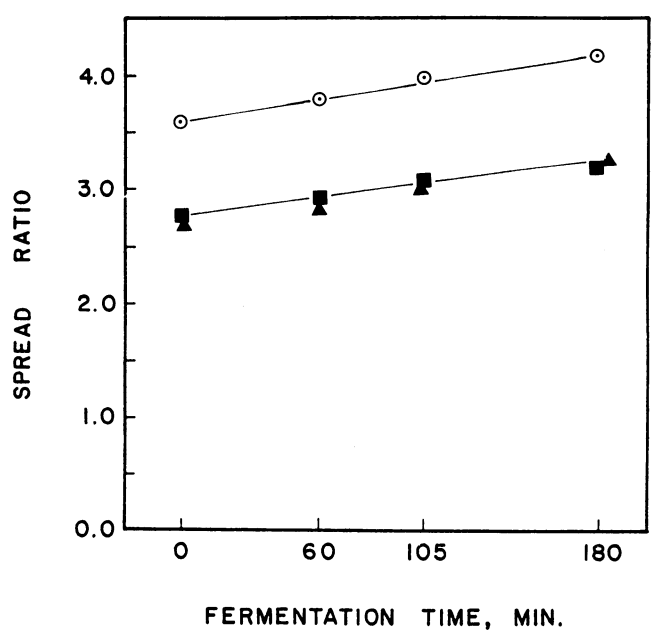

Fig. 8. Spread ratio of doughs containing L-cysteine (30 ppm) + dehydro-D-isoascorbic acid (100 ppm). ▲ = control, ⊙ = L-cysteine, and ■ = L-cysteine + dehydro-D-isoascorbic acid.

oxidized chemically to their dehydro forms during dough mixing. The reaction may be written as follows (Khan and Martell 1967):

$$AH_2 + O_2 \rightarrow A + H_2O_2.$$

Because ~20 ppm of hydrogen peroxide gives optimum oxidation of a dough (Dahle and Sullivan 1963), the equation for the chemical oxidation of ascorbic acid leads to the prediction that because all isomers would produce hydrogen peroxide, they would all behave as improvers of dough. Evidence also suggests (Mitsuda et al 1967) that the reaction of oxygen with glutathione, mercaptoethanol, and cysteine also produces hydrogen peroxide. The overall reaction for air-oxidation of SH compounds may be written:

$$RSH + O_2 \rightarrow RSSR + H_2O_2.$$

The hydrogen peroxide generated by these reactions in dough is probably destroyed by the catalase that occurs naturally in flour. Honold and Stahmann (1968) reported that two hard wheat flours and two spring wheat flours contained averages of 0.7 and 3.5 units (1 μmol per min at 30° C), respectively, of catalase activity per gram of flour. Thus, 100 g of hard wheat flour would contain about 60 units of catalase activity. If the flour contained 100 ppm of ascorbic acid, its chemical oxidation during mixing could release as many as 60 μmols of hydrogen peroxide. With a 3-min mixing period, the maximum rate of release of H_2O_2 would theoretically be about 20 μmols per minute.

The data in Table III show that adding catalase to a dough containing 100 ppm of L-AA had no effect on the spread ratio of the dough. On the other hand, addition of hydrogen peroxide (20–1200 ppm) produced an effect typical of a fast-acting oxidant. We believe that these results show that the concentration of hydrogen peroxide generated during dough mixing remains too low to give a noticeable oxidizing effect; the hydrogen peroxide is destroyed either by catalase or by its reaction with substances in dough that have no effect on flow.

We postulate the immediate reaction, whereby the reduction in dough flow by L-AA is probably due to the ability of its dehydro form to counteract the action of SH groups in a dough. Further, the reaction does not require a catalyst. Figure 8 shows that dehydro-D-isoascorbic acid (100 ppm) completely eliminates the increase in dough flow caused by adding 30 ppm of cysteine. The presence of glutathione dehydrogenase may cause a slightly lower level (~10% after 20 and 80 min of lay time) of SH in the presence of dehydro-L-ascorbic acid (Tsen 1965) than in the presence of dehydro-D-isoascorbic acid. Whether that small change explains the stereospecific action of L-AA on dough flow requires more investigation.

The mechanism of the time-dependent reduction of dough flow by L-AA remains unknown. It is probably a reaction in which DE-L-AA acts either as a specific cofactor for an enzyme or as an activator or deactivator of an enzyme in flour.

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