gallol and other phenolic antioxidants, which were effective for carotene in oil solution but relatively ineffective in alfalfa meal (*2, 3*). A notable exception is 6-hydroxy-2,2,4-trimethyl-1,2-dihydroquinoline, which, although the most effective compound tested in mineral oil solution, was only moderately effective in the meal (compound 40). As has been pointed out (*2, 3*), if a compound is to be capable of preventing carotene loss in a complex substance such as alfalfa meal, it must not only be an effective antioxidant, but must also satisfy a number of other conditions, including solubility in that phase which it is designed to protect. The oily nature of the alkoxy derivatives seems to facilitate their passage through the meal to the site of the carotene. The hydroxy derivative, on the other hand, is a crystalline solid.

The sensitive positions on the quinoline ring were shown to be the 2-, 4-, and 6-positions. Furthermore, the specificity at these positions was very pronounced. Thus, alkyl substitution of all three positions enhanced antioxidant activity, and alkoxy substitution on the 6-position was even more effective than alkyl substitution.

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GARVAN MEDAL ADDRESS

PROTEINS IN FLOUR

Review of the Physical Characteristics of Gluten and Reactive Groups Involved in Change in Oxidation

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The present state of our knowledge of the proteins of flour is reviewed. The reactive groups of gluten involved in the oxidation and reduction of flour are, so far as is known, the sulfur-containing amino acids, cysteine, cystine, and methionine. Maturing agents exert their beneficial effect by action on free and combined mercapto groups of the protein, but the mechanism of their action is still unsolved.

WHEAT FLOURS vary widely in their baking characteristics and in their response to oxidation. These variations are due, in large measure, to the amount and the physical properties of the proteins of the flour.

Osborne (*27*), in his classical work on the wheat proteins, separated the proteins of flour into five main fractions based on solubility: gliadin, a prolamine soluble in 70% ethyl alcohol; glutenin, soluble in dilute acid and dilute alkali; a neutral salt-soluble globulin; a water-soluble, heat-coagulable albumin; and an ill-defined "proteose." The classification of these proteins on the basis of solubility behavior has not proved very satisfactory. Gortner (*18, 19*), Blish (*8–10*), McCalla (*23–25*), Rich (*33*), and others have shown that, in the separation of wheat flour glutens, one is not dealing with true solubility effects, but rather with a complex mixture of components which possess different degrees of peptizability with varied ionic environment. The globulin fraction of the wheat flour proteins has since been shown to be made up of three individual components (*12, 30*) and the albumin fraction to consist of at least six individual components similar in molecular weight but differing in electrophoretic patterns (*29*).

Gluten

We are concerned in practice with a complex mixture of gliadin and glutenin, together with small amounts of lipides and starch, commonly known as gluten. Neither gliadin nor glutenin is a homogeneous protein and the so-called glutenin fraction, particularly, cannot be dispersed in any solvent sufficiently well to permit the use of the ultracentrifuge, electrophoresis, or usual physical techniques. It is consequently ill characterized.

Beccari (*2*) in 1745 first reported the separation of gluten from the starch of flour. Gluten is conveniently prepared by adding 60 to 65% water to a hard wheat flour, mixing and allowing the dough to rest about 30 minutes, then washing out the bulk of the starch and other more soluble constituents under a steady stream of water. An elastic, rubberlike material holding, roughly, two thirds of its weight of water is obtained.

Gluten has the approximate composition shown in Table I, naturally varying

with the type of flour and the washing procedure.

Flour contains 1 to 2% lipides, including phosphatides as well as glycerides. Practically all of the lipide is attached to the gluten by strong electrostatic forces which only alcohol will release; the lipides cannot be extracted from gluten by ethyl or petroleum ether.

Table I. Composition of Wheat Gluten

	%
Protein	85.0
Lipide	8.3
Starch[a]	6.0
Ash	0.7

[a] Starch is held more or less mechanically; the longer and more vigorous the washing, the less the starch.

Hess (20) has shown that the starch granules in the endosperm of wheat are completely surrounded by protein. As a small fraction of the protein is released as discrete particles in milling, it is possible to separate the starch and protein of flour without the use of water by reason of the difference in density of the two fractions. Appropriate mixtures of chloroform-ether or chloroform-benzene can be used to effect the separation. The isolated endosperm protein differs greatly from the gluten separated from flour by water. When the protein swelled without mechanical treatment, a volume increase of about 25% was obtained, whereas, when gluten swelled, the corresponding volume increase was 200%. Differences in the x-ray diagrams were especially marked.

