SOME INTERRELATIONSHIPS OF ASCORBIC ACID AND DEHYDROASCORBIC ACID IN THE PRESENCE OF FLOUR SUSPENSIONS AND IN DOUGH[1]

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ABSTRACT

In suspensions of flour (65% extraction) at pH 6.0, ascorbic acid (AA) is oxidized only slowly, and the evidence does not suggest the presence of a very active AA oxidase in the endosperm of wheat. But AA is oxidized rapidly in dough when the dough is mixed in air. Under these conditions most of the AA is oxidized to dehydroascorbic acid (DHA). When DHA is added to flour suspensions or to dough, there is only a slight reduction to AA, in 90 min. at 27°C. But if glutathione (GSH) is also present, then DHA is reduced to AA in a few minutes. This does not occur with flour made from steamed wheat. The observations confirm the presence of an active DHA reductase in the wheat grain. The distribution of this enzyme in the wheat grain has been determined. Preliminary observations have been made on the potential usefulness of measurements of the activity of the enzyme as an index of heat-damage to the endosperm of the wheat grain.

Ascorbic acid is of special interest among the flour improvers in that, first, it is a naturally occurring substance of metabolic importance and, second, it is a reducing agent. The usual improving agents are oxidizing agents; a widely held view at present is that their effect is due to oxidation of some of the thiol groups present in flour proteins (1,2,3). It is well known that ascorbic acid (AA) is readily oxidized, for example by air in the presence of small amounts of copper or iron, or enzymatically by certain oxidases. The first well-defined step in the oxidation of ascorbic acid is to dehydroascorbic acid (DHA), and this step is reversible. DHA may also be oxidized further, and irreversibly, to diketogulonic acid and other compounds. Earlier investigators have suggested that the apparent paradox of the reducing agent AA's being

[1]Manuscript received June 10, 1964. Communication from the Research Association of British Flour-Millers, Cereals Research Station, St. Albans, England. Presented at the 49th annual meeting, Toronto, Ontario, April 1964.

a flour improver may be explained by this oxidation to DHA, which is then the effective improving agent. The most pertinent and detailed observations were those of Sandstedt and Hites (4) in 1945. They found that extracts of a second clear flour oxidized AA fairly slowly but more rapidly than a boiled extract — suggesting enzymatic oxidation, and also suggesting that extracts containing DHA rapidly oxidized glutathione (GSH). However, oxidation of AA by extracts of a higher-grade flour (85% patent) was slight. Until very recently, no further detailed work on the problem appears to have been reported, and information on the fate of AA and DHA in dough, as distinct from flour extracts, has not been available. Because of renewed interest in AA as a "natural" flour improver and the current intensive study of the role of thiol groups, we have made some further observations on AA and DHA in flour suspensions and dough. Before this work was completed, the results of a rather similar investigation were reported by Kuninori and Matsumoto (5,6). Our work is in general agreement with their findings and extends their observations in certain directions.

Materials and Methods

Wheat. Samples of Manitoba and of 15 varieties of wheat grown in the United Kingdom were used.

Flour. Samples of flour were milled in the laboratory on a Buhler mill. The extraction rate of flour from the wheat was about 65%.

Wholemeal samples were prepared by grinding the wheat in a coffee mill.

AA was obtained from Hopkins & Williams Ltd., GSH from Lights Ltd.

DHA was either made by the method of Kenyon and Munro (7), or prepared immediately before use by dropwise addition of the exact equivalent of iodine to a solution of AA.

Thiogel was obtained from Schwarz Bio-Research Inc., and other reagents were obtained from Hopkins & Williams Ltd. Glass-distilled water was used in the preparation of all solutions.

General Methods. Measurements were made in flour or wholemeal slurries and extracts of flour or wholemeal at pH 6.0, McIlvaine's buffer, and in unyeasted dough. Doughs were made by mixing 28 g. flour with sodium chloride solution (2.5%) in a Minorpin mixer at 26.6°C. When required, AA, DHA, GSH, and thiogel were dissolved in the salt solution immediately before the dough was mixed. Observations were made both in air and under nitrogen.

