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## REVIEWS

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### Food Browning and Its Prevention: An Overview<sup>†</sup>

Mendel Friedman

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture,  
800 Buchanan Street, Albany, California 94710

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Enzymatic and nonenzymatic browning reactions of amino acids and proteins with carbohydrates, oxidized lipids, and oxidized phenols cause deterioration of food during storage and processing. The loss in nutritional quality and potentially in safety is attributed to destruction of essential amino acids, decrease in digestibility, inhibition of proteolytic and glycolytic enzymes, interaction with metal ions, and formation of antinutritional and toxic compounds. Studies in this area include influence of damage to essential amino acids on nutrition and food safety, nutritional damage as a function of processing conditions, and simultaneous formation of deleterious and beneficial compounds. These compounds include kidney-damaging Maillard reaction products, mutagens, carcinogens, antimutagens, antioxidants, antibiotics, and antiallergens. This overview covers the formation, nutrition, and safety of glycated proteins, characterized browning products, and heterocyclic amines. Possible approaches to inhibiting browning reactions and preventing adverse effects of browning during food processing and food consumption, including protection against adverse effects of heterocyclic amines by *N*-acetylcysteine, caffeine, chlorophyll, conjugated linoleic acid, lignin, and tea extracts, are also described. This research subject covers a complex relationship of the chemistry, biology, and pathology of browning products and the impact on human nutrition and health. Future study should differentiate antinutritional and toxicological relationships, define individual and combined potencies of browning products, and develop means to prevent the formation and to minimize the adverse manifestations of the most antinutritional and toxic compounds. Such studies should lead to better and safer foods and improved human health.

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**Keywords:** *Browning prevention; food browning; food safety; glycated proteins; glycosylation; heterocyclic amines; human health; Maillard products; nutrition*

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#### INTRODUCTION

Amino-carbonyl and related interactions of food constituents encompass those changes commonly termed browning reactions. Specifically, reactions of amines,

amino acids, peptides, and proteins with reducing sugars and vitamin C (nonenzymatic browning, often called Maillard reactions) and quinones (enzymatic browning) cause deterioration of food during storage and commercial or domestic processing. The loss of nutritional quality is attributed to the destruction of essential amino acids and a decrease in digestibility and inhibition of proteolytic and glycolytic enzymes. The production of antinutritional and toxic compounds may further

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reduce the nutritional value and possibly the safety of foods (Carpenter, 1991; Finley and Friedman, 1973; Friedman, 1977a,b, 1982a; Friedman et al., 1989; Hurrell, 1990; Mauron, 1990; Percival and Schneeman, 1979; Schumacher and Kroh, 1994; Smith and Friedman, 1984). A special class of browning reactions results in the formation of heterocyclic amines in meat and fish. These are derived from the interaction between amino acids, carbohydrates, and creatinine, which is present only in animal food. Such reactions occur widely in foods subjected to heat processing and storage.

To develop rational approaches to minimize adverse consequences of browning reactions and optimize beneficial ones, studies are needed to relate compositional changes to nutritional and toxicological consequences. To catalyze progress, a need exists to define known chemical, biochemical, nutritional, and toxicological indices of browning and its prevention. This limited review addresses the nutritional and safety consequences of the browning reaction from selected studies in the widely scattered literature. Specifically covered are (a) formation, nutritional values, and safety of specific browning products; (b) formation and nutritional value of glycated proteins; (c) antimutagenic, antioxidant, antibiotic, and antiallergenic browning products; (d) formation and risk to human health of heterocyclic amines; and (e) possible approaches to minimizing and preventing food browning and the resulting adverse consequences to food quality and safety.

The following outline of categories and examples gives an indication of the complex dynamics of browning in food and *in vivo*.

#### 1. nonenzymatic browning

(a) heat catalyzed protein– and amino acid–carbohydrate reactions, *e.g.*, wheat gluten plus glucose (bread crust); lactalbumin plus lactose (stored milk powder); free amino acids plus glucose (fried potatoes and foods for parenteral nutrition)

(b) *in vivo* protein–carbohydrate reactions, *e.g.*, hemoglobin plus glucose in diabetics or eye lens protein plus glucose (cataracts)

(c) protein-oxidized fatty acid reactions, *e.g.*, casein plus oxidized linoleic acid

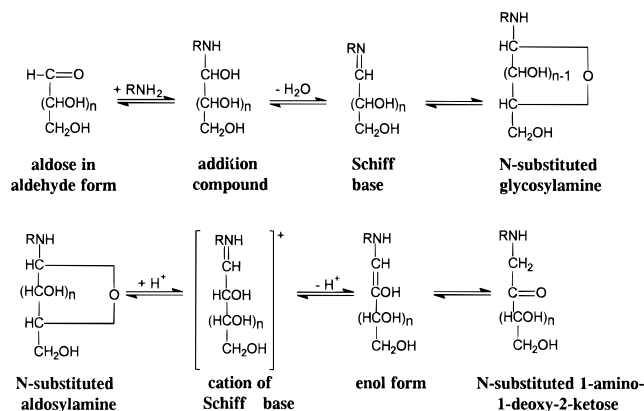
(d) heterocyclic amine formation, *e.g.*, heat-catalyzed reactions of amino acids, glucose, and creatinine to form polycyclic amines in cooked meat and fish

#### 2. enzymatic browning

(a) polyphenol-oxidase-catalyzed oxidation of polyphenol compounds in fruits and vegetables to quinones, which then polymerize to dark melanin pigments of unknown structure, *e.g.*, formation of brown or black spots from chlorogenic acid in bananas, pears, lettuce, and potatoes and the browning of fruit juices

(b) reaction of polyphenol-derived quinones with free amino acids and proteins to form dark polymers, *e.g.*, reactions of casein with oxidized chlorogenic acid in mixed foods containing both casein and potatoes

Since the discovery of browning reactions over 80 years ago (Maillard, 1912), food scientists have been studying the mechanisms of browning and its effects on organoleptic properties, appearance, nutritional quality, and safety. In contrast, medical scientists have only been exploring relationships between *in vivo* browning and disease and aging for about 15 years. When the medical researchers determined that the *in vivo* reactions were quite similar to those occurring in food, they utilized the available knowledge discovered by food



**Figure 1.** Molecular events in the initial stages of the Maillard reaction (Finot et al., 1977; Friedman, 1982).

scientists. From such broad-based cross-fertilization of ideas, we can expect further progress that will benefit both food science and medicine.

Since more than one type of browning can occur simultaneously in food, this integrated overview attempts to develop a better understanding of the complex overlapping aspects of browning to help stimulate further progress on the pervasive impact of browning on human nutrition and health.

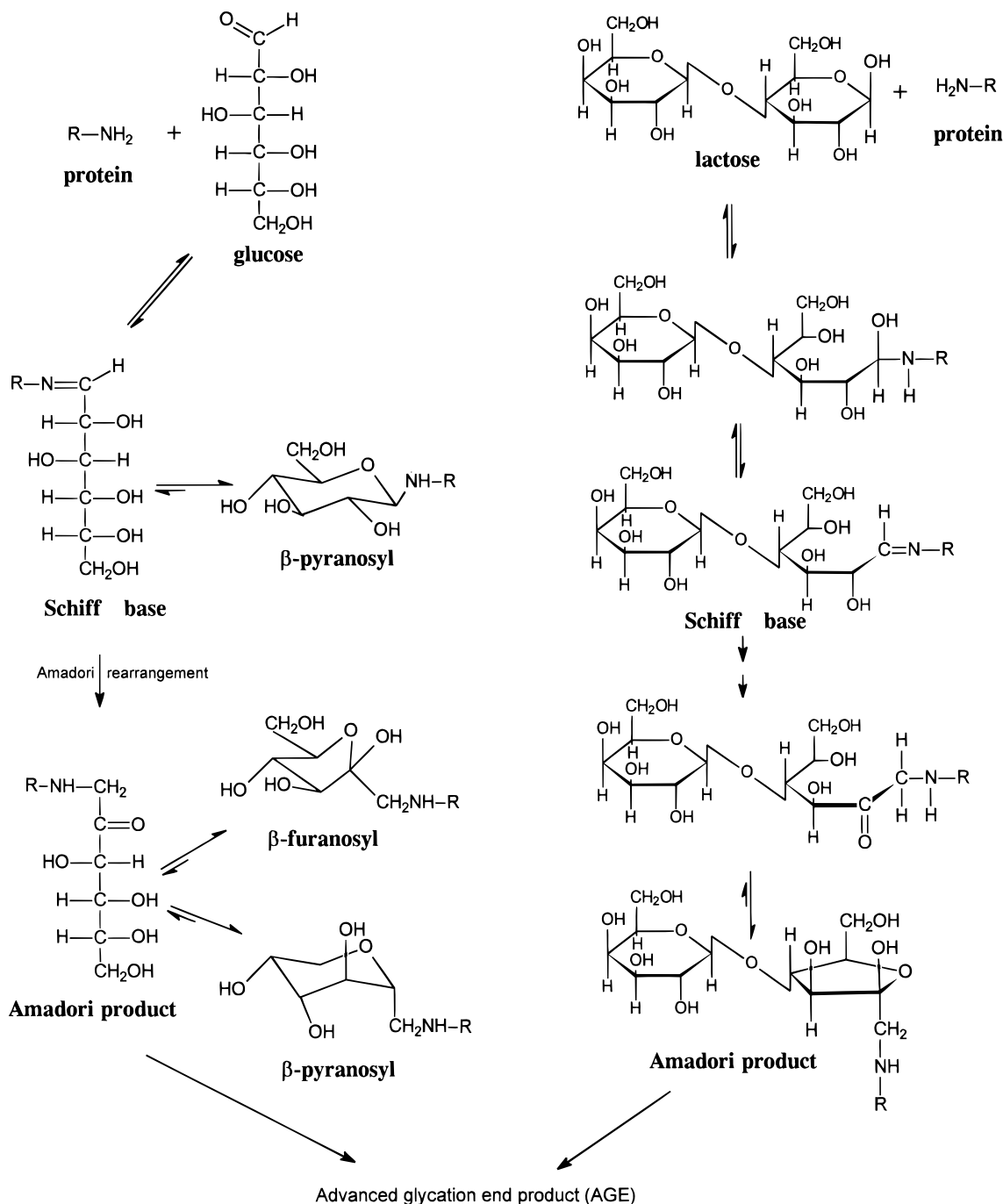
Figures 1–7 illustrate the formation and structures of nonenzymatic browning products. Figure 8 illustrates enzymatic browning and its prevention and Figures 9 and 10 show the formation and structures of heterocyclic amines.

