

## **Studies of the Role of Ascorbic Acid in Chemical Dough Development. I. Reaction of Ascorbic Acid with Flour-Water Suspensions**

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### **ABSTRACT**

At the 60-p.p.m. level, nearly all the L-ascorbic acid that was added to a flour-water suspension disappeared in approximately 15 min. This level is within the effective range for chemical dough development. At higher levels the rate was nearly independent of ascorbate concentration so that the time required for it to disappear increased directly with the amount. The rate was rapid enough to associate ascorbic acid oxidation with the function of this additive in chemically developed doughs. The pH optimum was 6.3, suggesting an enzymatic reaction. Salt at low concentrations was inhibitory. Cysteine was only slightly inhibitory. Bromate had no effect. Extraction of the chloroform-soluble lipids had no effect but extraction of the 1-butanol-soluble fraction caused 60% inhibition. Reconstituting the lipid fraction with the butanol-extracted flour did not result in any recovery in the rate at which ascorbate was oxidized, but supplementation of untreated flours with butanol-extracted lipids significantly enhanced this activity.

The conventional methods of bread production require a relatively long interval for dough development between mixing and baking. Time is needed, not only to allow the yeast to produce CO<sub>2</sub>, but also to permit certain changes that affect both the elasticity and the gas-retaining power of the dough. An inadequate development time results in an unsatisfactory loaf of bread.

If bread dough is subjected to a very vigorous mechanical mixing, as in the Chorleywood Process (1), the holding time that is otherwise required for dough development is greatly reduced. By combining very small amounts of both L-cysteine and L-ascorbic acid with the dough mixture, the power input that is ordinarily required for mechanical development is decreased to the extent that conventional dough mixers may be used (2). With these two additives included, the process is referred to as chemical dough development (3). Presumably, the effect of cysteine is that of a reducing agent for flour protein disulfide linkages, and perhaps it acts to promote disulfide interchange (4).

For some time, ascorbic acid has been recognized as an improving agent in conventional straight dough systems, where it undergoes oxidation to dehydroascorbic acid. It is the latter which is responsible for the improver effect

(4,5). Like bromate, the dehydroascorbic acid promotes the oxidation of flour protein sulfhydryl groups.

The manner in which ascorbic acid exerts its effect on chemically developed doughs is the subject of some controversy. It cannot be replaced by dehydroascorbic acid (5) so the mechanism is different from that which is encountered in conventional dough systems. Ascorbic acid does not reduce disulfide linkages (5). Two quite different hypotheses have been advanced. Johnston and Mauseth (4) and Zentner (6) have suggested that ascorbic acid causes the observed effects by interacting, in some way, with the hydrogen bonds in the dough system. In contrast, Dahle and Murthy (5) contend that the effect is related to the antioxidant properties of this compound, and speculate that it serves a protective function for certain of the lipid components which are known to have a great influence on loaf volume and bread quality (7).

Using flour-water suspensions and flour extracts, as well as doughs, several investigators (5,8-12) have examined the fate of added ascorbic acid. In either extracts or suspensions, it appears to be oxidized enzymatically, but the disappearance rates also appear to be slow—slow enough to question whether a mechanism that involved oxidation could be of any significance. In contrast, very little of the added ascorbic acid can be detected in doughs after short mixing periods (10,12). In spite of this latter observation it is not possible to conclude that rapid oxidation has occurred. Such a result may simply be a consequence of not being able to extract the ascorbic acid from the dough. The interpretation of the available data is complicated because different investigators have used widely different levels of ascorbic acid.

The rate at which ascorbic acid disappears from flour-water suspensions has been reinvestigated. The effects of several variables are reported.

### MATERIALS AND METHODS

Two different samples (A and B) of untreated bread flour were used. Both were freshly milled from Canadian HRS wheat in a commercial mill. Sample A, which was used for most of the experiments, had an ash content of 0.37% and a crude protein content ( $N \times 5.7$ ) of 12.0%. The respective values for sample B were 0.49 and 14.8%.

Glass-distilled water was used throughout.

All chemicals were reagent grade.