Anatomical parts of the grain were obtained by microdissection, as described by Hinton (8).

AA was determined by titration with phenolindo-2,6-dichlorophenol after addition of metaphosphoric acid solution to reaction mixtures. In the reduction of DHA in the presence of GSH the course of reaction was followed by indophenol titration and, in some instances, iodine titration. Details of procedure are given below with each of the tables of results.

Results

Stability of AA in Flour Suspensions at pH 6.0. The question of whether there is an AA oxidase system in flour active at pH 6.0 was examined by adding AA to flour extracts and flour slurries and measuring the AA content remaining at time intervals up to 6 hr. Typical results are given in Table I. As a control, flour from wheat steamed at 100°C. was used; this procedure inactivates the enzymes present in wheat.

TABLE I

RECOVERY OF ADDED AA FROM FLOUR SLURRIES

Reaction Time[a] at 20°C.	Indophenol Titration[b]		Iodine Titration[c]	
	Normal Flour	Flour from Steamed Wheat	Normal Flour	Flour from Steamed Wheat
hr.	*ml.*	*ml.*	*ml.*	*ml.*
0	3.10	3.12	1.46	1.46
2	2.90	3.10	1.33	1.42
4	2.76	3.0	1.24	1.36
6	2.73	2.95	1.21	1.31

[a] Reaction time (for each time point): 5 g. flour + 5 ml. pH 6.0 McIlvaine's buffer + 12.5 ml. AA solution (1.5 mg./ml.). Stopped with 12.5 ml. metaphosphoric acid solution (20%). Filtered and 2 ml. aliquots of filtrate titrated.
[b] 1 ml. = 0.375 mg. AA.
[c] 1 ml. = 0.80 mg. AA.

The results (Table I) show that with suspensions of low-extraction flour (65%) there is only very slow oxidation of AA. There is no evidence of very active oxidase action at this pH, since the slow rate of oxidation in the presence of normal flour is only slightly greater than that observed in the presence of flour from steamed, enzyme-free wheat. Nonenzymatic oxidation also appears to be sluggish in the presence of flour at this pH, since the observed loss is hardly greater than is observed in buffer in the absence of flour.

Stability of AA in Dough. The results with flour suspensions thus would not lend much support to the view that the improving effect of AA is due to its oxidation to DHA. But these results cannot be directly translated to behavior in dough. In dough-mixing, air is incorporated in the mix and a large surface area is continuously being exposed, which may favor an oxidative process. The stability of AA in dough was therefore examined. Typical results are given in Table II.

TABLE II

RECOVERY OF ADDED AA FROM UNYEASTED DOUGH[a]

CONDITION OF DOUGH	AA RECOVERED[b]
	%
After 1 min. mix	81
After 1 min. mix + 90 min. rest	49
After 5 min. mix	22

[a] Dough: 28 g. Manitoba flour, 15 ml. salt solution (2.5%) containing 3 mg. AA. For measurement of AA dough broken down in blender for 20 sec. with 100 ml. metaphosphoric acid solution (12.5%), centrifuged, 50-ml. aliquots of supernatant titrated with indophenol solution (400 mg./liter).
[b] As percentage of quantity added.

These results with dough are in marked contrast to those obtained with flour suspensions. The mixing process causes rapid disappearance of AA, and this continues, more slowly, during a subsequent rest period. Such experiments thus establish that AA may be transformed in dough, but they do not, of course, provide evidence that the change is one of oxidation to DHA. Before considering this possibility it is necessary to consider the behavior of DHA itself when it is present in flour and dough.