#### REACTIONS

**Protein Glycation.** Nonenzymatic glycosylation, or glycation, is the covalent attachment of sugars to  $\alpha$ - or  $\epsilon$ -NH<sub>2</sub> groups of amino acids and proteins to form glycated proteins (Figures 1–5). This should be differentiated from enzyme-catalyzed glycosylation, during which oligosaccharide chains are attached to asparagine or serine side chains through glycosidic linkages to form glycoproteins. The first glycation product or Schiff base rearranges to a more stable ketoamine or Amadori product. The Amadori products can then form cross-links between adjacent proteins or with other amino groups. The resulting polymeric aggregates are called advanced glycation end products.

Furth (1988) and Ahmed and Furth (1990, 1991) summarize several assays for glycated proteins. These include the thiobarbituric method, periodate oxidation, borohydride reduction, serum fructosamine assay, and other separation methods based on the use of boronate complexes of sugars, fluorescence spectroscopy, and immunoassays for specific derivatives. Hydrolysis of glycated proteins results in transformation of about 30% of the Amadori compound fructosyllysine to furosine. A minor cyclization product called pyridosine is also formed. About 50% of the fructosyllysine reverts to lysine. Furosine can be separated by HPLC and detected at 280 nm. An improved ninhydrin assay was developed by Friedman et al. (1984a) to measure NH<sub>2</sub> groups in proteins and in enzymatic digests (Pearce et al., 1988).

**Browning Reaction Products.** The availability of sophisticated chromatographic and analytical techniques made it possible to isolate, characterize, and analyze several specific compounds formed *in vitro* and *in vivo* in the early and advanced stages of the Maillard



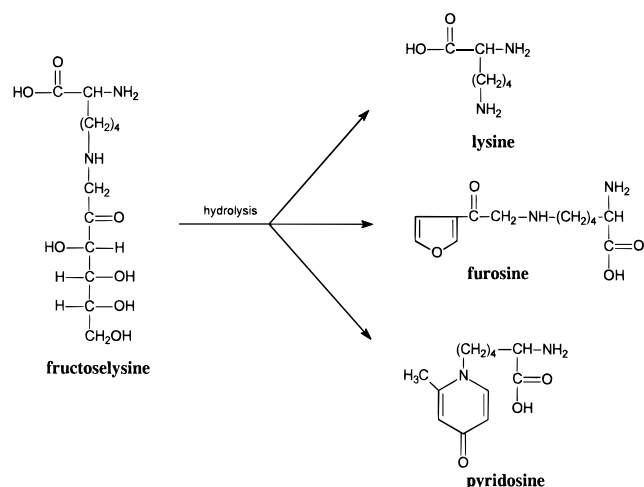
**Figure 2.** Intermediates formed during the initial glycation of protein  $NH_2$  groups by glucose or lactose (Furth, 1988; Matsuda et al., 1992).

reaction (Hodge et al., 1976; Ledl and Schleicher, 1990; Loscher et al., 1991; Westwood and Thornalley, 1995). Since the availability of such compounds makes it easier to define the nutritional, toxicological, and pathological significance of the Maillard reaction, I will summarize some of the reported findings on Maillard and related products.

**Fructoselysine and Furosine.** Chiang (1983) developed an HPLC method to measure furosine in heated and stored foods. This HPLC method complements the amino acid analysis method and appears to be fast, simple, and economical. All of the storage samples contained significant amounts of furosine. Levels decreased after prolonged storage or after overheating. Hartkopf and Ebersdobler (1993a,b, 1995) compared HPLC and ion-exchange chromatography methods for the determination of furosine. Henle et al. (1993a,b,

1995) describe a fast and sensitive ion-exchange chromatography method for the separation of furosine, pyridosine, and lysinoalanine, along with all other amino acids (Figure 3). They found that the molar ratios of furosine to pyridosine were different from previously published values. An unknown ninhydrin-positive compound was also detected in hydrolysates of heated casein/lactose and heated milk samples.

Hewedy et al. (1994) studied the production of chemical indicators of heat damage resulting from ultrahigh-temperature (UHT) treatment used to sterilize milk. Processing temperature and time induced increases in the following indicators of damage: furosine,  $N$ - $\epsilon$ -carboxymethyllysine, and (hydroxymethyl)furfural. Carboxymethyllysine was not a good general indicator of damage since it was detected in low concentrations only in the more severely heated samples. Since carboxy-



**Figure 3.** Acid hydrolysis products of fructoselysine (Corzo et al., 1994; Finot et al., 1977; Furth, 1988; Hartopf and Ebersdobler, 1994; Henle et al., 1995).

methyllysine is more stable than lactuloselysine (galactose-fructose-lysine), it could serve as an indicator of severe damage. The values for the three indicators of chemical damage were highly correlated (Corzo et al., 1994; O'Brien, 1995).

Furosine can also serve as an indicator of the freshness of shell eggs (Hidalgo et al., 1995).

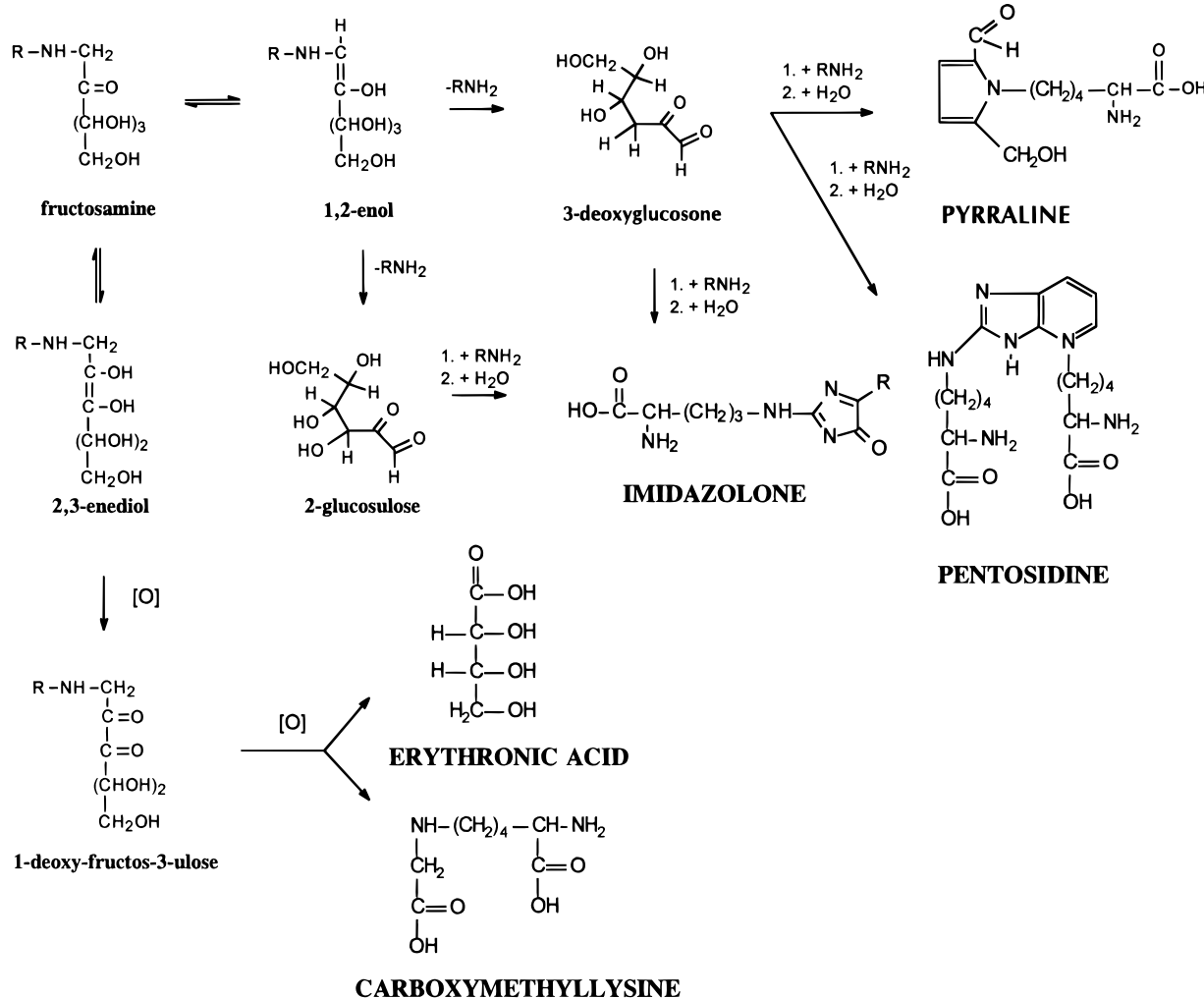
**Lactuloselysine.** In milk, the initial stage of the Maillard reaction involves interaction between the

$\epsilon$ -NH<sub>2</sub> of protein-bound lysine with lactose to form lactuloselysine [ $\epsilon$ -(deoxylactose)lysine].