The isolated protein, upon swelling in water, showed a reversible, discontinuous lattice expansion of from 45 to 55 A., up to 90 A., whereas gluten, under the same conditions, showed a reversible, continuous lattice expansion of from about 45 to 55 A. Hess concluded that, during the conversion of protein to gluten, mechanical treatments cause a structural chemical alteration in the protein molecule.

Gluten possesses viscoelastic properties. If stretched, it tends to spring back and, if made into a ball, it gradually flattens. When gluten is stretched, the elongation is the result of both viscous and elastic deformation. The feeling of the gluten is described as extensible, sticky, soft, elastic, short, or tough. Too great an extensibility, in its extreme resulting in softness or stickiness, or the opposite (too much shortness or toughness) is reflected in the machining properties of a flour dough where the gluten, together with the starch, forms a three-dimensional network that exhibits elastic, viscous, and plastic properties. The lipides have an important influence on the physical characteristics of gluten.

Chemists have long sought to explain the pronounced differences in the physical properties of gluten obtained from various wheat flours, but as yet there is no satisfactory answer. Fleurent (15) believed that the physical properties of gluten could be accounted for, in part, by the ratio of gliadin to glutenin; the larger the amount of gliadin, the more extensible the gluten. More recent experiments have indicated that the difference between these two protein fractions is too small to offer an adequate explanation of the observed variations in the physical properties of gluten.

It was hoped that, as the techniques for measuring amino acids improved, either the presence of some unique amino acid or a sufficiently outstanding difference in the amounts of one or more of the amino acids present in glutens of widely varying physical characteristics would offer some clue for the marked variations in the bread-making properties of flour.

The most recent compilation of the amino acid composition of gluten is listed in Table II.

The dicarboxylic acids, particularly glutamic acid, occur in large amounts. The quantity of ammonia formed on hydrolysis with 20% hydrochloric acid is approximately equal to that required for the dicarboxylic acids, and both glutamine and asparagine are found in enzymatically hydrolyzed gluten as well as the water extracts of flour (38). Hence, it can be reasonably well assumed that both glutamic and aspartic acids are present as amides. Gluten is relatively poor in basic amino acids and contains few ionizable groups.

Analyses of glutens obtained from flours with widely different dough characteristics and baking quality have shown no apparently significant divergence in the amounts or kinds of amino acids (11, 28). As is true with so many other proteins, amino acid composition has proved disappointing in explaining either the physical properties or the biological activity. Until we know more of the structure, the configuration, and the secondary valence forces of the amino acids, the unique properties of wheat gluten will elude explanation. The study of the gluten proteins is all the more frustrating because their inhomogeneity and relative lack of solubility in solvents that do not cause denaturation make present physical methods difficult to apply and interpret. The presence of lipides in firm combination with the proteins adds to the problem.

Effect of Oxidizing and Reducing Agents on Gluten

Gluten properties can be greatly modified by certain oxidizing and reducing agents. Most flours, especially when fresh or milled from recently harvested wheat, are underoxidized. In order to obtain the optimum dough-handling characteristics and the best baked products from the standpoint of volume, grain, and texture, it is common practice in the milling and baking industries to age the flour, effecting slow oxidation by air, or, more commonly, to add certain oxidizing agents to the flour or dough. Compounds that effect these beneficial results include o-iodosobenzoate, iodoacetic acid, iodoacetamide and cystine, although less specific oxidizing agents such as potassium bromate, potassium iodate, chlorine dioxide, and

Table II. Average Composition of Wheat Gluten (28)

Constituent	G. Amino Acid/100 G. Protein[a] Found	Moles Amino Acid/10 G. Protein[a]	Amino Acid Nitrogen of Total Nitrogen, %
Alanine	2.2	25	2.0
Ammonia	4.5	264	21.2
Arginine	4.7	27	8.6
Aspartic acid	3.7	28	2.2
Cystine	1.9	8	1.3
Glutamic acid	35.5	241	19.3
Glycine	3.5	47	3.7
Histidine	2.3	15	3.6
Isoleucine	4.6	35	2.8
Leucine	7.6	57	4.6
Lysine	1.8	12	2.0
Methionine	1.9	13	1.0
Phenylalanine	5.4	33	2.6
Proline	12.7	110	8.8
Serine	4.7	45	3.6
Threonine	2.6	21	1.7
Tryptophan	1.1	5	0.9
Tyrosine	3.1	17	1.4
Valine	4.7	40	3.2
Total	108.5	779[b]	94.5

[a] Computed for theoretical protein containing 17.5% nitrogen.
[b] Number of moles of ammonia omitted from total.

Table III. Compounds Showing Maturing Action on Flour

Potassium iodate[a]	Ammonium persulfate
Potassium bromate[a]	Nitrogen trichloride
Chlorine dioxide[a]	Cystine
Chlorine[a]	Alloxan
Iodoacetic acid	Ascorbic acid
Iodoacetamide	Copper
Iodosobenzoic acid	Iodine
Sodium chlorite	Peroxides

Sodium perborate

[a] Used commercially.

chlorine are used in practice. Table III lists some flour improvers or maturing agents.

