LABILE REDUCING MATERIALS FOUND IN WHEAT FLOUR

by

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INTRODUCTION

Small amounts of oxidizing agents exert a marked effect on the physical properties and baking characteristics of a flour dough. As little as .002 percent, based on the weight of flour, of an oxidizing agent such as potassium bromate will cause a fermenting dough to become tough, elastic and less extensible. The volume of the loaf produced from a given weight of such a dough increases to a maximum with increasing amounts of oxidizing agents that may be as high as 25 percent greater than the loaf volume from the unoxidized dough. The amount of oxidation required to produce this maximum loaf volume varies with different flours and has been shown by Larmour (1931), Ofelt (1939) and many others to be a function of protein content and variety.

Small amounts of reducing agents exert an equally great but opposite effect to that of oxidizing agents on the physical properties and baking characteristics of flour doughs. The addition of .1 percent of a substance such as glutathione which has reducing properties has been shown by Jorgensen (1936) to cause a dough to become very extensible and sticky. The volume of the loaf produced from a given weight of dough was 10 percent less than that from a normal untreated dough. The addition of small amounts of cysteine to a dough has the same effect as other reducing agents on its physical properties but does not greatly affect the final loaf volume as is shown by Ofelt and

Larmour (1940). This is probably due to the absorption of this amino acid by the yeast cells.

Numerous theories have been proposed to explain these effects, not any of which, however, have found complete acceptance.

According to the theory advanced by Jorgensen (1936), which will be referred to as the proteolytic theory, the proteinase enzymes of flour are activated by reducing agents and inhibited by oxidizing agents. The action of these activated proteinases in the dough causes a partial degradation of the protein fraction with a resulting increase in extensibility and decrease in elasticity. The addition of oxidizing agents, on the other hand, brings about an inhibition of the proteolytic activity.

This theory is supported by the work of Balls and Hale (1938) who have isolated a proteinase from wheat and have shown it to be of the papain type. It is well known that the enzymes of this type are activated by reducing agents and inhibited by oxidizing agents. The objections to this theory pointed out by Sullivan et al (1940) are that the increase in carboxyl or amino groups on the autolysis of flour are not great enough to indicate any great hydrolysis to amino acids and also that the amount of reductant required is too large to activate the small amount of proteinase present. Bungenberg de Jong (1938) expressed the belief that the action of reducing agents is entirely too rapid to be accounted for on the basis of enzymic hydrolysis of the protein. Another weakness of this theory is that

to this time there has been offered no good explanation of why the coherence of a dough should increase in the presence of oxidizing agents.

A second theory to explain these effects might be called the protein oxidation theory. According to this theory the changes in the physical properties of dough on oxidation or reduction are due to the direct oxidation and reduction of the sulfhydryl groups of the protein molecules. Reduction of these groups would effect a depolymerization of the protein structure and a resulting decrease in its coherence. Oxidation, on the other hand, would lead to a greater degree of polymerization of the protein and an increased coherence of the dough mass. Sullivan et al (1940) expressed belief in a theory of this type.

While this theory holds promise of satisfactorily explaining the observed phenomena, it is not supported by any adequate body of facts. As expressed by Sullivan et al (1940), "The explanation of the action of certain chemicals in improving the baking quality of flour hinges on the proof that SH or some similar reducing group is present in the dough."

Balls and Hale (1940) were the first to isolate a sulfhydryl bearing constituent from the petroleum ether extract of flour. The true nature of this substance is still uncertain as is also the role it might play in dough oxidation and reduction.

The theory that oxidation effects were primarily due to the effect on the phosphatides has been expressed by Working (1928) and by Bungenberg de Jong (1938).

In consideration of the facts it seems a reasonable premise that there is present in flour at least one reversible redox system that is of primary importance in dough oxidation and reduction.

Numerous workers have presented evidence showing that the lipid fraction of wheat flour is affected by oxidizing agents. Sullivan et al (1940) have presented evidence that the chemical composition of the lipid from fermented doughs is altered when oxidizing agents have been added. They do not, however, believe that the improving effect of oxidizing agents is due primarily to their effects on the lipids. Ofelt and Larmour (1940) have presented data indicating that the ether extraction of a Tenmarq flour resulted in the elimination of the bromate response and that after the same treatment a sample of Chiefkan flour retained a definite bromate response but that the optimum bromate level was somewhat decreased. These facts would suggest the existence of possibly even two oxidizable substances in flour, one of which was removed by ether extraction.

This work was, therefore, undertaken to establish the existence or absence of reducing material in various fractions from wheat flour which might be important in explaining the primary oxidation of flour doughs. Special emphasis was laid on the demonstration of reducing groups in the protein fractions for the purpose of obtaining evidence either supporting or contradicting the protein oxidation theories.

In surveying the literature of biological oxidations it soon became apparent that the most reactive reducing biologicals,

or conversely, those most readily oxidized, are sulfhydryl containing compounds, the phosphatides, ascorbic acid, and the fatty acids.