In order to measure the rate of disappearance of ascorbic acid, a concentrated suspension of flour in water was thoroughly mixed in a beaker. An aliquot of a freshly prepared aqueous solution of ascorbic acid was added to the suspension. Solution concentrations and liquid volumes were designed to give the desired levels<sup>1</sup> of reagent in a final mixture consisting of one part flour and three parts water w./v. The mixture was stirred vigorously and continuously with a magnetic stirrer unless otherwise noted. At selected time intervals, aliquots of approximately 12 ml. were removed using a fast-flowing pipet, and mixed with 10.0-ml. portions of a 6% w./v. aqueous solution of metaphosphoric acid. The exact amounts of suspension were determined by weighing the acidified aliquots. For those experiments involving a single fixed time, the entire suspension was

<sup>1</sup>Reagent levels refer to initial amounts and henceforth they are based on the amount of flour in the suspension.

acidified with an equal volume of the 6%  $\text{HPO}_3$  solution. If cysteine or bromate were included, aqueous solutions of these reagents were added to the suspension at the same time as the ascorbic acid. Sodium chloride, if included, was added to the suspension prior to the ascorbic acid.

When desired, the pH of the suspension was adjusted with small amounts of dilute NaOH or  $\text{H}_3\text{PO}_4$  solutions prior to addition of the ascorbic acid, but pH measurements were performed routinely after the addition of the ascorbic acid. The pH was not adjusted unless otherwise noted. Most experiments were at room temperature but a few were controlled at a higher temperature with a thermostatted water bath. Suspension temperatures were routinely measured.

The acidified suspension was centrifuged at  $15,000 \times g$  and the supernatant was subjected to analysis for ascorbic acid by the indophenol-xylene-extraction method (13). With the higher levels of ascorbic acid it was necessary to further dilute the supernatant solutions with 3%  $\text{HPO}_3$  solution before the analyses. The analytical procedure was modified slightly by diluting the acetate buffer to one-fifth of the recommended concentration. Procedures to eliminate interfering substances were not found to be necessary, except in those experiments that included added cysteine. In order to remove this amino acid, 20 ml. of the acidified extract was passed through a 6-cm. bed of cation-exchange resin (Dowex 50  $\times$  8). Freshly packed, 9-mm.-diameter columns of the resin were used for each 20-ml. sample. In preparation for packing, the resin was washed successively with NaOH solution and water, and equilibrated with 3%  $\text{HPO}_3$  solution.

Control experiments for the kinetic studies substituted boiled aqueous extracts of flour for the flour suspensions. Such extracts were prepared by the centrifugation of 1:3 w./v. flour-water suspensions. Other control experiments substituted buffered solutions for the flour suspensions.

Flour samples from which a substantial portion of the lipid components had been removed were prepared by solvent extraction using either water-saturated 1-butanol or chloroform. The 1-butanol was freshly distilled in the presence of sodium metal. This was a necessary precaution, for otherwise the impurities eventually interfered with the ascorbic acid analysis. The chloroform was also purified by successive washing with water, drying over  $\text{CaCl}_2$ , refluxing with anhydrous  $\text{Na}_2\text{SO}_4$ , and finally distilling. Untreated flour was extracted by stirring for 30 min. with 5 volumes of solvent at room temperature. The suspension was centrifuged at  $1,000 \times g$  and the insoluble residue was extracted once more in the same manner. The supernatant fractions were combined. The solvent was evaporated from part of the extract under a stream of air at  $40^\circ$ . The remaining extract was concentrated 10-fold with a rotary vacuum evaporator at a temperature of less than  $40^\circ\text{C}$ . The flour residue was spread out on a polyethylene sheet and air-dried, ground to a floury texture, and further dried at room temperature for 24 hr. in a vacuum dessicator, with continuous pumping.

For certain of the control experiments, solvent-treated flours were prepared by mixing untreated flour with either water-saturated 1-butanol or chloroform in a 1:1 w./v. ratio. The resulting thick suspension was dried in the same manner as described above for the flour residues.

To add isolated flour lipids back to the solvent-extracted flours, or to add them to untreated flour, a solution of those lipids in 1-butanol was mixed with the flour in a 1:1 w./v. ratio. The resulting suspension was dried as described above.