DHA in Flour Suspensions. It is known that DHA can oxidize some relatively simple –SH compounds such as GSH nonenzymatically, but this only occurs at a significant rate at pH values above 7.0, i.e., considerably higher than the normal pH of dough. But Hopkins and Morgan (9) and Crook and Morgan (10) showed that many plants contain an enzyme system which catalyzes this reaction at pH values below 7.0:

$$2\ \text{GSH} + \text{DHA} \underset{\text{reductase enzyme}}{\rightleftharpoons} \text{GSSG} + \text{AA}$$

As GSH is oxidized, the DHA is concomitantly reduced to AA. The reaction can be followed by observing the disappearance of GSH and the appearance of AA. If DHA is added to a flour suspension or an extract of flour at pH 6.0 containing GSH, the reaction shown above occurs very rapidly. In 10 to 15 min. at room temperature most of the added DHA is reduced to AA. This appears to be due to the reductase enzyme, since if flour from steamed wheat is used, reaction in 10 min. is negligible (Table III).

Similar results were obtained in a series of experiments using a wide variety of flours. If GSH was omitted from the reaction mixture, the reduction of DHA in 10 min. was less than 4%. The reaction was dependent on pH increasing in rate as the pH was increased to 7.0, and it was inhibited if the flour was heat-treated. It therefore appeared from these results that flour contains the reductase enzyme of Morgan and Hopkins which catalyzes rapid oxidation of GSH by DHA.

TABLE III
REDUCTION OF DHA, IN FLOUR SLURRIES CONTAINING GSH, AT PH 6.0[a]

FLOUR	DHA REDUCED TO AA (10 min. at 20°C.)
	% of DHA added
From normal unheated wheat	89
From same wheat previously steamed	5

[a] 5 g. flour, with 15 ml. McIvaine's buffer, pH 6.0; 1 ml. GSH solution (10 mg./ml.), 5 ml. DHA solution (0.48 mg./ml.). Reaction stopped with 5 ml. metaphosphoric acid solution (25%). Centrifuged; 10-ml. aliquot of supernatant titrated with indophenol.

The next step was to examine whether this enzymatic oxidation of GSH by DHA occurred in dough. DHA and GSH were added to the dough liquid; the dough was mixed for 1 min. and allowed to stand. Determinations of AA were made immediately after the 1-min. mixing and at subsequent time intervals by dispersing the dough in metaphosphoric acid, centrifuging, and titrating aliquots of the supernatant with indophenol. Table IV shows the results of a typical experiment.

TABLE IV
REDUCTION OF DHA BY GSH IN DOUGH[a]

CONDITION OF DOUGH	AA FORMED[b]
	mg.
After 1 min. mix	1.8
After 15 min. rest	1.6
After 30 min. rest	1.55
After 60 min. rest	1.4
After 120 min. rest	1.3

[a] Each determination on 44 g. dough containing 2.4 mg. DHA and 10 mg. GSH. Dough mixed in air and held at 27°C.
[b] From 2.4 mg. DHA present initially.

It will be seen that under these conditions there is very rapid reduction of DHA in dough. After 1 min. of mixing, more than 70% of the added DHA is reduced to AA. As the dough stands, the quantity of AA found decreases – presumably because it is reoxidized, once the GSH has itself undergone oxidation.

The fact that the enzyme was active in dough suggested a method which could be used to obtain an answer to the question left posed by the data of Table II. These data showed that AA disappeared when mixed in dough, but did not establish that it was oxidized to DHA. This could now be tested by taking dough into which AA had been mixed, breaking it down, and adding GSH. Any DHA formed would then be reduced to AA. If the disappearing AA was mainly oxidized to DHA, then after addition of GSH most of it should be recoverable as AA. The results of such an experiment, in which AA was added to

TABLE V

ADDITION OF AA TO DOUGH: RECOVERY OF UNCHANGED AA PLUS AA OBTAINED BY ENZYMATIC REDUCTION OF DHA FORMED IN THE DOUGH FROM THE ADDED AA[a]