Hopkins (1925) in his work on glutathione showed that in an acid medium of pH 3-4 the sulfhydryl compounds act as oxygen carriers for the oxidation of fatty acids but are not themselves oxidized until the fatty acid oxidation has continued to a considerable extent. At pH 7.4-7.6, however, the sulfhydryl compounds are oxidized immediately by oxygen with only a slight oxidation of the fatty acids.

Meyerhof (1923) has shown that the oxidation of lecithin in the presence of sulfhydryl compounds is very similar to that of fatty acids but that the oxidation of even such unsaturated glycerides as linseed oil in the presence of sulfhydryl compounds is preceded by an induction period. In all cases, however, the rate of oxidation of fatty acids and glycerides was increased by the presence of sulfhydryl containing compounds.

Hopkins and Morgan (1936) and Szent-Györgyi (1928) have shown that in the presence of ascorbic acid, glutathione will first be oxidized followed by the oxidation of the ascorbic acid. Likewise, if dehydro-ascorbic acid is added to glutathione, the former will be reduced to ascorbic acid while the latter will be oxidized to the disulfide form.

These facts would indicate that any free sulfhydryl bearing compounds present in flour would certainly be readily oxidizable or would be of importance in explaining the oxidation of such substances as the phosphatides and fatty acids. The fact that

dehydro-ascorbic acid, an extremely weak oxidizing agent, is able to effect an improvement in loaf volume, comparable on a weight-for-weight basis to potassium bromate, as shown by the work of Melville and Shattock (1938) seems to indicate that the important oxidizable compounds of flour are comparable in reactivity to the sulfhydryl bearing compounds.

These considerations, therefore, narrowed the scope of this work to an investigation of the existence or absence of sulfhydryl groups, or of groups with comparable reducing reactivity, in the various fractions of flour in general and in the protein fractions in particular.

METHODS

The time-honored and most specific qualitative test for the indication of the presence of free sulfhydryl groups is the nitroprusside test. This test is made by adding three to five drops of freshly prepared five percent sodium nitroprusside to a few drops of the solution in question followed by three to five drops of ammonium hydroxide. The presence of ammonium ions increases the intensity of the color. The solution turns pink or red in the presence of sulfhydryl bearing compounds but a yellow color develops in the absence of these groups. The pink color fades to yellow quite rapidly in the presence of small amounts of sulfhydryl compounds and some judgment is required in determining whether the color is not due to the original pink of the reagent. The nitroprusside test is positive with all sulfhydryl compounds known but not with hydrogen sulfide. A positive

test is also obtained in the presence of acetone, and the work of this investigation led to the belief that it was not wholly reliable in alcoholic solutions.

Because of the lack of sensitivity of the nitroprusside test and because of the inability to use it as a quantitative test, a second method for the qualitative identification and quantitative determination of sulfhydryl groups was adapted.

This test is based on the fact that, according to Anson (1939b), cysteine is the only amino acid which is known to give a definite stoichiometric reaction with ferricyanide or which is known to react with dilute ferricyanide at all. It has also been shown that in dealing with proteins, ferricyanide in dilute neutral buffered solution reacts completely, immediately, stoichiometrically and solely with the sulfhydryl groups of the protein molecule. A complete discussion and proof of these statements are given by Mirsky and Anson (1936) and by the outstanding work of Anson (1939a)(1939b)(1940)(1941).

The ferricyanide method has the advantage of being extremely sensitive. Its specificity when dealing with purified protein preparations is highly desirable. Its lack of specificity when dealing with non-protein preparations is also desirable in that it offers a delicate qualitative test for the presence of reactive reducing materials other than, but comparable in reactivity to, the sulfhydryl groups.

The reagents used in this determination are as follows:

Duponol PC. A 10 percent solution was prepared and after standing several days was filtered and stored at room tempera-

tures.

Phosphate buffer. A buffer solution of pH 6.8 was prepared by mixing equal parts by volume of one molar sodium dihydrogen phosphate with one molar sodium monohydrogen phosphate.

Ferric sulfate reagent. This reagent was prepared by the directions of Folin and Malmros (1929) with the exception that gum arabic was used in place of gum ghatti as a protective colloid. According to these authors, the keeping quality of the reagent is enhanced by the use of gum ghatti. No decomposition was encountered during the several months when this work was being carried on. Twenty g of gum arabic were dissolved in 1000 cc of distilled water and the solution was filtered. A solution of 5.0 g of annhydrous ferric sulfate in 75 cc of 85 percent phosphoric acid plus 100 cc of distilled water was then added to the gum arabic solution. One percent potassium permanganate solution was then added in small increments to this mixture to oxidize any organic reducing materials present in the gum arabic. About 15 cc of permanganate solution were required and the reaction was judged to be complete when the addition of one drop gave a color to the solution which persisted for 10 minutes. This solution was very turbid but cleared completely after standing two weeks.

2.0 N sulfuric acid solution.

.01 molar potassium ferricyanide solution stored in a dark bottle.

.001 molar cysteine hydrochloride solution prepared fresh

when required.

.001 molar potassium ferrocyanide solution prepared fresh when required.