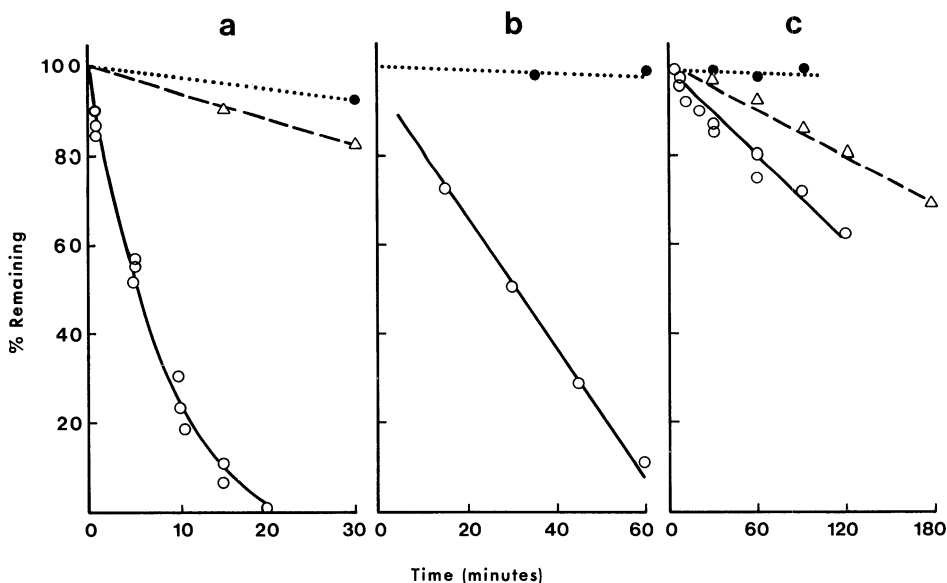


Fig. 1. The effect of L-ascorbic acid concentration on the rate of its disappearance from aqueous suspensions of flour A. a) 60 p.p.m. initial ascorbic acid concentration; b) 400 p.p.m.; c) 3,000 p.p.m. The dotted lines are for control experiments using a boiled flour extract in place of the flour suspension. The dashed lines are for further control experiments using 0.02M phosphate buffer in place of the flour suspension. The pH was  $5.9 \pm 0.1$  and the temperature was  $24^\circ \pm 2^\circ\text{C}$ .

## RESULTS AND DISCUSSION

The two samples of flour showed significant differences in their ability to catalyze the oxidation of L-ascorbic acid. With an aqueous suspension of untreated flour A, to which 120 p.p.m. of ascorbic acid was added, 50% of the additive remained after 15 min. of reaction time. The pH was 5.9 and the temperature  $24^\circ\text{C}$ . Under the same conditions using flour B, only 30% of the ascorbic acid remained.

The amount of naturally occurring substances in untreated patent flour, which were capable of reducing the indophenol dye, was very small. Within the limits of the analytical procedure, this quantity did not change with variation in the time of mixing. All the data have been corrected for this factor.

The rate at which added ascorbic acid disappeared from a 1:3 w./v. flour-water suspension was found to be dependent upon several factors.

### Effect of Ascorbic Acid Concentration

It has been the practice of other investigators (5,8-12) to report the fraction of added ascorbic acid that disappeared within a given time interval. Meaningful comparisons among their data are difficult because of the rather wide variation in the levels of ascorbic acid that they have used. Many of the apparent discrepancies among their results can be related to the variation in the initial ratios of ascorbic acid to flour.

The disappearance of ascorbic acid from suspensions of otherwise untreated flour at room temperature and at three different levels of the added reagent is illustrated in Fig. 1. The pH of an untreated suspension was  $5.9 \pm 0.1$ . At the two higher levels of added ascorbic acid it was necessary to adjust the pH to bring it within these limits. At 60 p.p.m., which is within the range of concentrations that are effective for chemical dough development (2), the ascorbic acid disappeared from a flour suspension considerably faster than it did in the control experiments. The rate decreased as the reaction progressed. These results support the hypothesis that the major process was enzymatic (8). When the reaction was stopped by acidifying the suspension immediately after adding the ascorbic acid, nearly all of the latter was recovered from the mixture. Thus it does not appear that any significant quantity of this compound was strongly adsorbed onto the insoluble flour proteins, for presumably such adsorption would be a very rapid process. The rate of ascorbic acid disappearance at the 60-p.p.m. level was in reasonable agreement with that reported for doughs (5,10,12).