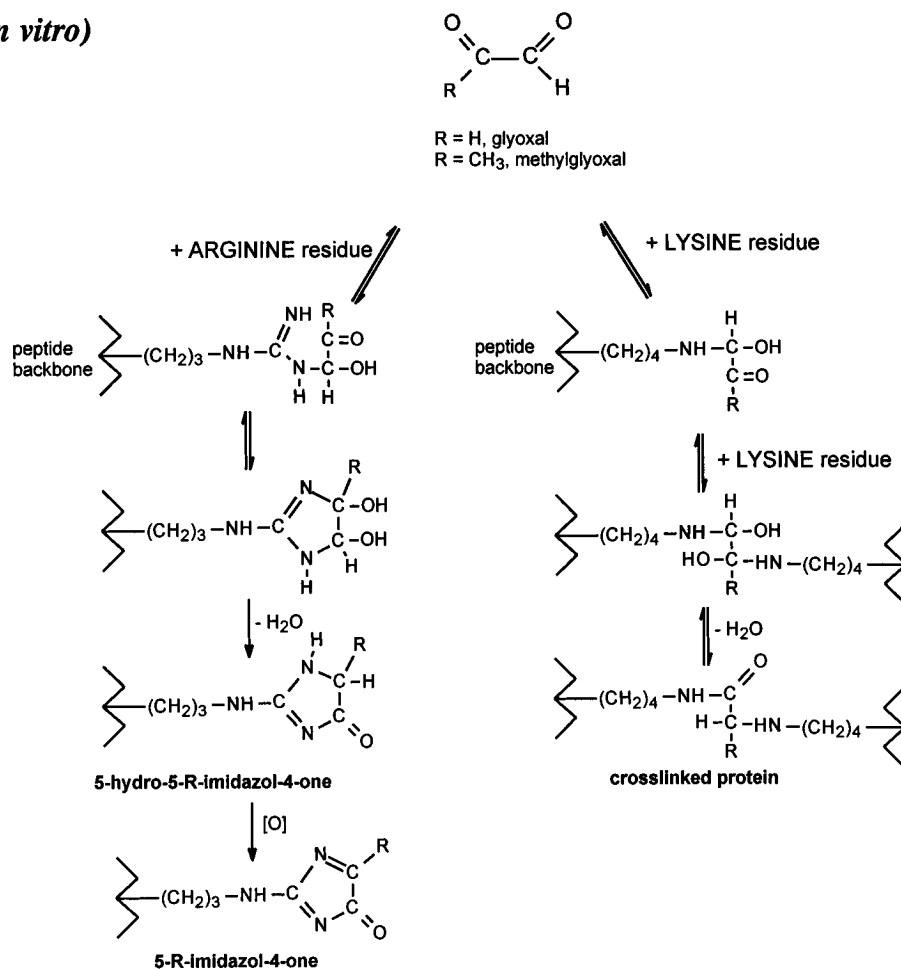
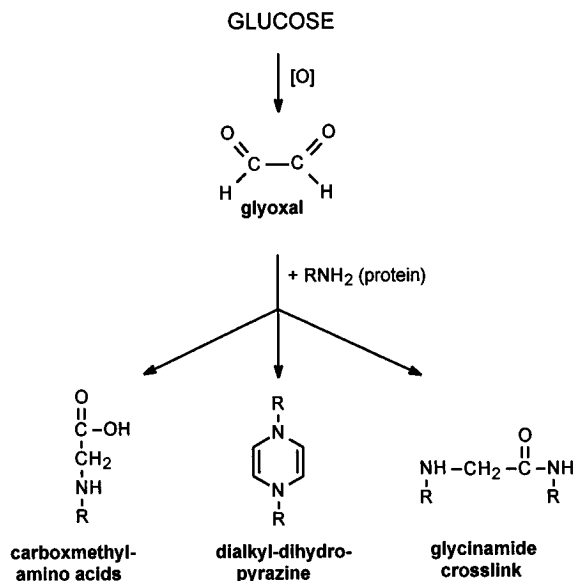
To overcome problems with furosine analysis, Henle et al. (1991a) developed a method to measure lactuloselysine and unmodified lysine directly via ion-exchange chromatography following complete enzymatic hydrolysis of the milk products. The relative amounts of lactuloselysine and lysine in milk products were calculated by a mathematical relationship without knowledge of the initial lysine concentration before storage or heat treatment. The values for lactuloselysine determined according to the enzyme-hydrolysis-ion-exchange method for a number of industrial milk-based baby food products were 3–6 times higher than those obtained with the furosine method. These findings suggest that the furosine method may underestimate the extent of lysine modification for Amadori compounds such as lactuloselysine formed in the early stages of the Maillard reaction.

Evangelisti et al. (1994) found that for infant milk formulas the quantity of lysine blocked as lactuloselysine increased with the lactose to protein ratio. Partial substitution of lactose with dextrans decreased blocked lysine measured as furosine.

Dehn-Muller et al. (1991) showed that heat treatment of milk also induces the formation of lysinoalanine (Figure 7), the concentration of which ranged from 10 to 80 mg/kg of protein, depending on conditions used. Mechanisms governing formation of cross-linked amino



**Figure 4.** Pathways toward Maillard reaction products derived from a glycated protein (Ahmed et al., 1986; Hayase et al., 1989; Sell and Monnier, 1989).

A (*in vitro*)B (*in vivo*)

**Figure 5.** Possible products derived from reaction of protein NH<sub>2</sub> groups with glyoxals (Amrani-Hemaimi et al., 1995; Wells-Knecht et al., 1995; Westwood and Thornalley, 1995; Whitfield and Friedman, 1972).

acids such as lysinoalanine in structurally different proteins and the resulting nutritional and toxicological consequences are described in detail elsewhere (Friedman, 1977a,b, 1982b,c; Friedman et al., 1982a, 1984b,c, 1986; Liardon et al., 1991).

*N-ε-Carboxymethyllysine.* Ahmed et al. (1986) found that incubation of *N-α*-formyl-*N-ε*-fructoselysine, under physiological conditions, resulted in the formation of carboxymethyllysine in 40% yield. Since *N-ε*-carboxy-

methyllysine and the accompanying erythronic acid were not produced under a nitrogen atmosphere, an oxygen-dependent free radical pathway best explains the mechanism of pH- and phosphate-ion-dependent formation of the lysine derivative. Carboxymethyllysine was also detected in human lens tissue proteins, in tissue collagen, in urine of diabetics, and in hydrolysates of ribonuclease glycosylated in phosphate buffer (see below). The authors suggest that (a) since both the lysine

derivative and erythronic acid are metabolically inert, oxidative cleavage of Amadori compounds *in vivo* may limit protein glycation; (b) carboxymethyllysine may be a useful marker for assessing the age of extracellular proteins in the body and/or the cumulative exposure of tissue proteins to glucose; and (c) the formation of this marker may be enhanced by locally high phosphate concentrations present in connective tissues and by free radical species in areas of inflammation.

Liardon et al. (1987) showed that rats fed heat-processed proteins containing either fructoselysine had significant levels of carboxymethyllysine in their urine. Since the lysine derivatives can originate both from processed food sources (Ruttkat and Ebersdobler, 1995) and *in vivo*, urinary carboxymethyllysine levels may not be a good indicator either of protein damage *in vitro* or of *in vivo* glycation of tissue proteins.

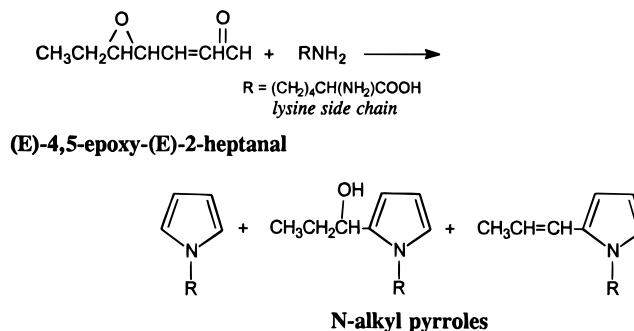
Because of the possible overestimation of the carboxymethyllysine in food (since it can form both during the Maillard reaction and during acid hydrolysis of the glycated protein), Hartkopf et al. (1994) developed an HPLC method to measure carboxymethyllysine derived specifically from food processing. They recommended that a borohydride reduction of fructoselysine to hexitollysine, which does not produce carboxymethyllysine on hydrolysis, should precede the hydrolysis. Sodium cyanoborohydride may be a preferred reducing agent (Chauffe and Friedman, 1977; Friedman et al., 1974).

***ε*-Pyrrolylsine.** Nakayama et al. (1980) isolated  $\epsilon$ -[2-formyl-5-(hydroxymethyl)pyrrole-1-yl]-L-norleucine, also known as *pyrraline*,  $\epsilon$ -pyrrolylsine, and lysylpyrrolealdehyde, from the Maillard reaction between D-glucose and L-lysine. The product of the early Maillard reaction, deoxyfructosyllysine, decomposes as the reaction progresses to the so-called advanced Maillard reaction products. This transformation is accompanied by a decrease in furosine levels and  $\epsilon$ -pyrrolylsine. Since the furosine method cannot accurately indicate long-term storage or overheating, Chiang (1988) developed an HPLC method with electrochemical detection to monitor  $\epsilon$ -pyrrolylsine.  $\epsilon$ -Pyrrolylsine was more stable in dry food systems and is, therefore, a better indicator of long-term storage at elevated temperatures than furosine.

Resmini and Pellegrino (1994) devised an HPLC method to measure protein-bound  $\epsilon$ -pyrrolylsine in dried pasta. They also simultaneously measured the parallel formation of furosine after enzymatic hydrolysis of the pasta followed by solid-phase extraction and ion-pair chromatography. They report a close relationship between the accumulation of these two compounds as a function of heat treatment. The findings suggest that pasta should be exposed to minimal heat treatment to avoid protein damage, especially loss of lysine, the formation of furosine, and the formation of digestive-enzyme-inhibiting and potentially mutagenic pyrrolealdehydes.

***Aspartyl- and Glutamyllysines.*** Heat catalyzes the interaction between  $\epsilon$ -NH<sub>2</sub> groups of lysine and  $\beta$ -aspartyl-COOH and CONH<sub>2</sub> side chains in proteins (Figure 7). Corresponding reactions between  $\epsilon$ -NH<sub>2</sub> groups of lysine and  $\gamma$ -COOH groups or  $\gamma$ -CONH<sub>2</sub> groups of glutamic acid or glutamine lead to the formation  $\gamma$ -glutamyl-L-lysine derivatives (Hurrell and Carpenter, 1977; Otterburn et al., 1977). Such isopeptide bonds may not be as readily digested as naturally occurring peptide bonds (Friedman and Finot, 1990; Oste, 1991).