The ferricyanide test was carried out quantitatively by the following procedure. To 2.0 cc of the solution being tested were added .5 cc of the phosphate buffer solution, .5 cc of the 10 percent Duponol PC solution and 1.0 cc of the .01 molar ferricyanide solution. The buffer solution established the pH at which the oxidation took place at 6.8. The Duponol PC denatured the protein thus rendering the bound sulfhydryl groups reactive and also preventing the precipitation of the protein by the buffer solution or the other reagents added later. In applying this procedure as a qualitative test for the presence of free sulfhydryl groups the Duponol PC was omitted. The ferricyanide effected the oxidation of the sulfhydryl groups to the disulfide form.

After the above mixture had stood for five minutes, 1.0 cc of 2.0 N sulfuric acid solution was added, followed by .5 cc of the ferric sulfate reagent. The sulfuric acid halted the reaction and prevented it from proceeding past the oxidation of the sulfhydryl groups. The ferric sulfate reacted with the potassium ferrocyanide formed by the oxidation to form Prussian blue. The gum arabic acted as a protective colloid and prevented the precipitation of the Prussian blue.

Twenty minutes were allowed for the color to develop after which the solution was diluted to 100 cc to stop the color development. A blank solution was prepared in exactly the same

manner with the exception that the addition of the ferric sulfate reagent is omitted.

The light transmission of the colored solution was then compared with the blank which is taken as unity. A Wilkens-Anderson KWSZ photometer was used for the comparisons in this work. To eliminate the effect of the yellow color in this comparison, a red filter with a maximum transmission at .650 microns was used in the light source. The decrease in light transmission was then due to the light absorbed by the Prussian blue present. A discussion of this colorimetric estimation was presented by Folin and Malmros (1929).

EXPERIMENTAL DATA

Because of the importance of the gluten fraction in the dough structure, an investigation was first made to establish the presence or absence of reducing material there.

The Gluten Proteins

Twenty-five g of an unbleached, straight grade Blackhull flour were mixed with 15.0 cc of distilled water. The gluten was washed from this dough under running tap water, cut into fine pieces and dispersed completely in 200 cc of .005 N acetic acid. To this dispersion were then added 10 cc of the phosphate buffer described above. The gluten flocculated and was collected on a stirring rod by stirring the solution. The solution from which the gluten had been removed did not show any appreciable reducing power when tested by the ferricyanide procedure

described. Considerable foam had collected on the surface of this solution, however, and when tested by this method showed the presence of reducing material both in the presence and absence of Duponol PC reagent. The nitroprusside test was so weak as to be uncertain.

The gluten was again dispersed in 200 cc of .005 N acetic acid solution and precipitated by the addition of 10 cc of phosphate buffer. Very little foam collected at the surface of this solution after the precipitation of the protein and it did not show any reducing power. The gluten was collected and again dispersed in 200 cc of .005 N acetic acid.

When this purified gluten dispersion was tested for reducing power with ferricyanide, a negative response was obtained both in the presence and absence of Duponol PC reagent. A negative nitroprusside test was obtained with this dispersion both before and after boiling. The boiling treatment was intended to denature the protein and thus render any bound sulfhydryl groups reactive. Duponol PC is unsatisfactory as a denaturing agent in conjunction with the nitroprusside test since it depresses the color formation.

The work of Sullivan et al (1940) has shown that if a solution containing 2,6-dichlorophenol-indophenol is boiled in the presence of gluten, it will eventually be decolorized, indicating a reducing action by the gluten. When the above gluten dispersion was boiled in the presence of ferricyanide in buffered solution at pH 6.8 and in the presence of Duponol PC, the reduction of part of the ferricyanide to ferrocyanide was indicated

by a color formation of Prussian blue.

To determine if this reducing power at boiling temperatures was due to the reactions of sulfhydryl groups or of other protein groups, a series of quantitative estimations was made of the reducing power of gluten as compared with the reducing power under identical conditions of egg albumin of known sulfhydryl content.

Egg albumin was prepared by dissolving the whites of three eggs in an equal volume of water. This solution was made one-half saturated with ammonium sulfate by adding an equal volume of saturated ammonium sulfate solution and the precipitated globulins were separated by filtration. Saturated ammonium sulfate was added to the filtrate to the point of incipient turbidity. .2 N sulfuric acid was then added with rapid stirring to the point where the precipitate formed by the addition of acid could not be quite completely dissolved with continued stirring. Sufficient saturated ammonium sulfate solution was added to bring the solution to three-fourths saturation. After the precipitate was allowed to come to equilibrium with the mother liquor by standing for 12 hours in the cold the suspension was centrifuged and the supernatant liquid discarded. The precipitated albumin was washed with three-fourths saturated ammonium sulfate, dissolved in distilled water and filtered. When this albumin had been twice more precipitated from three-fourths saturated ammonium sulfate, centrifuged, washed, redissolved and filtered it was redissolved and dialyzed in a collodion bag against distilled water until no barium sulfate precipitate was obtained when the dialysate was treated with barium chloride.