At the 3,000 p.p.m. level, the disappearance rate was independent of the ascorbic acid concentration and when presented as in Fig. 1, c, the process appears to be relatively slow. At this level the disappearance rate in a 0.02M phosphate buffer at pH 6.0 was approximately one-half of that observed with the flour suspension. The rate using a boiled flour extract was much lower. These latter observations support the conclusions of Sandstedt and Hites (8) that there is a protective effect of dissolved flour proteins against non-enzymatic decomposition of ascorbic acid.

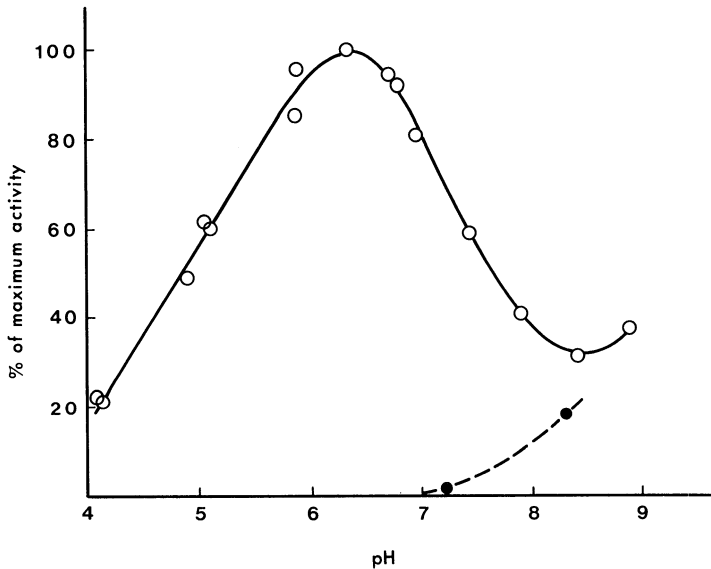
A level of 3,000 p.p.m. is much higher than the amount used in chemical dough conditioning. Such high levels were employed in an attempt to approximate the conditions used in a similar study by Carter and Pace (10).

At the intermediate 400-p.p.m. level an apparent intermediate rate for the disappearance of ascorbic acid was observed (Fig. 1, b). As at the higher level, the rate remained constant as the ascorbic acid content decreased.

Absolute rates for the enzymatic reaction which caused the disappearance of ascorbic acid have been calculated. The results depend upon the data that were used to correct for the non-enzymatic reaction. It was assumed that the control experiments using boiled extracts of flour would provide the best data for making such corrections. On this basis, for flour A at a temperature of  $24^{\circ}\text{C}$ . and at pH 5.9, the absolute rates were 10.0  $\gamma$  ascorbic acid per min. per g. of flour at the 3,000-p.p.m. level of ascorbic acid; 6.0  $\gamma$  per min. per g. of flour at the 400-p.p.m. level, and an average of 4.0  $\gamma$  per min. per g. over the first 5 min. at the 60-p.p.m. level. When the rate of ascorbic acid disappearance from phosphate buffer solutions is used to estimate the non-enzymatic reaction, the calculated rate for the enzymatic reaction was 5.0  $\gamma$  per min. per g. of flour at both the 3,000 and 400 p.p.m. levels and it remained at 4.0  $\gamma$  per min. per g. for the 60-p.p.m. level. In any event, it can be concluded that above 60 p.p.m., the order of the reaction was very nearly zero with respect to ascorbic acid concentration. This suggests that the amount of substrate was sufficient to saturate the enzyme and the rate-limiting factor was the amount of enzyme present in the flour.