Schmitz et al. (1976) found that heating milk, milk



**Figure 6.** Reaction of fatty-acid-derived epoxyeneheptanal with protein NH<sub>2</sub> groups to form *N*-alkylpyrroles (Hidalgo and Zamora, 1995).

products, and milk proteins for 24 h at 0 °C in the dry state transformed 2–5% of lysine residues to *N*- $\epsilon$ -( $\gamma$ -glutamyl)-L-lysine. No *N*- $\epsilon$ -( $\beta$ -aspartyl)-L-lysine was detected after an enzymatic hydrolysis and analysis by ion-exchange chromatography. Weder and Scharf (1981) demonstrated the formation of aspartyllysine and glutamyllysine cross-links, in addition to minor amounts of lysinoalanine, in tryptic digests of heated ribonuclease. Heat treatments used in daily practice do not seem to lead to extensive isopeptide bond formation.

Yasumoto and Suzuki (1990) isolated an enzyme, *N*- $\epsilon$ -( $\gamma$ -glutamyl)-L-lysine hydrolase, from a *Flavobacterium* microorganism. This enzyme can hydrolyze glutamyllysine isopeptide bonds to glutamic acid and lysine.

***Amadori Prolines.*** Proline interacts with reducing sugar to form a glycosylated proline derivative called Amadori proline [1-(2'-carboxy)-1-deoxy-D-fructose] (Mills and Hodge, 1976). Amadori proline is transformed to an oxazine under the influence of heat (Tressl et al., 1994).

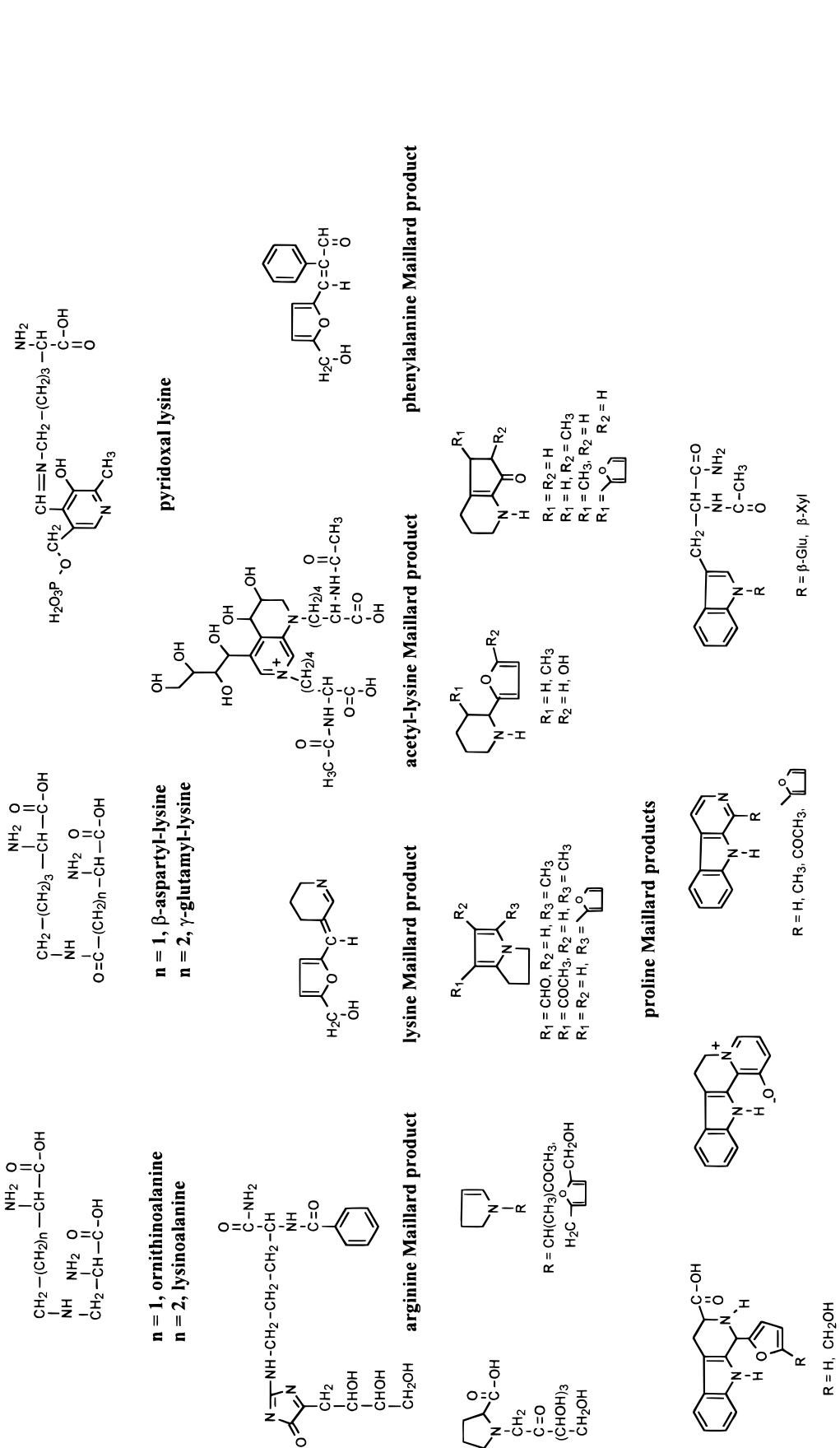
Yaylayan and Mandeville (1994) proposed a mechanism for this transformation initiated by base-catalyzed 2,3-enolization of Amadori proline compound without formation of deoxysones (Huyghues-Despointes et al., 1994).

***Tryptophan Derivatives.*** Saito et al. (1986) showed that *N*-acetyltryptophan, a model compound for tryptophan residues in proteins, reacts with glyoxal at pH 6.8 and 50 °C to form *N*-( $\alpha$ -acetyl)-1-(2-hydroxy-1-oxoethyl)-L-tryptophan. An analogous compound, *N*-( $\alpha$ -acetyl)-1-(1-hydroxy-2-oxopropyl)-L-tryptophan, was isolated from the reaction mixture with methylglyoxal (Figure 7; Friedman and Cuq, 1988; Oste and Friedman, 1990).

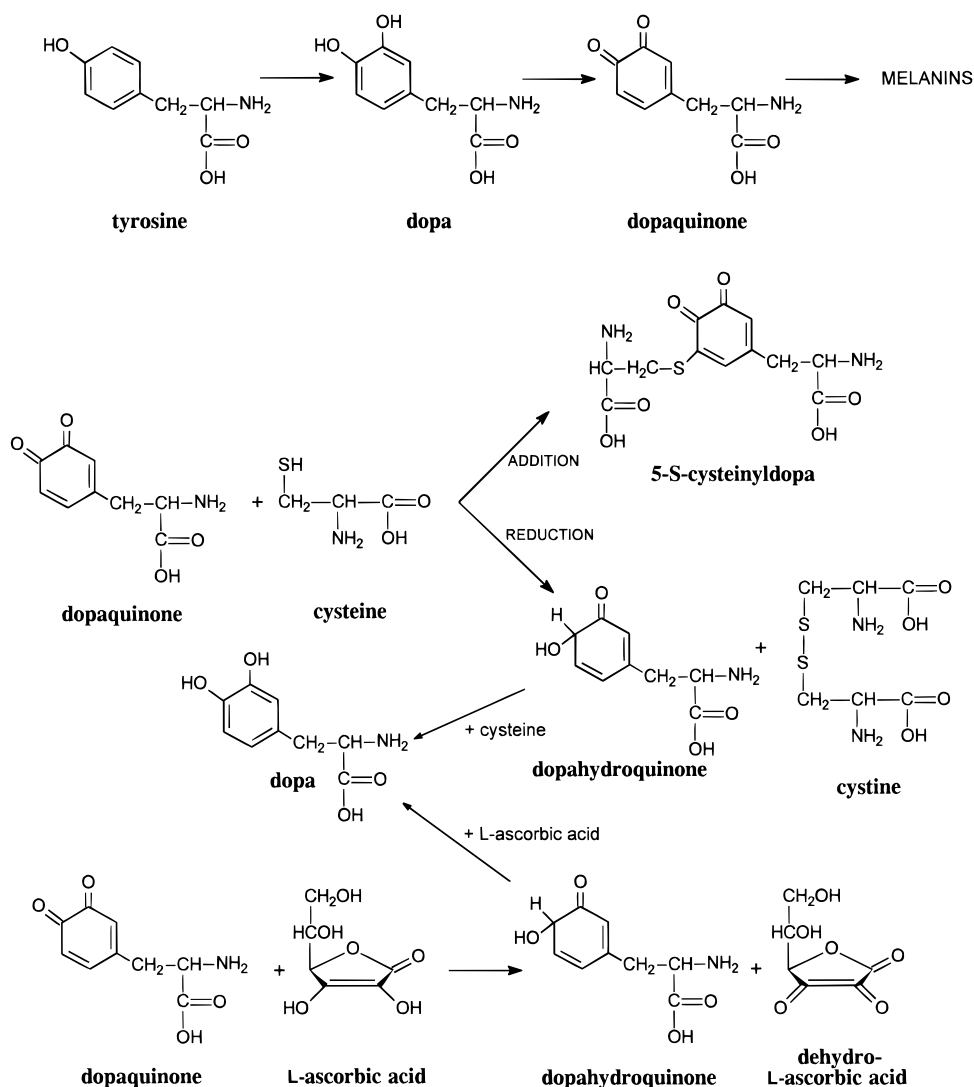
***D-Amino Acids.*** Small amounts of racemization of L- to D-amino acid residues also sometimes occur under the influence of heat. As is the case for lysinoalanine, significant racemization of protein-bound amino acids takes place mainly at high pH (Borg and Wahlstrom, 1989; Friedman, 1991; Masters and Friedman, 1980). This aspect is also beyond the scope of this review.