CONDITION OF DOUGH	AA RECOVERED[b]
	%
After 1 min. mix	100
After 1 min. mix + 90 min. rest	90
After 5 min. mix	91

[a] 48 g. unyeasted dough broken down with 100 ml. McIlvaine's buffer, pH 6.1, for 20 sec. in blender. Aliquot of 50 ml. incubated with 1 ml. GSH (5 mg./ml.) at 20°C. for 10 min., 20 ml. of metaphosphoric acid solution (25%) added, centrifuged, 25-ml. aliquots of supernatant titrated with indophenol (200 mg./liter).
[b] As percentage of quantity added initially.

dough, the dough mixed, and the total AA determined after incubation of the dough with GSH, are shown in Table V.

If these results are taken in conjunction with those given in Table II, it is apparent 1) that in dough AA becomes oxidized and the oxidation product is mainly DHA, and 2) that DHA can enzymatically oxidize GSH when this is present in dough. In low-extraction flour there is little if any GSH naturally present; and if the improving action of DHA is to be ascribed to the oxidation of thiol groups, it is the thiol groups of proteins in the flour which presumably are implicated. It is uncertain, because of the usual specificity of enzyme action, whether the reductase which is involved in the oxidation of GSH will also catalyze the oxidation of protein thiol groups. Observations on this point were made by adding DHA to dough without added GSH, to dough with added GSH, and to dough with added thiolated gelatin — which provides protein thiol groups and which also reduces some flour protein disulfide groups to sulfhydryl. The results of such an experiment are given in Table VI.

TABLE VI

REDUCTION OF DHA IN UNYEASTED DOUGH[a]

SYSTEM	AA FORMED[b]
	%
Dough	
After 1 min. mix	0
After 1 min. mix + 90 min. rest	3.8
Dough containing Thiogel (5 mg./44 g. dough)	
After 1 min. mix	0.8
After 1 min. mix + 90 min. rest	4.6
Dough containing GSH (5 mg./44 g. dough)	
After 1 min. mix	72

[a] 44 g. dough containing 2.4 mg. DHA, mixed and held at 27°C., broken down, for determination of AA, in 100 ml. metaphosphoric acid (12.5%) for 20 sec. in blender. Centrifuged, aliquots of supernatant titrated with indophenol.
[b] As percentage of DHA added initially.

These results confirm that in dough there is very rapid reduction of DHA in the presence of sufficient GSH, but that in a normal dough using a low-extraction flour the reduction measured as above is very small. This is also found when the dough contains added thiolated gelatin. Whether or not this small reduction is sufficient to account for the improving action of DHA involving protein –SH cannot be decided without a more sensitive method of measurement. This is now being investigated.

These results specifically confirm the observations of Kuninori and Matsumoto (5,6) that AA is rapidly oxidized to DHA during dough mixing and that flour contains a DHA reductase which is active at a pH of about 6.0. Their findings are extended by the observations that the DHA reductase is active in dough in the presence of GSH, and that in the absence of GSH there is a slight increase in activity when the protein thiol groups in dough are increased.

Ascorbic Acid and the Oxidation of Unsaturated Fatty Acids. Wilbur *et al.* (11) have shown that AA catalyzes the oxidation of the unsaturated fatty acids linolenic and linoleic, as shown by the development of a colored compound with thiobarbituric acid. In previous work from this laboratory (Moran, Pace, and McDermott, 12), it was shown that oxidation of flour lipids by chlorine dioxide could be detected by the thiobarbituric acid test. If AA or DHA catalyzed the oxidation of flour lipids, then the oxidized fats might be implicated in the improving action. In some preliminary trials using dough with and without added AA and DHA, we have used the thiobarbituric acid test to detect oxidation of unsaturated fatty acids. Under the conditions used, we have not so far found any indication of greater oxidation in the doughs containing AA and DHA than in the control doughs.