This preparation of egg albumin is in accord with the classical method of Sorenson with the exception that no attempt was made to obtain a crystalline product. The albumin concentration was determined by the Kjeldahl method to be 20.8 mg per cc. This solution was stored in the cold and was preserved further by the addition of a drop of toluol.

A calibration curve of the light absorbed in the presence of given amounts of Prussian blue was prepared by adding various increments of potassium ferrocyanide to the reagents. The fraction of light transmitted through the colored solution when compared to that transmitted through the blank solution is designated as I/I_o. The optical density of the solution is designated as log I_o/I. The values of I/I_o and log I_o/I with various concentrations of ferrocyanide are shown in Table 1. These data are shown graphically in Plate I.

Table 1. The fraction of the incident light transmitted (I/I_o) through a solution containing the Prussian blue formed from various amounts of potassium ferrocyanide.

cc of .001 M $K_4Fe(CN)_6$*	I/I_o**	Log I_o/I
0.00	1.000	.000
0.50	.844	.074
1.00	.736	.133
1.50	.589	.230
2.00	.488	.312

* After formation of Prussian blue, the solution was diluted to 100 cc.

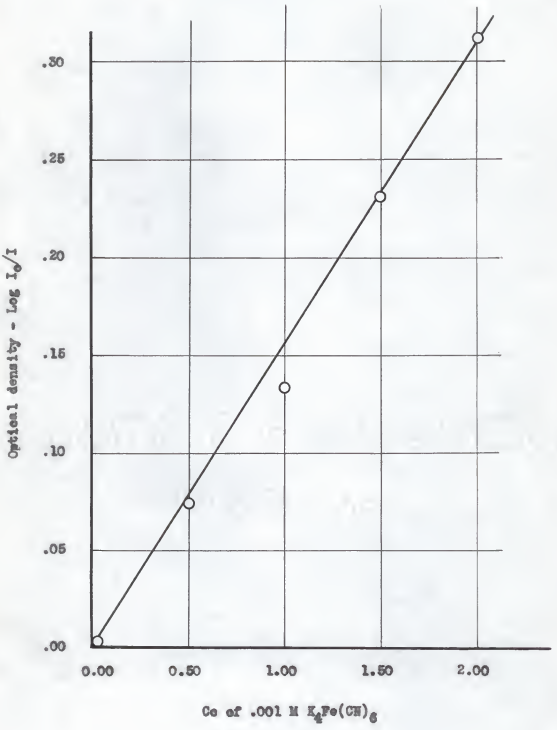
** Average of duplicate determinations.

When the log I_o/I is plotted against the concentration of ferrocyanide, an approximately straight line is obtained. This

EXPLANATION OF PLATE I

The relation between the optical density (Log I_o/I) and the amount of potassium ferrocyanide added to the solution.

PLATE I


.30
.25
.20
.15
.10
.05
.00
Optical density - Log I_o/I
0.00
0.50
1.00
1.50
2.00
Cc of .001 M $K_4Fe(CN)_6$

indicates that the absorption follows Beer's law, which is:

$$I = I_o e^{-klc}$$

where I_o is the intensity of the light striking the absorbing medium, I is intensity after passing through the distance l, c is the concentration of the absorbing material, and k is a constant which is characteristic of the dissolved material and of the wave length. Therefore, since both k and l are constant for this technique,

$$\text{Log } I_o/I = k'c$$

which is the equation of a straight line passing through the origin.

The amounts of Prussian blue formed from the ferrocyanide produced by the oxidation of known amounts of cysteine hydrochloride by ferricyanide was also determined and is given in Table 2. From these data it is evident that the reaction between ferricyanide and cysteine is equimolecular and quantitative and is represented by the equation,

$$2\ GSH + 2\ K_3Fe(CN)_6 \rightarrow GSSG + 2\ K_4Fe(CN)_6\ .$$

Table 2. A comparison of the amounts of ferrocyanide formed during oxidation of cysteine with the amounts of cysteine added to reaction.

cc of .001 M Cysteine-HCL *	I/I_o **	Log I_o/I	Equivalent cc of .001 M $K_4Fe(CN)_6$	Ratio of Cysteine to $K_4Fe(CN)_6$
1.00	.735	.133	1.00	1.00
1.50	.601	.220	1.47	1.02
2.00	.480	.318	2.06	0.97
			Average	1.00

* After formation of Prussian blue, the solution was diluted to 100 cc.

** Average of duplicate determinations.

Being thus satisfied as to the accuracy of the method, the sulfhydryl groups of denatured egg albumin were determined. The values expressed as percent of cysteine in the molecule are given in Table 3.

Table 3. The sulfhydryl content of egg albumin expressed as percent of cysteine.

cc of albumin*	mgm of albumin	I/I_o**	Log I_o/I	Cysteine equivalent: Millimoles	Mgm	% cysteine in albumin
1.00	20.8	.723	.140	.00110	.133	0.64
1.50	31.2	.582	.235	.00155	.185	0.60
2.00	41.6	.417	.265	.00225	.264	0.63
					Average	0.62

* After formation of Prussian blue, the solution was diluted to 100 cc.