#### Effect of Stirring

The precision of the data from replicate analyses was highest when the suspensions were stirred continuously. However, if the stirring was stopped 30



**Fig. 2.** The effect of pH on the rate of disappearance of L-ascorbic acid from flour-water suspensions. The initial concentration of ascorbic acid was 60 p.p.m. and the temperature was  $24^{\circ} \pm 2^{\circ}\text{C}$ . The dashed line is for the control experiment, using a boiled flour extract in place of the flour suspension.

sec. after addition of ascorbic acid, the results were generally the same as those obtained with continuous stirring. In a few experiments the unstirred samples showed approximately a 5% reduction in the rate at which ascorbic acid disappeared. The small effect of differences in the mode of stirring may suggest that dissolved oxygen does not play any substantial role in the overall reaction, but such a conclusion probably is not justifiable. In mixing the flour suspension, it may be assumed that the system became saturated with  $\text{O}_2$ . That amount would be sufficient to oxidize the 120 p.p.m. of ascorbic acid that were added in these particular experiments.

#### Effect of pH

The rate at which ascorbic acid disappears was sensitive to pH as illustrated in Fig. 2. The shape of the curve is typical of an enzymatic process. The optimum pH was  $6.3 \pm 0.2$ . A range of pH optima from 4.5 to 6.5 has been reported for the ascorbate oxidases of other species (14).

#### Effect of NaCl

Sodium chloride had an inhibiting effect on the rate of ascorbic acid disappearance (Fig. 3). This effect was further influenced by pH. Greater inhibition occurred as pH increased, particularly at the lower salt levels. The incremental response to increasing salt concentration diminished progressively. This was also more obvious as the pH increased.

The extent to which the reaction was inhibited may depend more upon the

concentration of salt in solution rather than the salt to flour ratio. The experimental design did not permit a conclusion in this regard and further experiments are planned to evaluate this aspect of the problem.

When dough is developed mechanically, delayed addition of salt results in a reduction of the mixing requirement (15). Some of the effects of salt on dough properties are undoubtedly due to its direct action on gluten proteins. However, there is the further possibility that the requirement for additional mixing may be associated with the inhibitory effect upon the enzymatic oxidation of ascorbic acid.

#### **Effect of Cysteine**

The presence of L-cysteine had a rather small effect on the disappearance rate for ascorbic acid at the 120-p.p.m. level. In the presence of an equimolar amount of cysteine, this rate ranged from 75 to 85% of that observed in its absence. Detectable amounts of ascorbic acid had disappeared in 30 sec. after the start of the experiment and the rate did not diminish until more than 50% of the added ascorbic acid was gone.

It has been established elsewhere that cysteine is quite rapidly oxidized in such a system (16). Although no attempt was made here to measure the extent of such oxidation directly, the fact that steps had to be taken to remove cysteine in order to prevent substantial interference with the ascorbic acid determination suggests that considerable amounts of this amino acid did remain at the end of the experiment.

Any possible interrelationship between cysteine and ascorbic acid in chemical dough development remains obscure. If ascorbic acid oxidation is an important feature of the mechanism, it appears unlikely that the major role of cysteine is to protect the ascorbic acid from such oxidation. Evidence for a glutathione-dependent dehydroascorbate reductase in wheat flour has been reported (8). The present study indicates that any analogous cysteine-dependent activity must be small in comparison to the rate at which ascorbic acid was oxidized.