Distribution of Reductase Enzyme in Wheat Grain. It was found that the activity of the enzyme involved in DHA reduction could be measured in as little as 10 mg. of flour. This suggested the practicability of investigating its distribution in the various parts of the grain, using the small quantities of material which are obtained by microdissection. The relative activity of DHA reductase in dissected fractions of the wheat grain (var. Cappelle Desprez) is shown below.

Fraction	*Activity* γ *AA/min./10 mg. at 25°C.*
Pericarp + testa	0.6
Aleurone	11.2
Endosperm	14.3
Embryo	70.7
Scutellum	71.0

These figures show that, while the concentration of activity is

highest in the germ, there is also appreciable activity in the endosperm. Indeed, it follows from the figures given above, together with the fact that the endosperm comprises about 82% of the grain, that most of the total activity of the grain is located in the endosperm. This accounts for the relatively high activity found in white flour.

Activity of the Enzyme in Relation to Heat-Damage of Grain. DHA reductase activity is sensitive to heat. For example, wheat at 17% moisture content lost over half its reductase activity when held at 70°C. for 30 min. Since the enzyme is present in the endosperm, heat-damage sufficient to have a deleterious effect on the endosperm proteins, and hence on those of flour, might possibly be indicated by a fall in reductase activity. Because of the farm-drying practiced in a wet season in the United Kingdom which sometimes results in heat-damage to the grain, a quick test for such damage has a useful practical value. Determination of reductase activity can be carried out quickly, and the possibility of using it as a test of heat-damage is now being explored. If it is to be of any value, it is obviously essential first to know the degree of variation in reductase activity in different sound, unheated wheats. This is being examined by determining the activity in samples of wholemeal. Preliminary results indicate that the test will pick out severely overheated samples, but whether it will be adequate for less severely heated samples is still uncertain.

Literature Cited

1. Sullivan, Betty, Howe, Marjorie, Schmalz, F. D., and Astleford, G. R. The action of oxidizing and reducing agents on flour. Cereal Chem. **17:** 507–528 (1940).
2. Mecham, D. K. Effect of sulfhydryl-blocking reagents on the mixing characteristics of doughs. Cereal Chem. **36:** 134–145 (1959).
3. Frater, R., Hird, F. J. R., Moss, H. J., and Yates, J. R. A role for thiol and disulphide groups in determining the rheological properties of dough made from wheaten flour. Nature **186:** 451–454 (1960).
4. Sandstedt, R. M., and Hites, B. D. Ascorbic acid and some related compounds as oxidizing agents in doughs. Cereal Chem. **22:** 161–187 (1945).
5. Kuninori, Toyo, and Matsumoto, H. L-Ascorbic acid oxidizing system in dough and dough improvement. Cereal Chem. **40:** 647–657 (1963).
6. Kuninori, Toyo, and Matsumoto, H. Dehydro-L-ascorbic acid reducing system in flour. Cereal Chem. **41:** 39–46 (1964).
7. Kenyon, J., and Munro, N. Isolation and some properties of dehydroascorbic acid. J. Chem. Soc. **1948:** 158–161.
8. Hinton, J. J. C. The distribution of vitamin B_1 and nitrogen in the wheat grain. Proc. Roy. Soc. **B134:** 418–429 (1947).
9. Hopkins, F. G., and Morgan, E. J. Some relations between ascorbic acid and glutathione. Biochem. J. **30:** 1446–1462 (1936).
10. Crook, E. M., and Morgan, E. J. The reduction of dehydroascorbic acid in plant extracts. Biochem. J. **38:** 10–15 (1944).
11. Wilbur, K. M., Bernheim, F., and Shapiro, O. W. The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acids by various agents. Arch. Biochem. Biophys. **24:** 305–312 (1949).
12. Moran, T., Pace, J., and McDermott, E. E. Interaction of chlorine dioxide with flour: certain chemical aspects. Nature **171:** 103–106 (1953).