** Average of duplicate determinations.

The value of .62 percent cysteine in albumin is in good agreement with the values determined by other workers using various oxidizing agents as reported by Kuhn and Desnuelle (1938) and by Greenstein (1938).

Worker	Oxidizing Agent	Percent Cysteine
Todrick and Walker	Dichlorophenol-indophenol	.63
Mirsky and Anson	Cystine	.55-.62
Kuhn and Desnuelle	Porphyrindin	.58
Greenstein	Porphyrindin	.50

After obtaining this information a series of determinations were made in which the oxidation of the denatured albumin by ferricyanide was allowed to proceed under the same conditions which showed reducing power with gluten proteins, namely, at boiling temperatures for extended periods of time. The amount of ferricyanide reduced is indicated in the "apparent percent of

Table 4. The "apparent cysteine content" of egg albumin from oxidation with ferricyanide at 100° C for various periods of time.

cc of albumin *	Time of heating	Mgm of albumin	I/I_o **	Log I_o/I	Cysteine equivalent: Millimoles	Cysteine equivalent: Mgm	"Apparent % cysteine"
1.00	0 min.	20.8	.723	.140	.00110	.133	0.62
1.00	20 min.	20.8	.620	.207	.00140	.170	0.82
1.00	40 min.	20.8	.593	.228	.00155	.188	0.90

* After formation of Prussian blue, the solution was diluted to 100 cc.
** Average of duplicate determinations.

Table 5. The "apparent cysteine content" of gluten proteins from oxidation with ferricyanide at 100° C for various periods of time.

cc of gluten *	Time of heating	Mgm of gluten	I/I_o **	Log I_o/I	Cysteine equivalent: Millimoles	Cysteine equivalent: Mgm	"Apparent % cysteine"
1.00	0 min.	8.9	1.000	.000	.00000	.000	0.00
1.00	60 min.	8.9	.913	.037	.00030	.036	0.41
1.00	75 min.	8.9	.869	.061	.00045	.055	0.61
2.00	75 min.	17.8	.753	.124	.00090	.109	0.61
2.00	105 min.	17.8	.668	.176	.00125	.151	0.85

* After formation of Prussian blue, the solution was diluted to 100 cc.
** Average of duplicate determinations.

cysteine" in albumin. The results of these determinations are given in Table 4.

Another series of determinations were then made with the purified gluten dispersion under similar conditions. The results of this series are given in Table 5.

From the fact that the purified gluten dispersion did not give a positive nitroprusside test and from the fact that it did not reduce dilute neutral buffered ferricyanide in the absence of Duponol PC reagent, it is concluded that the gluten proteins do not possess any free sulfhydryl groups.

Since the gluten dispersion did not give a positive nitroprusside after boiling and did not reduce ferricyanide in the presence of Duponol PC it seemed likely that there were no bound sulfhydryl groups in these proteins. That the reducing power exhibited by the gluten proteins at high temperatures was not due to the liberation of sulfhydryl groups is indicated by the facts that under these conditions, other protein groups than the sulfhydryl of albumin become reactive, and that the reduction is not stoichiometric but varies with the time of oxidation and is otherwise uncharacteristic of the reactions of the sulfhydryl groups. It seems a reasonable conclusion, therefore, that there exist neither free nor bound sulfhydryl groups in the gluten proteins.

It is also indicated that there is some reducing matter in crude gluten that is removed on purification.

The Soluble Proteins

One hundred g of unbleached, straight grade Blackhull flour were dispersed in 200 cc of distilled water and digested with frequent shaking for one hour. The suspension was then centrifuged to remove the gluten and starch. Both in the presence and absence of Duponol PC, the supernatant liquid showed the presence of reducing material when tested by the ferricyanide technique. The nitroprusside test was uncertain. The 125 cc of supernatant were saturated by the addition of 80 g of solid ammonium sulfate and the proteins which were salted out were separated by filtration. Both the filtrate and precipitate gave a positive test with ferricyanide. The precipitate gave a faint but definite test with nitroprusside without preliminary denaturation of the proteins. This precipitate, which consisted chiefly of albumins and globulins, was dispersed in distilled water, reprecipitated from saturated ammonium sulfate, redispersed, reprecipitated and redispersed. To remove the ammonium sulfate, the purified dispersion was electrodialyzed against parchment membranes.

This purified dispersion of soluble proteins gave a positive test for reducing power in the presence and absence of Duponol PC. It also gave a faint positive test with nitroprusside. From these facts it was concluded that this dispersion probably contained free sulfhydryl groups.

These proteins were precipitated by the addition of an equal volume of 95 percent alcohol, filtered and the precipitate

washed with absolute alcohol and ether. When this precipitate was redispersed in water it failed to give a positive test with ferricyanide or with nitroprusside. The above filtrate was evaporated to dryness under vacuum. The residue consisted of only a trace of oily material. This material gave a positive test with ferricyanide and with nitroprusside.