#### **Effect of Bromate**

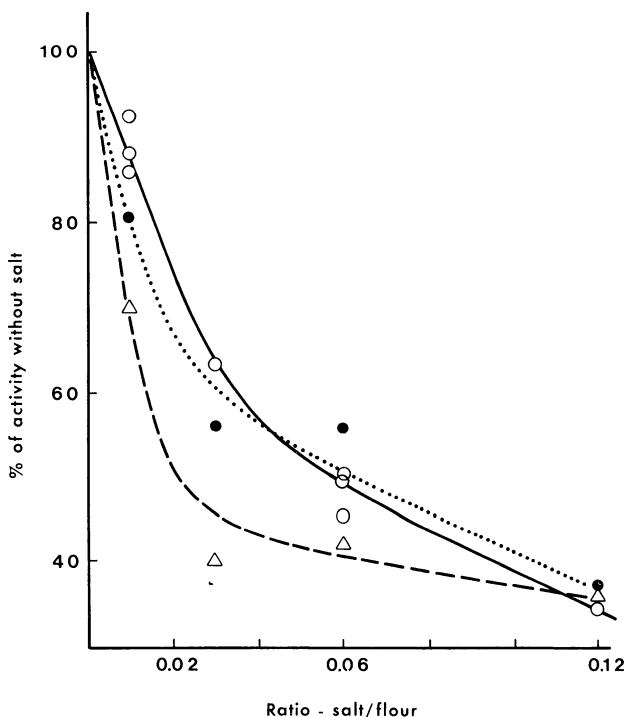
The inclusion of  $\text{KBrO}_3$  along with ascorbic acid in a flour suspension, with both additives at the 120-p.p.m. level, had no effect whatever on the rate at which ascorbic acid disappeared. Nor did the bromate affect the control experiments. The results suggest that these two additives act independently insofar as their effects on loaf characteristics are concerned.

#### **Effect of Temperature**

Only preliminary results have been obtained. At the 120-p.p.m. level of ascorbic acid and at pH 5.85 with no other additives, the rate at 35°C. was 1.4 times as fast as at 23°C. This corresponds to an activation energy of 5.0 kcal. per mole as calculated by the Arrhenius equation.

#### **Effect of Flour Lipids**

If the hypothesis of Dahle and Murthy (5) is correct, the extraction of lipid components from the flour might be expected to affect the rate at which ascorbic acid is oxidized. Chloroform has recently been recommended as a solvent for extracting flour lipids (7). The rate at which 120 p.p.m. of added ascorbic acid disappeared from a suspension of chloroform-extracted flour was identical to



**Fig. 3.** The effect of NaCl on the rate of disappearance of L-ascorbic acid from flour water suspensions. The initial concentration of ascorbic acid was 60 p.p.m. and the temperature was  $24^{\circ} \pm 2^{\circ}\text{C}$ . Solid line = pH 5.9; dotted line = pH 6.8; dashed line = pH 7.7.

that observed in suspensions of untreated flour. The chloroform-extractable lipids accounted for 1.0% of the flour weight.

The components extractable with water-saturated 1-butanol accounted for 1.8% of the flour weight. The rate at which ascorbic acid disappeared from a suspension of butanol-extracted flour was only 40% of that observed with untreated flour. The decrease was probably not a consequence of the solvent denaturing the enzyme, for the butanol-treated flour (see experimental) showed the same activity as the untreated flour. Adding the lipids back to the butanol-extracted flour did not restore the original activity. Such reconstituted flour gave results identical to those observed with the butanol-extracted flour. On the other hand, adding some of the butanol-extracted lipid material to untreated flour, so as to approximately double the lipid content, did result in considerable enhancement of the rate at which ascorbic acid disappeared from a flour suspension. Supplementation with an extract which had been evaporated under a stream of air caused an increase in the rate of only 10% but when the extract was concentrated under conditions less conducive to lipid oxidation, the rate was 140% of that observed with untreated flour.

It is difficult to interpret these results in any mechanistic fashion. They suggest



that the polar lipids rather than the nonpolar lipids were involved. In a general way they seem to lend support to the hypothesis of Dahle and Murthy (5).

### GENERAL DISCUSSION

Except at levels of ascorbic acid that were below those found to be most effective for chemical dough development, the factor that limits the rate at which it disappears was not the amount that was present in the flour suspension. There are several alternative explanations. The rate-limiting factor may be the amount of some enzyme in the flour, presumably the amount of ascorbic acid dehydrogenase. The level of dissolved O<sub>2</sub> may also be rate-limiting under some circumstances, but this does not appear to be true at the levels of ascorbic acid that are found to be effective for dough development. The oxidation of ascorbic acid may be coupled in some way to the protection of a polar lipid, so that the rate at which the former disappears could depend upon the amount of the latter. In any event, whatever the true rate-limiting factor is, it is highly probable that the reaction which results in the disappearance of ascorbic acid is intimately associated with the process of chemical dough development.

### Acknowledgments

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