The optimum amount of such oxidizing agents is critical and rather small—frequently of the order of a few parts per million based on the weight of the flour. Their action is primarily on the gluten. Too extensible a gluten can be made more elastic and tougher by oxidation. Overoxidation will result in shortness or brittleness (loss of elasticity or coherence). As would be expected, reducing agents produce the opposite effect. Glutathione, thioglycolic acid, cysteine, and sodium sulfite cause a drop in the viscosity of the gluten proteins and produce extreme extensibility, softness, and stickiness. Because most flours are naturally underoxidized and consequently do not perform at their optimum, the mechanism of this oxidation-reduction has been the subject of numerous investigations, including many from this laboratory.

Reactive Groups in Oxidation And Reduction of Flour

The reactive groups involved in the changes in the physical properties of gluten and dough on oxidation and reduction belong to the sulfur-containing amino acids—methionine, cysteine, and cystine.

The work of Reiner et al. (26, 31, 32) in this country and Bentley et al. (4-6) in England showed that the toxic substance formed by the action of nitrogen trichloride on gluten is methionine sulfoximine. However, neither this compound nor the sulfoxide or sulfone of methionine seems to have any influence on the beneficial effects observed on flour oxidation. Moreover, none of the flour improvers now in use forms the sulfoximine. Although the thio ether group is rather readily oxidized, the methionine content of flours and of bread baked from flours with and without oxidation shows no apparently significant variation.

Practically all mercapto (sulfhydryl) reagents are flour improvers. While certain of the effective oxidizing or alkylating reagents and the reversibility of the reaction would implicate the mercapto group, it has not been possible

to obtain a satisfactory correlation between mercapto determinations and response to oxidation. Water extracts of flour give extremely small reducing values on titration with iodate, ferricyanide, or iodosobenzoate. The lower the grade of flour, the higher the reducing value and the greater the response to oxidation. Some years ago glutathione was isolated from wheat germ (36, 37). Lesser amounts were found in wheat bran. Because the flours of longer extraction contain more germ and bran particles, such lower grade flours would be expected to show higher reducing values. Some investigators have assumed that the compound that responds to oxidation in all flours is free glutathione. This is true only in the case of low grade flours containing relatively large amounts of bran and germ particles. Glutathione is water-soluble and, if present, should be readily identified in water extracts. However, most flours that respond to oxidation do not give a nitroprusside test for a free mercapto group although it is readily demonstrable by other techniques. Moreover, the total reducing values of flours, as measured at present, do not correlate with the amount of oxidant required or with the observed changes of the physical characteristics of dough on oxidation.

The reaction of gluten with reducing agents demonstrates the important role of the S—S linkages. Small amounts of reducing agents such as thioglycolic acid, glutathione, cysteine, and sodium sulfite or bisulfite, as well as other compounds such as potassium cyanide, produce extreme extensibility and softness. With sufficient amounts of these reagents and mechanical stirring, gluten can be readily solubilized, especially at higher pH levels. Marked loss of cystine can be observed under these conditions. However, there is no proof that cystine is involved on oxidation of flours. In general, flours milled from wheats higher in protein possess stronger, more elastic glutens. Tough gluten is obtained from many flours very high in protein. Because of the effects observed with thiol compounds and because the higher the protein of a flour, the higher the content of all amino acids including cystine, it is reasonable to assume that cystine has an influence on

the strength and elasticity of a gluten. The S—S group of cystine probably forms crosslinks between the polypeptide chains or helixes and the more crosslinks, the greater the rigidity, toughness, and gas retention of the gluten. As some of the cystine may be held within single chains, the total percentage of cystine would not necessarily indicate the number of cross linkages between chains.

An interesting aspect of the oxidation of flour doughs, by potassium bromate particularly, is that the effects observed depend on the work done on the dough: mixing, rounding, molding, or braking. Any given flour which responds to oxidation demands a rather critical amount of the reagent for any given formula and procedure. Overoxidation can produce worse results than no treatment at all. Freilich and Frey (16) have shown that an overtreated dough showing signs of shortness and age can be brought back by remixing. Work in this laboratory has shown that the effects of overoxidation by bromate can be ameliorated by certain mechanical treatments such as braking, and that, for such mechanical procedures, the optimum level of oxidation is higher than for conventional machining (37). Baker and Mize (7), Freilich and Frey (17), and Smith and Andrews (35) have shown that the physical characteristics of dough are affected by oxygen of the air which is incorporated in the dough during mixing.