To determine the nature of this material more exactly, 1200 g of freshly milled, unbleached straight grade flour from a commercial mixture of wheat were dispersed in 2400 cc of distilled water. This dispersion was digested for one hour with frequent shaking and then centrifuged. The supernatant liquid was saturated with ammonium sulfate and the precipitated proteins filtered. The precipitate was extracted with 95 percent alcohol, absolute alcohol and ether and the combined washings evaporated under vacuum to dryness. The fatty residue was taken up with absolute alcohol. A small amount of white gelatinous material from this residue which was insoluble in absolute alcohol and ether gave a negative test with ferricyanide and nitroprusside. The alcohol-ether solution of the lipid material was evaporated to dryness under vacuum. Approximately 2 g of this lipid were obtained. This material gave a positive test for reduction with ferricyanide and with nitroprusside, indicating the presence of free sulfhydryl groups. It is probable that this reactive material is the same as that described by Balls and Hale (1940) and Balls, Hale and Harris (1942).

To ascertain if this material might be especially rich in phosphatide content or was representative of the whole flour fat, the nitrogen content was determined and the phosphatide

content estimated by the amount of material precipitated with acetone. The values obtained are shown in Table 6 along with the values reported by Sullivan et al (1940) for both flour fat and for the phosphatide fraction. From these comparisons it is evident that this material isolated was merely a representative fraction of the fat that was removed from flour by water extraction and was carried down with the flocculated proteins that were salted out.

Table 6. Analysis of fatty material precipitated with the soluble proteins.

Determination	Precipitated fat	Values from Sullivan	
		Flour fat	Phosphatide
Kjeldahl % N	1.65	1.65	4.28
Precipitated from acetone %	20.4	25.0	100.

To verify the absence of reducing material other than that due to the lipid material in the soluble proteins, 50 g of alcohol-ether extracted, unbleached Blackhull flour were dispersed in 100 cc of water, digested for one hour and centrifuged. The supernatant liquid was saturated with ammonium sulfate and the precipitated proteins were filtered and redispersed in distilled water. A negative test for reducing matter was obtained with the ferricyanide test. The nitroprusside test was also negative.

From the fact that the alcohol-ether extracted soluble proteins and also the soluble proteins prepared from alcohol-ether extracted flour did not give a positive nitroprusside test and

did not reduce ferricyanide under normal conditions, it was concluded that the major soluble protein fractions of wheat flour, namely, the albumins and globulins, do not contain any reactive sulfhydryl groups.

It was also shown that the crude preparation of soluble proteins contained a small amount of reducing material which was due to the presence of fatty material. This reducing material gave a positive nitroprusside test for free sulfhydryl groups and is probably identical with the lipoprotein of Balls, Hale and Harris (1942).

The Lipids

In view of the work done on the soluble proteins, it seemed a foregone conclusion that there existed a material in the lipid fraction of flour which carried free reducing groups, probably sulfhydryl. Since it was felt that the product obtained by Balls and Hale (1940) was a hydrolytic derivative of a more complex molecule, an attempt was made to isolate this sulfhydryl bearing substance in its native and reduced state. These attempts only substantiated the previous conclusions of those authors that this material possessed solubilities similar to those of the phosphatides, and even more, was in all probability a peptide containing phosphatide. A late article by Balls, Hale and Harris (1942) also expressed this view.

It was found, however, that considerably greater quantities of this material seemed to be extracted by alcohol-ether treatment than by ether or petroleum ether extraction alone.

The Non-protein Water Soluble Material

In the work on the soluble proteins it was mentioned that the filtrate from which the albumins and globulins were precipitated by saturated ammonium sulfate possessed the ability to reduce ferricyanide. One hundred g of alcohol-ether extracted flour were dispersed with 200 cc of distilled water. After one hour of digestion with frequent shaking, the suspension was centrifuged and the supernatant was concentrated under vacuum to one-fifth its original volume. This concentrate gave a strong positive test for reducing matter with ferricyanide in the absence of Duponol PC. The test with nitroprusside was only faintly positive, not nearly comparable in strength to the strong test with ferricyanide.

The concentrated extract was treated with three volumes of 95 percent alcohol, the flocculated proteins were filtered. After one more precipitation these proteins gave a negative test for reducing power with ferricyanide in the absence of Duponol PC. When the filtrate had been concentrated nearly to dryness under vacuum, it was taken up in a small volume of absolute alcohol and concentrated again. After this process had been repeated twice more, the alcohol soluble material was decanted and evaporated to dryness under vacuum. This nearly colorless, gum-like material gave a negative test for reducing matter with ferricyanide and a negative nitroprusside test. The alcohol insoluble material, which comprised the main bulk of the non-

protein water soluble matter, gave a fairly strong positive test for reducing power with ferricyanide but only a faint nitroprusside reaction. This material was brown in color and of a very gummy nature.

From this work it was concluded that there probably existed a small amount of some sulfhydryl bearing substance in this fraction. There is also present a considerable amount of a reducing substance whose reactivity is probably not due to, but is comparable to, that of sulfhydryl compounds.

Baking Tests

To study the effect of the various fractions investigated on the bromate response during baking, the following work was undertaken.