***Oxidized Lipid Reactions.*** Lipids are a major component of the diet. Unsaturated lipids are oxidized to hydroperoxides, cyclic peroxides, epoxides, and aldehydes (Karel, 1973). These lipid derivatives not only adversely affect flavor but also interact with proteins and other food ingredients, reducing nutritional quality and safety.

For example, Nielsen et al. (1985a,b) report that a peroxidized methyl lineolate induces significant losses of lysine, tryptophan, methionine, and cysteine residues in whey proteins. Rat feeding studies of the modified protein revealed a reduction in protein efficiency ratio



**Figure 7.** Structures of characterized lysine derivatives (Arcelli et al., 1980; Finley and Friedman, 1977; Friedman et al., 1984a,b; Hurrell and Carpenter, 1977; Ziegler et al., 1977) and Maillard products (Friedman and Cug, 1988; Ledl and Schleicher, 1990; Konishi et al., 1994; Nakamura et al., 1994; Saito et al., 1986) formed during food processing.



**Figure 8.** Inhibition of polyphenol oxidase-catalyzed enzymatic browning by trapping the dopaquinone intermediate with cysteine or ascorbic acid (Fahey, 1977; Friedman, 1973; Friedman and Bautista, 1995; Voldrich et al., 1995).

(PER), nitrogen digestibility, and other indices of protein quality. The authors suggest that restricting free access to oxygen during food packaging and maintaining low water activity will protect the protein in a mixed food from damage due to reactions with peroxidized lipids.

Studies are beginning to appear on the nature of the compound formed during lipid–amino acid reactions. For example, Hidalgo and Zamora (1995) characterized the pyrrole derivatives formed from the reaction of lysine with an epoxyenealdehyde (Figure 6).

Reactions of wool with dicarbonyl compounds such as glyoxal could serve as a model for establishing both the extent of modification and the nature of the products formed. For example, Whitfield and Friedman (1972) examined the effect of 13 structurally different dicarbonyl compounds on the arginine and lysine content of wool. Amino acid analysis of acid hydrolysates of treated wool samples showed that glyoxal modified only arginine side chains, whereas some of the other compounds modified both arginine and lysine. Westwood and Thornalley (1995) characterized protein–methylglyoxal derivatives formed *in vitro* and Wells-Knecht et al. (1995) protein–glyoxal products formed *in vivo* (Figure 6).

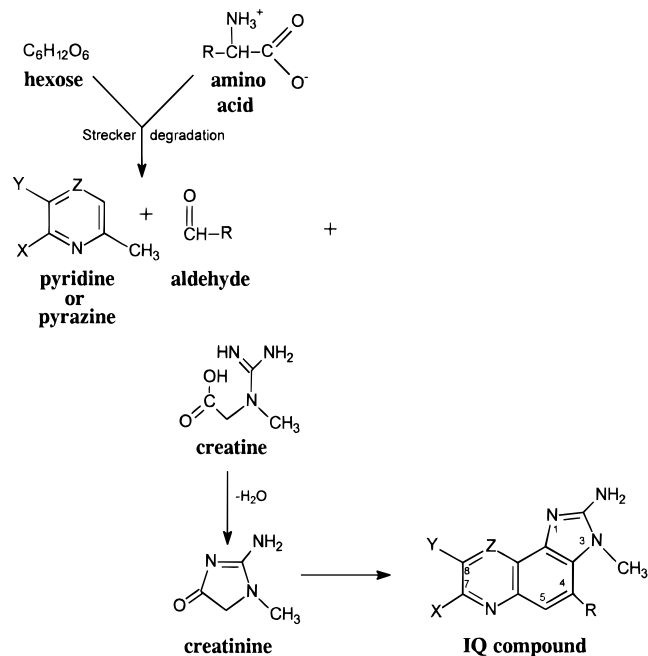
**Oxidized Polyphenol (Quinone) Reactions.** The enzyme polyphenol oxidase (PPO) catalyzes two reactions—hydroxylation of tyrosine to *o*-dihydroxyphenylalanine

(DOPA) and oxidation of DOPA and other *o*-phenols such as chlorogenic acid to *o*-quinones (Figure 8). The quinone can then either undergo further oxidation to brown melanin pigments or participate in addition–polymerization reactions with protein functional groups to form cross-linked polymers (Dao and Friedman, 1992; Deshpande et al., 1984; Golan-Goldhirsh et al., 1984; Wong, 1989). Enzymatic browning is ubiquitous in fruits and vegetables, adversely affecting color, taste, nutrition, and safety (Hurrell and Finot, 1984; Matheis and Whitaker, 1984; Sapers, 1993).

It is not widely recognized that porphyrins such as heme and melanins can also be oxidized to dehydroporphyrin and dehydromelanin quinone-like intermediates, which can undergo related browning-type reactions (Chauffe et al., 1975; Friedman, 1973).

***In Vivo* Products.** According to Dunn et al. (1989) and Sell and Monnier (1989), proteins are glycosylated (nonenzymatically glucosylated) *in vivo*, forming fructosyllysine derivatives which can be transformed oxidatively to *N*- $\epsilon$ -(carboxymethyl)lysine and hydrolytically to furosine (Figures 3 and 4). Levels of fructosyllysine increase in hemoglobin, plasma proteins, collagen, hair, lens, etc. *in vivo* in direct proportion to the degree of hyperglycemia in diabetic patients. Dunn et al. (1990) showed that carboxymethyllysine rather than furosine may be the major glycation end product in adult human





**Figure 9.** Postulated reaction route for formation of heterocyclic amine (IQ) compounds. R, X, and Y may be H or Me; Z may be CH or N (Jägerstad and Skog, 1991).

lens protein during aging. Measurement of fructosyllysine alone may not adequately indicate the extent of the modification of long-lived lens and other proteins *in vivo*. Rather, measurement of accumulation of carboxymethyllysine and pentosidine appears to be a useful indicator of age-dependent oxidative modification of proteins (Dyer, 1991).

Glucosone (D-arabino-hexos-2-ulose) is an important intermediate in the Maillard reaction formed by oxidative degradation of glucosides and by  $\gamma$ -radiolysis of

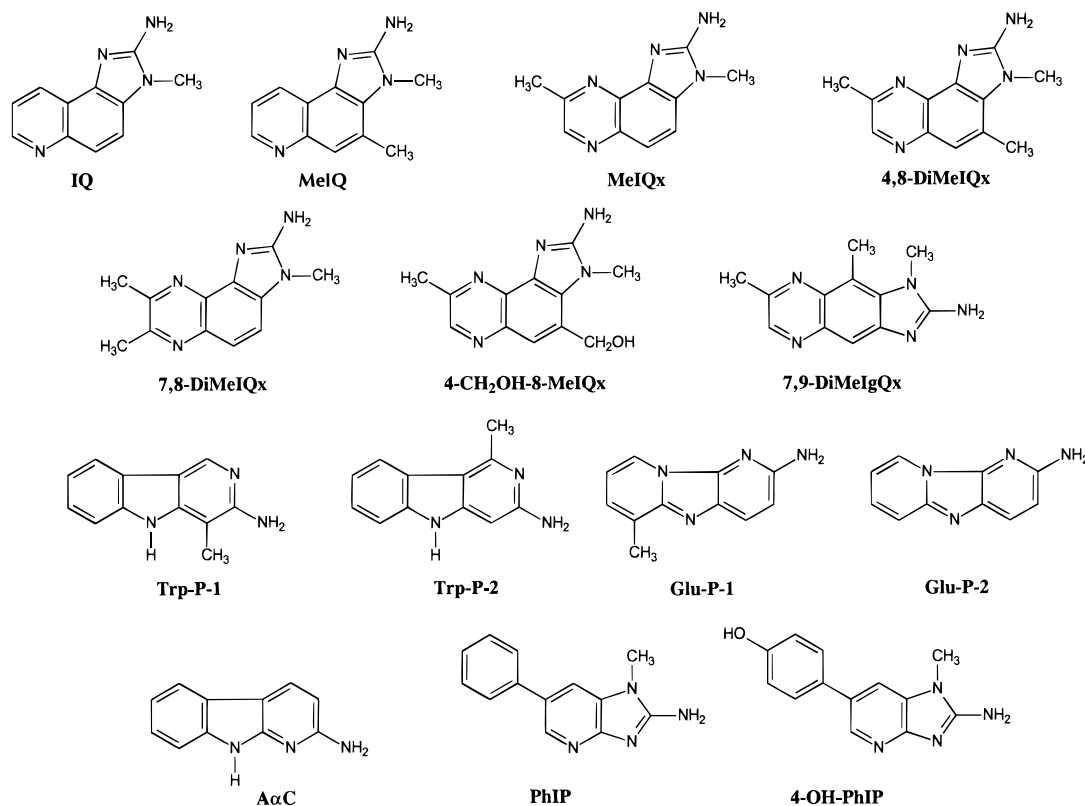
**Table 1.** Effect of Processing Temperature on the Protein Efficiency Ratio (PER) of Cereal Products [Adapted from Hansen et al. (1975)]

product	max processing temp ( $^{\circ}C$ )	PER	N digestibility (%)
puffed wheat	>260	-0.87	69
wheat granules	204	0.36	80
wheat flakes	149	0.46	72
wheat shreds	121	1.67	75
bread crust	175	0.62	85
bread crumb	100	1.36	88
wheat flour		1.11	89
casein		3.27	94

sugars. Konishi et al. (1994) describe an arginine derivative of 3-deoxyglucosone. Nakayama et al. (1992a-c) report that glucosone was cytotoxic to Chinese hamster V79 lung cells in the presence of cupric ions. The enzyme catalase inhibited the cytotoxicity. These authors propose a mechanism for the cupric ion-catalyzed oxidation of glucosone which leads to the formation of hydrogen peroxide, presumably the active toxicants.