More recently an interesting series of papers from the Grain Research Laboratory of Canada (13, 14, 21), has shown that the internal stresses set up in unbromated doughs relax at a rate that is independent between mixing and working. But, in bromated doughs, the rate of relaxation of internal stresses decreases linearly with reaction time and with increasing concentration of bromate. Moreover, changes in physical dough properties appear only when the dough is worked after a time of reaction. Structural relaxation of doughs was found to be characterized by a rate constant. Rate constants increase slightly with time in unbromated doughs, but decrease in bromated doughs. The higher the bromate concentration, the more pronounced is the time-dependent change in the rate constant.

Role of Mercapto Group in Rheological Changes

Flours that are benefited by oxidation give small reducing values on titration with iodate, ferricyanide, or iodosobenzoate, although the nitroprusside test is usually negative because of interference with color development by other constituents of flour. A most satisfactory method for the determination of the free mercapto groups of flour is the recently published amperometric

procedure of Kolthoff et al. (22), using the rotating platinum electrode with mercuric chloride as the titrating solution. When flour (40 grams) and water (100 ml.) are mixed 3 minutes under nitrogen in a Waring Blendor and an aliquot of the centrifuged solution is titrated, closely reproducible figures are obtained. Straight grade flours of the 1953 spring wheat crop averaged 0.0012% mercapto, calculated as cysteine. When ether-extracted, almost the same figure is obtained, showing that the mercapto groups do not originate from the ether-soluble fraction. Higher values for free mercapto are found when flour is extracted with $0.05M$ borax (pH 9.2) or with salt solutions at lower hydrogen ion concentrations. The use of any peptizing reagent above pH 9.0 with flour or gluten may increase the mercapto groups, owing to destruction of cystine. At higher alkalinities, hydrogen sulfide and ammonia are evident on neutralizing the solution.

Although there is a small amount of free mercapto groups present in the albumins of flour that can be accurately measured by the mercurimetric titration, there are probably more mercapto groups bound in some manner in the water-insoluble gluten. This is indicated by mixing minute amounts of potassium iodate (2.5 mg.) with wet gluten (30 grams). After 1 hour, the untreated gluten decreases in viscosity and begins to spread, whereas the treated gluten holds its orginal shape. The reducing groups in gluten are exceedingly difficult to measure. Although gluten can be readily peptized by such reagents as guanidine hydrochloride, borax, ammonia, Dupanol, Naccanol, and acetic acid, titration of the centrifugate with either silver nitrate in alcoholic medium or mercuric chloride in water solution results in precipitation of protein on the platinum electrode by alcohol or the supporting electrolyte. As a result, no satisfactory measurement can be obtained.

When lipide-free gluten is peptized with various reagents, phosphorus is liberated and the lability of the linkage suggests the possibility that mercapto groups may be combined with phosphoric acid. Recently Binkley (7) synthesized S-phosphocysteine. He found that hydrolysis of the compound with acid released hydrogen sulfide, whereas hydrolysis with alkali or enzymes (extract of rat kidney) produced cysteine and phosphate. Hydrolysis of gluten with acid does produce hydrogen sulfide, as measured by the blackening of lead acetate paper, and hydrolysis with dilute alkali does release mercapto and phosphate. While it is possible that mercapto groups may be linked with phosphoric acid, the presence of both together may be coincidental. Proof awaits further work and the nature of the

mercapto compound and its linkage is still being investigated.

Dough made from wheat flour is a complex, dynamic mixture of water, starch, proteins, enzymes, and inorganic salts. In large measure, its rheological behavior is dependent on the physical properties of gluten. Changes in bonding occur on fermentation, oxidation, and work (mixing, rounding, molding, braking), thus modifying the structural properties of the three-dimensional network. Some of these bonds are in primary valence chains, some are hydrogen bonds with oxygen and nitrogen, and some, in all probability, are weaker hydrogen bonds in which the sulfur atom is involved. Urea, sodium salicylate, certain detergents, and inorganic salts can sever hydrogen-bonded structures and all of them can peptize gluten. Hydrogen bond energies are weak compared to ordinary valence bonds; this accounts for the relative ease in breaking the bond as the temperature is raised or energy supplied by work. The strength of the bond is known to decrease with decreased electronegativity of the atom bridged to hydrogen, and hydrogen-bonded sulfur would be the easiest to break.

As mercapto groups are formed, the gluten becomes more extensible. It is possible that, when work is done on a dough in the presence of an oxidizing agent such as potassium bromate, those mercapto groups that are in close proximity are oxidized and realigned by the shearing force to form S—S cross linkages or other stronger bonds that produce greater strength of the gluten. Proof of this hypothesis is difficult.

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