One thousand g of dried freshly milled flour from a commercial wheat mixture (Milling Dept. No. 2014) were digested for two hours with 1500 cc of 95 percent alcohol. The supernatant was decanted and the flour extracted six times with 250 cc and twice with 500 cc portions of petroleum ether. The lipid extract was evaporated under vacuum to dryness. The residue was taken up with a small quantity of ether, filtered and the filtrate evaporated to dryness under vacuum. One-half of this lipid was taken up with a small amount of petroleum ether and the majority of the phosphatides precipitated by the addition of five volumes of cold acetone. For convenience this fraction is hereinafter referred to as the phosphatide fraction and the residue as the phosphatide-free fat fraction, although much phosphatide would

remain after a single precipitation with acetone. The phosphatide fraction, the phosphatide-free fat fraction and the untreated lipid material were placed under a high vacuum until ready for baking. Because of the possible interaction between acetone and the sulfhydryl compounds, little importance can be assigned without greater investigation to the results from fractions which have been treated with this solvent.

The alcohol-petroleum ether extracted flour from above was digested for one hour with 2000 cc of distilled water and centrifuged. The supernatant was concentrated under vacuum to 250 cc. One thousand cc of 95 percent alcohol were added and the precipitated proteins were separated by filtration. The precipitate was twice taken up in 100 cc of distilled water and reprecipitated by the addition of 300 cc of 95 percent alcohol. The combined filtrates were concentrated under vacuum to 100 g. Twenty g of this material were added to each loaf in which this variable was being studied. Unfortunately the techniques developed and the equipment available were such that several days were required in the preparation of such large amounts of this material, making it quite possible that some oxidation or decomposition had taken place.

The flour used in this baking was the same as that from which the above fractions were prepared. An equivalent amount of each fraction was superimposed on the baking formula as was removed by the extractions. Thus, for example, in the fat tests, a bromate series was baked in which no addition was made to the flour and another series in which the fat extracted from

100 g of flour was added to the dough made from 100 g of flour. In this manner, the effect of doubling the amount of fat on the bromate series was determined.

The formula used in this baking is as follows:

Flour	100.0 g
Salt	1.5 g
Sugar	6.0 g
Malt	.25 g
Yeast	2.0 g
Shortening	3.0 g
Water	65.0 g
Dry milk	4.0 g
$KBrO_3$	As indicated

The dough was mixed for 3 ½ minutes, which gave the optimum consistency, at 100 r.p.m. in a Swanson-Working type mixer. The doughs were fermented at 30° C for three hours. They were punched 102 minutes and 154 minutes after mixing by passing through a National Manufacturing Company Sheeting roll. The doughs were molded with a Thompson Laboratory Moulder, panned, proofed for 55 minutes at 30° C, and baked for 25 minutes at 250° C in a Despatch revolving hearth oven. The loaves were weighed immediately after removal from the oven and the volume measured. The loaves were cut, photographed and graded the following day. The baking data are given in Table 7 and are arranged graphically in Plate II. The photographs of these loaves are shown in Plates III and IV.

Inasmuch as the baking values represent only single loaves, it is impossible to draw extensive conclusions from them. The assumptions necessary to the interpretation of the results would not be warranted without considerable investigation. There seems to be some evidence, however, indicating that the alcohol-petro-

leum ether extract of flour is responsible for at least a part of the oxidation response during baking.

Table 7. Baking data of loaves containing various fractions isolated from wheat flour.

Loaf no.	Sample	Mg of $KBrO_3$	Weight of loaf (g)	Loaf vol. (cc)	Grain-texture	Crumb color
1	Control	0	153.5	725	90	90
2		3	153.0	860	95	95
3		6	153.3	855	90	95
4		9	153.7	800	85	90
5	Control + whole fat	0	157.5	650	90	75 cr***
6		3	155.2	770	95	80 cr
7		6	154.8	830	100	85 cr
8		9	154.4	870	95	85 cr
9	Control + phosphatide	0	154.7	765	90	90
10		3	154.3	860	95	95
11		6	153.9	865	90	95
12		9	154.4	850	85	85
13	Control + phosphatide-free fat	0	156.0	670	85	75 cr
14		3	154.3	810	90	80 cr
15		6	154.4	845	95	85 cr
16		9	153.7	850	90	85 cr
17	Control + non-protein water soluble matter	0	159.3	695	90	90
18		3	159.2	795	95	95
19		6	159.8	805	95	95
20		9	161.4	750	90	95

*** cr = creamy

It is of interest to note that the doughs containing added whole fat and added phosphatide-free fat exhibited gas bubbles on the surface of the fermenting doughs. This phenomenon is also generally observed in doughs of Chiefkan and other flours which have a high bromate requirement. The doughs containing added phosphatide, on the other hand, were even smoother in feel and appearance than the control doughs.

EXPLANATION OF PLATE II

Fig. 1. The effect of adding whole flour fat on the bromate response of a flour.

Fig. 2. The effect of adding the phosphatide fraction on the bromate response of a flour.

Fig. 3. The effect of adding the phosphatide-free fat fraction on the bromate response of a flour.

Fig. 4. The effect of adding non-protein water soluble material on the bromate response of a flour.