According to Knecht et al. (1992), glucose causes browning and protein cross-linking at a rate 10 times greater than fructose. Both fructose- and glucose-derived Maillard products are potential precursors for the dicarbonyl compound 3-deoxyglucosone. This compound appears to be a potent cross-linking agent and may be important in the development of age- and diabetes-related changes in tissue proteins. The ability to detoxify 3-deoxyglucosone may provide a genetic basis for differences in age-related pathologies and complications of diabetes.

Taneda and Monnier (1994) devised an immunoassay for pentosidine. They used the ELISA to show that the amount of pentosidine in hydrolyzed skin collagen increases with age and with diabetes and uremia.



**Figure 10.** Structures of heterocyclic amines (Starvic, 1994a,b; Wakabayashi et al., 1994).

Shaw et al. (1995) report that nonspecific binding of Maillard products to macrophages may be more important than specific receptor binding in cellular recognition of such products. This finding implies that food-derived Maillard products may compromise the effectiveness of binding and removal by macrophages of Maillard products formed *in vivo* in individuals of advanced age or suffering from diabetes.

#### NUTRITION AND SAFETY

**Glycated Proteins.** As mentioned, chemical modification of proteins by carbohydrates results in the formation of glycated proteins. Such reactions may adversely affect the nutritional value and occasionally the safety of the proteins (Friedman, 1996; Table 1). This category of browning reactions will be briefly examined with selected food proteins.

Gumbmann et al. (1983) showed that loss of nutritional quality of heat-treated casein, casein-glucose, and casein-starch can be related to decreased nitrogen digestibilities, as opposed to just destruction of essential amino acid. These changes impair intestinal absorption and nutritional quality in general. The possible formation of toxic compounds might also impair nutritional quality (Schneeman and Dunaif, 1984).

Chuyen et al. (1991) examined nutritional and physiological effects of casein modified by glucose at 50 °C and at 95 °C in aqueous solution. They report that (a) weight gain of rats fed the heated caseins decreased with the extent of modification; (b) biochemical parameters such as hematocrit, erythrocyte, leucocyte, GOT, GPT, triglycerides, and cholesterol in serum were not affected by the treatments; (c) serum glucose levels of rats fed the browned caseins were significantly lower than those of unheated controls; and (d) supplementation of the heated samples with amino acids lost during the Maillard reactions did not restore the nutritional quality.

On the basis of the utilization by rats of <sup>14</sup>C-labeled casein browned with glucose at 37 °C, Mori and Nakatsuji (1977) concluded that the main reason for the observed reduction in nutritive value may be the reduction in absorption from the intestine of browning-induced lysine derivatives. Diets of casein and cod fish proteins sterilized in an autoclave at 121 °C in the presence of glucose resulted in lower digestibility and growth in rats when compared to unheated protein diets (Lipka and Ganowiak, 1993). The authors suggest that urinary taurine may serve as an indicator of nutritional damage for food proteins with inadequate sulfur amino acid content.

Keyes et al. (1979) studied the effects of autoclaving at 121 °C for 30 and 90 min in the presence of lactose on the protein quality of casein and lactalbumin. They found that the PER of casein was less susceptible to damage than that of lactalbumin and that the available lysine (but not total lysine) correlated with protein quality as measured by PER. This finding suggests that casein may be a better reference protein than lactalbumin for studies of the nutritional value of foods and feeds.

Tanaka et al. (1977) and Lee et al. (1982) carried out long-term studies on the nutritional and toxicological effects of nonenzymatic Maillard browning of egg albumin and D-glucose stored at 37 °C for 10 days. The brown product was freeze-dried and incorporated in dietary formulations. The PER value of the control egg albumin decreased from 3.0 to 1.1 after browning.

Long-term feeding studies revealed that rats on brown and control diets gained weight equally for the first month. Then those on the browned diet began to lag behind the control diet group even though the PER values of both diets was the same. After 3 months, rats on brown diets weighed 30% less than those on the control diet. This difference after 6 or 12 months was about 25%. These results show that the rats did not adapt themselves to the brown diet over the 1-year feeding period and that other factors associated with the brown diet affected nutritional quality.

These factors could be the formation of toxic compounds during browning. This suggestion is reinforced by the observation that rats fed brown diets had enlarged hearts, livers, kidneys, and spleens. This was accompanied by increased activities of liver enzymes in the serum, suggesting adverse effects on liver function. Also, black-brown pigments of an unknown nature were found in the liver. The observed higher serum glucose levels with the brown diets also suggests physiological and toxicological stress. Brown proteins may induce damaging physiological and toxicological effects that are not detectable by chemical or short-term nutritional studies.

A key question in assessing the nutritional value of heated protein-carbohydrate mixtures is the relative susceptibility of different proteins to different carbohydrates. To address this issue, Knipfel et al. (1975) investigated the effects of carbohydrate-heat interactions on the nutritive value of casein, soy, and egg proteins. They fed rats proteins that were autoclaved in the presence of 10% carbohydrates for various time periods. The following is a brief summary of this important study: (a) weight gain and food intake of rats fed egg protein were reduced more rapidly by autoclaving, regardless of carbohydrate present, than were those of rats fed casein and soy; (b) NPR of egg protein rapidly decreased with heating to the same value as soy, while casein was more resistant to protein damage; (c) digestibility of egg protein was reduced more severely than that of casein or soy protein; (d) the presence of glucose, fructose, or sucrose during autoclaving of the proteins reduced the nutritive value more than autoclaving the pure proteins alone, while starch or cellulose had little effect on nutritional quality. These results show that egg protein was much more susceptible to damage than casein or soy proteins and that reducing sugars such as glucose are more damaging to nutritive value than the nonreducing polysaccharides cellulose and starch.

Rhee and Rhee (1981) evaluated the protein quality of defatted flours and protein isolates from cottonseed, peanut, and soybeans mixed with glucose or sucrose and heated at 100 °C. They reported that (a) sucrose-complexed proteins changed very little in *in vitro* digestibilities, available lysine, total amino acids, and computed PER (C-PER); (b) the glucose complexes decreased substantially in the quality indices mentioned; (c) lysine-rich soy protein lost a greater percent of its lysine than lysine-poor peanut and cottonseed proteins; and (d) protein digestibility, available lysine, and C-PER were all highly correlated with a browning index, and available lysine was highly correlated with C-PER.

Hansen et al. (1975) studied the effects of thermal processing of wheat flour proteins on susceptibility to digestion and nutritional value (Table 1). Wheat products such as puffed wheat that were heated at high temperatures had little protein nutritional value. In

contrast, low-temperature-processed products such as bread crumb and wheat shreds had minimal damage. They also found good correlations between lysine released by enzyme digestion and PER. To maintain nutritive value, product temperature should not exceed 125 °C, as is the case for bread crumbs though not for bread crust (Friedman, 1992; Friedman et al., 1987; Ziderman and Friedman, 1985; Ziderman et al., 1989).

Friedman and Finot (1990) compared the growth of mice fed (a) an amino acid diet in which lysine was replaced by four dietary levels of  $\gamma$ -glutamyllysine; (b) wheat gluten diets fortified with lysine; (c) a wheat-bread-based diet (10% protein) supplemented before feeding with lysine or glutamyllysine, not co-baked; and (d) bread diets baked with these levels of lysine or  $\gamma$ -glutamyllysine. With the amino acid diet, the relative growth response to glutamyllysine was about half that of lysine. The effect of added lysine on the nutritional improvement of wheat gluten depended on both lysine and gluten concentrations in the diet. With 10 and 15% gluten, 0.37% lysine hydrochloride produced markedly increased weight gain.

The nutritive value of bread crust, fortified or not, was markedly less than that of crumb or whole bread. Lysine or  $\gamma$ -glutamyllysine at the highest level of fortification, 0.3%, improved the protein quality (PER) of crumb over that of either crust or whole bread, indicating a possible greater availability of the second-limiting amino acid, threonine, in crumb.

**Browning Products.** For nutritional utilization, lysine must be liberated from a food protein by digestion. It is therefore important to establish factors that affect digestibility as well as the nutritional availability and safety of lysine derivatives formed in the Maillard and related reactions.

Friedman et al. (1982a) and Friedman and Gumbmann (1979, 1981) studied the biological utilization of lysine derivatives in mice using all-amino-acid diets in which all lysine was replaced by an equimolar amount of the derivative. Lysine derivatives were utilized to some extent as a nutritional source of lysine. The authors suggest that all such studies should use amino acids in which the derivatives can serve as the only source of lysine. This is not the case with glycosylated proteins.

Finot (1990) showed that the early Maillard product, deoxyketosyllysine (*N*- $\epsilon$ -fructosyllysine), was utilized by the rat as a source of lysine to the extent of about 5–15% compared to lysine. Hurrell and Carpenter (1977) showed that protein-bound fructosyllysine formed in stored albumin–glucose mixtures did not serve as a lysine source.

Ebersdobler et al. (1989, 1991) studied dose-response excretion in humans of protein-bound *N*- $\epsilon$ -fructosyllysine. Glycosylated casein containing *N*- $\epsilon$ -fructosyllysine was consumed orally by 42 human volunteers. More than 90% of the lysine derivative was not recovered in the urine or feces. It may have been metabolized by microflora of the lower gastrointestinal tract, making it unavailable as a nutritional source of lysine.

Sherr et al. (1989) showed that monofructosyllysine was absorbed in significant amounts by rats and incorporated into liver microsomes. In contrast, the difructosyllysine derivative was not absorbed to any extent. About 72% of absorbed monofructosyllysine competitively retarded the absorption of free lysine while the difructosyl derivative reduced lysine absorption by blocking the absorption site.

Plakas et al. (1988) investigated the bioavailability of lysine in browned-fish-protein isolate by measuring the lysine content in plasma of rainbow trout fed the isolate. They demonstrated an 80% loss in bioavailable lysine following heating the fish isolate under mild conditions (40 days at 37 °C). The authors concluded that rainbow trout are similar to other animals in their inability to utilize the deoxyketosyllysine formed in the early stages of the Maillard reaction and that the plasma response in trout was a good indicator of biologically available lysine.