PLATE II

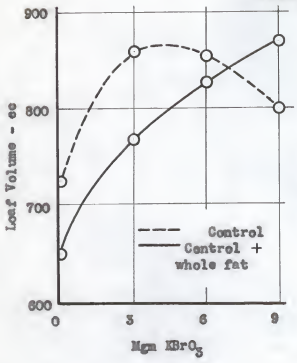


Fig. 1

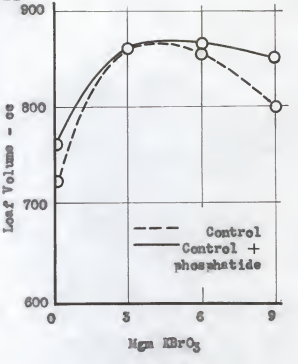


Fig. 2

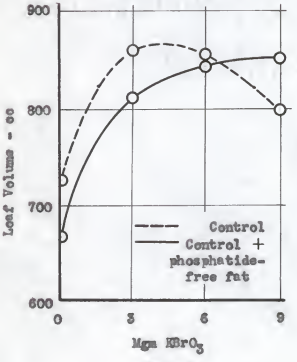


Fig. 3

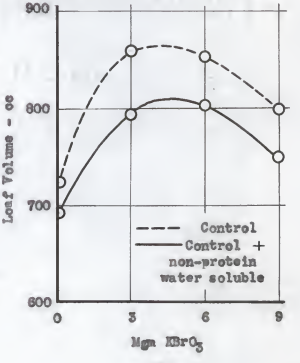


Fig. 4

EXPLANATION OF PLATE III

Loaf No.		Mg. $KBrO_3$
5	Control + whole fat	0
6		3
7		6
8		9
1	Control	0
2		3
3		6
4		9
9	Control + phosphatide	0
10		3
11		6
12		9

PLATE III

EXPLANATION OF PLATE IV

Loaf No.		Mg. $KBrO_3$
13	Control + phosphatide-free fat	0
14		3
15		6
16		9
1	Control	0
2		3
3		6
4		9
17	Control + non-protein water soluble material	0
18		3
19		6
20		9

PLATE IV

The only explanation which can be offered for the decrease in loaf volume at the optimum bromate level of loaves containing added non-protein water soluble material is that this fraction had become oxidized or partially decomposed as suggested above.

SUMMARY AND CONCLUSIONS

This investigation was undertaken to demonstrate the presence or absence of labile reducing groups in the protein fractions of wheat flour whose oxidation would explain commonly observed phenomena or to demonstrate the presence of other reactive reducing matter which might be important in this respect. Various fractions which were separated from wheat flour were investigated for reducing power, with especial emphasis being laid on the demonstration of the presence of free sulfhydryl groups.

As a result of this work, the following conclusions were drawn:

1. No free or bound sulfhydryl groups in the gluten proteins could be demonstrated by this work.

2. The reducing effects of gluten at high temperatures were found to be due probably to the reactivity of groups other than the sulfhydryl.

3. Free sulfhydryl or other reducing groups in the albumins and globulins of wheat flour could not be demonstrated in this study.

4. The presence of free sulfhydryl groups in the lipid

fraction of wheat flour was indicated as highly probable.

5. It was shown that free sulfhydryl groups probably exist in the non-protein water soluble material of wheat flour.

6. It seems likely that there is also present in the non-protein water soluble material of flour a reducing substance whose reactivity is not due to the presence of sulfhydryl groups. The reactivity of this material, however, is comparable to that of those groups.

7. Baking data indicate the possibility that the alcohol-petroleum ether extractable lipid fraction of wheat flour is responsible for at least a part of the response to oxidation during baking.

The assumption has been made in the protein oxidation theory of dough oxidation and reduction that free sulfhydryl groups or other reactive reducing groups exist in the protein molecules of the dough. None of the evidence presented in this work would tend to substantiate this assumption, but rather, would tend to discredit it.

The fact that the most reactive reducing materials were found in the lipid fraction and in the non-protein water soluble fraction would seem to offer support to those theories which postulate that the effects of oxidation in doughs is due to secondary surface effects resulting from the primary oxidation of surface reactive agents.

The work of Hopkins (1925) and of Meyerhof (1923) as cited has shown the possible reactivity of the fatty acids and phosphatides in any oxidation process. The later work of Tait and

King (1936) has shown that at pH 3.5, lecithin is oxidized aerobically in the presence of sulfhydryl compounds more rapidly than fatty acids or glycerides, or even the hydrolytic products of lecithin. The latter workers have further shown that at pH 8.4, oxidized glutathione is able to transfer hydrogen from lecithin to o-cresol indophenol and effect its reduction anaerobically.

A consideration of these facts leads to the formulation of the hypothesis that the role of the sulfhydryl groups of flour, and especially those contained in the lipid fraction, in dough oxidation might be primarily that of hydrogen carriers for the oxidation of the phosphatides and unsaturated fatty acids. The alteration of the properties of the phosphatides and the unsaturated fatty acids by oxidation is proposed to account for at least a part of the change in the physical properties and baking characteristics of doughs after oxidation.

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