These and related studies on structure–nutritional utilization of lysine derivatives suggest that not only steric bulk of the molecules but charge and basicity must influence the lysine derivatives' susceptibility to enzymatic hydrolysis and their transport and utilization as a nutritional source of lysine.

Finot (1990) offers a useful summary of physiological and pharmacological effects of Maillard products which may adversely affect protein, mineral, and vitamin nutrition. Briefly, these effects include (a) inhibition of processes such as growth, protein and carbohydrate digestion, amino acid absorption, and activity of intestinal enzymes including aminopeptidases, proteases, and saccharidases, and pancreatic enzymes such as chymotrypsin; (b) induction of cellular changes in the kidneys (karyomegaly and hypertrophy), the liver (brown spots, hypertrophy, and decrease in enzyme production), and the stomach cecum (hypertrophy); (c) adverse effects on mineral metabolism (Ca, Mg, Cu, and Zn); and (d) variable effects on allergic response and cholesterol metabolism.

Von Wagenheim et al. (1984) examined kidney histopathology of rats fed casein heated with glucose for 4 days at 65 °C. Enlarged epithelial cells and nuclei were observed after 2 weeks of feeding. The average size of the nuclei increased with feeding time, with significant differences being observed after 6, 8, or 10 weeks of feeding.

Furniss et al. (1986) extended the above findings about browning-product-induced nephrotoxicity. They report that feeding either lysinoalanine or casein heated with glucose at 37 °C for 3 or 15 days induced nephrocytomegaly and was associated with urinary loss of Zn. Increased urinary loss of Cu was also reported. Kidney levels of Zn and Cu were higher than controls in the group fed heated casein–glucose. These observations suggest that alteration of the mineral content of the diet could minimize browning-induced kidney damage (Friedman and Pearce, 1989; Pearce and Friedman, 1988; O'Brien and Walker, 1988; O'Brien et al., 1994; Rehner and Walter, 1991). Studies are needed to define the role in nutrition and food safety of Maillard products such as the arginine derivative 2-(4-benzoylamino)-4-carbamoylbutylamino-5-(2,3,4-trihydroxybutyl)-4-imidazoline shown in Figure 7.

In a related study, Friedman et al. (1984b) showed that the presence of glucose during alkaline treatment of soybean proteins significantly lessened the amount of lysinoalanine formed. A possible explanation for this effect is that glucose-blocked  $\epsilon$ -NH<sub>2</sub> groups of lysine were unable to combine with dehydroalanine to form lysinoalanine. The potential of carbohydrates to prevent formation of kidney-damaging lysinoalanine merits further study.

The extent of severity of these effects will be influenced by the severity of the heat treatments, by the content and structure of different Maillard and cross-

linked products formed on heating, and by the health and susceptibility of the consumer. We are challenged to define processing conditions to minimize the formation of the most antinutritional and toxic compounds including those listed in Figures 3–7. This will only be accomplished by detailed chemical and nutritional studies with specific, well-defined Maillard products that will permit defining a relative potency scale of antinutritional properties. It should then be possible to reduce the content of the most antinutritional compounds in the diet.

**Beneficial Maillard Products.** *Antimutagens.* Antimutagens can be classified into desmutagens and bioantimutagens. Desmutagens inactivate mutagens by chemical or enzymatic modification (Kada et al., 1985). Biomutagens suppress the mutagenesis after the DNA has already been modified by the mutagen (*i.e.*, they suppress DNA metabolism).

Yen and Hsieh (1994, 1995) explored the effect of Maillard products prepared by heating lysine and xylose and lysine at pH 9 and 100 °C for 1 h on the mutagenicity of the heterocyclic amine 2-amino-3-methylimidazo-4,5-quinoline (IQ) using the *Salmonella* microsome assay (see Figures 8 and 9 and section below on these compounds). The mutagenicity of IQ in *Salmonella typhimurium* TA98 and TA100 was strongly inhibited. The authors discovered that the antimutagenic effect resulted from the reaction of the Maillard product with metabolites of IQ to form inactive adducts (desmutagenic effect), not by direct inhibition of hepatic microsomal activation, which transforms inactive IQ to a DNA biological alkylating agent (bioantimutagenic effect). The inhibitory effect of the glucose–tryptophan Maillard product can also be described as a desmutagenic effect.

Other mechanisms that have been proposed to explain the antimutagenicity of Maillard products include (a) scavenging of free radicals by melanoidin type compounds and (b) inhibition of enzyme activity of the S9 microsomal mixture needed to activate mutagens.

Flavones also inhibit the mutagenicity of IQ and Trp-P-2 by directly reducing the hepatic microsomal activation (Lee et al., 1992). These observations imply that cooking meat with plant foods such as carrots and potatoes which contain flavones and flavonoids may reduce or eliminate formation of heterocyclic amine-type mutagens. The mechanism of the inhibition may be the result of both bioantimutagenic and desmutagenic effects.

Kato et al. (1987) report that nondialyzable melanoidins derived from the Maillard reaction of glucose and glycine degrade nitrite and prevent mutagenic nitrosamine formation.

*Antioxidants.* Controlled browning is often used to develop desirable flavor, odor, and color properties in foods including coffee, bread, and soybean sauce. Such browning reactions often lead to the formation of naturally occurring antioxidants (Hodge et al., 1976). For example, Chiu et al. (1991) describe the formation of antioxidants during the Maillard reaction between tryptophan and fructose or glucose. Specifically, they synthesized a fructose–tryptophan Maillard product ( $\epsilon$ -N-1-deoxyfructosyl-L-tryptophan) and evaluated its effectiveness in preventing lipid oxidation in sardines. Using peroxide value (POV) as an index of lipid oxidation, they report that the Maillard product was a potent inhibitor of oxidation. The naturally occurring antioxi-

dant tocopherol had a synergistic effect on fructose–tryptophan against lipid oxidation.

Glucose–tryptophan Maillard products exerted antioxidative effects toward linoleic acid only after a long induction period (Tanaka et al., 1992). Additional studies revealed that advanced stage glucose–tryptophan Maillard products had a shorter induction period and were also effective antioxidants for sardine lipids. Antioxidative effects were measured with the aid of an oxygen electrode, which gives a reading of the amount of dissolved oxygen in the absence and presence of the Maillard product. The antioxidative effect was calculated as  $T(a)/T(c)$ , where  $T(a)$  is the elapsed time for a reduction of dissolved oxygen in the reaction mixture containing the antioxidant and  $T(c)$ , the corresponding time of the control sample without it.

Several properties of glucose–tryptophan Maillard products prepared under different conditions of time and temperature were correlated with antioxidative effects. These include (a) extent of browning, determined as absorbance at 420 nm; (b) fluorescence, with excitation at 452 nm and emission at 530 nm; and (c) reducing power, measured as absorbance at 700 nm after exposure of the Maillard product to dipycrylhydrazyl (DPHH). Reducing power, electron-donating ability, and all of the mentioned parameters correlated with the antioxidative effects. This implies that the more advanced stage Maillard products are stronger antioxidants than those formed at the early stages of the reaction.

Tanaka et al. (1992) found that oxidation of singlet oxygen generated by exposing methylene blue to light was strongly inhibited by fructose–tryptophan Maillard products and by tryptophan. This observation suggests that the scavenging of active oxygen species by Maillard products is an important mechanism of the antioxidative effects. Lipid oxidation takes place at the unsaturated part of the triglyceride. It is an autocatalyzed chain reaction consisting of initiation, propagation, and termination. The resulting aldehydes and ketones are responsible for the development of off-odors and off-flavors in a variety of fat-containing foods. Since the oxidation is catalyzed by oxygen, exclusion of oxygen suppresses oxidation. Lipid oxidation is enhanced by heat, light, heavy metals, the presence of pigments, and the degree of unsaturation in the lipid molecules. Antioxidants exert their effect by donating electrons or hydrogen atoms to free-radical-containing lipids and by forming antioxidant–lipid complexes. Any substance that inhibits the propagation step in the chain reaction, decomposes lipid hydroperoxides, chelates heavy metal ions, or prevents light- and/or radiation-induced initiation of the chain reaction can, in principle, serve as an antioxidant.

You (1993) isolated an antioxidative compound from the browning reaction mixture of L-ascorbic acid.

Bedinghaus and Ockerman (1995) found that reducing sugars and free amino acids generate antioxidants in cooked ground pork patties.

*Antibiotics.* Einarsson (1987) and Einarsson et al. (1983, 1988) carried out extensive studies on the inhibition of bacterial growth by Maillard reaction products. The products were prepared by refluxing solutions containing either arginine and xylose or histidine and glucose. The effects were measured by determining the minimum concentration that inhibits the growth of the microorganisms. The bacteria included *Lactobacillus*, *Proteus*, *Salmonella*, and *Streptococcus faecalis* strains, *i.e.*, both pathogenic and spoilage organisms found in

foods. The results demonstrate a wide range of susceptibility among the 20 strains tested for growth inhibition by the Maillard product. *Bacillus cereus* strains were not inhibited, whereas *Bacillus subtilis* strains were strongly inhibited. The different *Escherichia coli* strains showed marked differences in the extent of inhibition. The Gram-negative enterobacteria *Salmonella* strains were not inhibited to any great extent, while the Gram-positive bacteria, *B. subtilis*, *Lactobacillus*, and *Staphylococcus* strains, were strongly inhibited. Fractionation of the Maillard products permitted isolation of higher molecular weight compounds by dialysis. The higher molecular weight fractions had a greater inhibitory effect than the lower ones.

Evaluation of antibiotic potencies of Maillard products revealed that (a) products formed from arginine were more potent than those derived from histidine; (b) arginine mixtures with either glucose or xylose had the same inhibitory effects; (c) formation of antibacterial compounds was favored when the reaction between amino acid and carbohydrate was carried out at a slightly alkaline pH of 9; (d) the antibacterial effect increased with increase in reaction time in the mixture containing arginine; and (e) increasing the temperature of reaction for histidine-glucose from 80 to 120 °C enhanced the rate of formation of the antibacterial compounds. Although pentoses are usually more reactive than hexoses in the Maillard reaction, no differences in antibacterial properties were observed with mixtures containing either xylose or glucose.

Potential targets for the action of antibacterial antimicrobial Maillard compounds include (a) cellular membranes, (b) genetic material, and (c) bacterial enzymes. Einarsson et al. (1988) cite evidence in support of the involvement of all three targets. First, the Maillard products influence the solubility of iron, acting as an iron-chelating agent in analogy with EDTA. Second, because iron in bacterial cells is a cofactor in enzymes involved in metabolism of oxygen and its products, the presence of Maillard products should also inhibit oxygen uptake. This is indeed the case. Third, Maillard products also inhibited the uptake of glucose and serine by the bacteria. This effect could adversely alter energy metabolism of the bacteria. Finally, the reported inhibition of digestive enzymes by Maillard products and their ability to precipitate proteins suggests another pathway for the inhibition of bacterial growth. All of the cited events are probably operative in the inhibition.

**Antiallergens.** The Schiff base formed in the first step of the Maillard reaction is biologically available (Finot et al., 1977). Therefore, it does not adversely affect protein nutrition. Many foods present a favorable medium for the Maillard reaction, including allergenic foods such as milk. Antigenic sites of food proteins responsible for adverse allergic responses could be selectively altered by modification with reducing carbohydrates under mild conditions. Chemical and structural modification during food processing could be at least part of the basis for the observation that cow's milk is less antigenic *in vivo* after heat treatment and for apparent differences in the allergenicities of liquid and powdered soybean infant formulas. Relatively mild conditions of heating food proteins with carbohydrates can reduce the antigenicity and possibly modify sites known to elicit allergic responses. For example, Oste et al. (1990) heated a solid mixture of soybean trypsin inhibitor (KTI) and carbohydrates in an oven at 120 °C and analyzed the dialyzed product by enzyme-linked

**Table 2. Mutagen Formation in a Variety of Foods (Barnes et al., 1983)**

food	sample	cooking procedure	cooking time (min)	revertants per sample
white bread	slice	broiling	6	205
pumpernickel bread	slice	broiling	12	945
biscuit	each	baking	20	735
pancake	each	frying	4	2500
potato	small slice	frying	30	200
beef	patty	frying	14	21700

immunosorbent assays (ELISA). Glucose, lactose, and maltose decreased the antigenicity of KTI by 60–80%, compared to a control sample heated without carbohydrate. Starch was less effective than the three reducing sugars. The decrease was rapid, occurring within 10 min when glucose was heated with KTI, with retention of 60% of the chemically available lysine. Longer heating times increased browning and reduced the level of available lysine in KTI, without further reducing antigenicity (Brandon et al., 1993).

Matsuda et al. (1992) evaluated the carbohydrate binding specificity of monoclonal antibodies raised against lactose-protein Maillard adducts. They found that (a) different components of the lactose-bovine serum albumin adduct produced as the reaction proceeds could serve as antigenic determinants; (b) the carbohydrate residue of the Amadori compound  $\epsilon$ -deoxy-lactulosyllysine formed in the early stage of the Maillard reaction was specific for the haptenic antigen of the lactose-proteins Maillard complex; (c) the terminal galactose residue in the lactose-protein Maillard reaction product appears to be important for antibody recognition; (d) the antibodies did not recognize the glucose-protein Maillard reaction products such as  $\epsilon$ -deoxyfructosyllysine, possibly because their size is too small to fill the antibody combining sites; and (e) the monoclonal antibodies did not react with milk glycoproteins of fresh raw milk but did react with proteins in pasteurized milk. The cited observations suggest that monoclonal antibodies could detect lactose-protein Maillard adducts as possible milk allergens in pasteurized milk and milk products.

The magnitude of antigenicity changes is probably highly dependent on the experimental conditions. In addition, because of the relative importance of the Maillard reaction and the reactions of nonreducing carbohydrates, further studies is merited. Nevertheless, the results of the cited studies suggest that the early stages of the Maillard reaction can significantly affect protein antigenicity. It should be noted however, that these reactions can also introduce new antigenic determinants into a food protein (Laligant et al., 1995).

## MUTAGENS AND CARCINOGENS

**Mutagenic Maillard Products.** Mutagenic and carcinogenic products in cooked protein-rich foods (Tables 2 and 3) are formed by several mechanisms, including carbohydrate caramelization, protein pyrolysis, amino acid/creatinine reactions, and amino-carbonyl (Maillard) reactions, in which free amino groups condense with reducing sugars to produce brown melanoidins, furans, carbolines, and a variety of other heterocyclic amines (Friedman and Cuq, 1988; Friedman and Henika, 1991; Friedman et al., 1990a,b; Vagnarelli et al., 1991).

Amino acid-reducing sugar mixtures have been widely used to study mutagen formation. Less fre-

**Table 3. Carcinogenic Potency of Heterocyclic Aromatic Amines (HAAs) in Rats and Mice (Starvic, 1994a,b)**

HAA	concn in diet (%)	TD <sub>50</sub> <sup>a</sup> (mg kg <sup>-1</sup> /day <sup>-1</sup> )	
		rats	mice
IQ	0.03	0.7	14.7
	0.03		
MeIQ	0.03	0.1	8.4
	0.04		
MeIQx	0.04	0.7	11.0
	0.06		
Trp-P-1	0.015	0.1	8.8
	0.02		
Glu-P-1	0.05	0.8	2.7
Glu-P-2	0.05	5.7	4.9
AαC	0.04		5.8
PhIP	0.04	0.9	31.3

<sup>a</sup> TD<sub>50</sub> is the daily dose that induces tumors in 50% of the animals tested when given throughout their lives.

quently, nonenzymatic browning of some foods also may occur by chemical reaction of acidic and neutral amino acid residues with nonreducing sugar or even with polysaccharide carbohydrates. These precursors are in fact much more abundant in foods than the reactants required for classical Maillard reactions. Such an alternative source of food browning would involve chemical mechanisms quite different from the amino-carbonyl reaction, and correspondingly different mutagens may be formed.

Friedman et al. (1990b) heated gluten, carbohydrates, and gluten-carbohydrate blends in a simulation of low-moisture crust baking. The baked materials were then assayed by the Ames *Salmonella* his-reversion test to evaluate the formation of mutagenic browning products. An aqueous acetonitrile extract of heated gluten was highly mutagenic when assayed with *Salmonella typhimurium* strain TA98 with metabolic activation. Weak mutagenicity was also observed with strains TA100 and TA102. Gluten heated in a vacuum oven yielded less extract than did the air-baked protein, but its mutagenic activity (revertants per milligram of extract) was similar. Baked D-glucose, maltose, lactose, sucrose, wheat starch, potato amylose, cellulose, microcrystalline hydrocellulose, sodium ascorbate and L-ascorbic acid, sodium (carboxymethyl)cellulose, or (hydroxypropyl)methylcellulose were moderately mutagenic in strain TA98 with microsomal (S9) activation and were weakly mutagenic without microsomal activation in strains TA100, TA102, and TA1537. Heated blends of gluten with 20% of these carbohydrates were also mutagenic, but the total activity recovered did not exceed levels of the individual ingredients baked separately. Maillard-type melanoidins prepared from L-lysine and D-fructose were very weakly active with strain TA98 but were mutagenic without S9 activation in strains TA100, TA102, and TA2637.

Knize et al. (1994a,b, 1995) investigated the mutagenic activity from amino acids heated at cooking temperatures. They failed to find any known heterocyclic amine commonly found in cooked meats that would explain the observed mutagenicity. They suggest that several new aromatic amine mutagens formed by heating amino acids with arginine may be responsible for the reported mutagenicity of baked grain-based foods.

Hiramoto et al. (1993) showed that Maillard products of 1:1 solid glucose-amino acid mixtures heated at 200 °C for 5 min induced single-strand breaks of DNA incubated overnight at 37 °C and pH 7.4. The Maillard products did not induce double-strand breaks in the DNA. The authors suggest that the chemiluminescent Maillard products exert their DNA-breaking effect by generating singlet oxygen, which attacks the DNA.

**Nonclastogenic Browning Products.** Heated sugar-amino acid reaction mixtures known to contain products clastogenic and/or mutagenic to cells were evaluated for clastogenic activity in mice using the erythrocyte micronucleus assay (MacGregor et al., 1989). Heated fructose-lysine reaction mixtures were also evaluated in the *Salmonella* his-reversion assay and the Chinese hamster ovary cell (CHO) chromosomal aberration assay to confirm and extend previous *in vitro* observations. Significant mutagenicity of fructose-lysine mixtures was observed in *Salmonella* strains TA100, TA2637, TA98, and TA102, with greater activity in mixtures heated at pH 10 than at pH 7. Both pH 7 and pH 10 reaction mixtures of the fructose-lysine browning reaction were highly clastogenic (chromosome damaging) in CHO cells. Heated mixture of fructose and lysine, and of glucose or ribose with lysine, histidine, tryptophan, or cysteine, did not increase the frequency of micronucleated erythrocytes in mice when administered by the oral route. This indicates the absence of chromosomal aberrations in erythrocyte precursor cells. Evidently, the genotoxic components of the browned mixtures are not absorbed and distributed to bone marrow cells in amounts sufficient to induce micronuclei when given orally or are metabolized to an inactive form. Because sugar-amino acid browning reactions occur commonly in heated foods, it is important to evaluate further the *in vivo* genotoxicity of browning products in cell populations other than bone marrow (Kitts et al., 1993a,b).

**Heterocyclic Amines.** The relationship between dietary content and human disease such as cancer is a major concern for human health. A need exists to define the relationship between specific diet components and cancer and to devise strategies to minimize the formation of the most toxic compounds. Of special interest is the formation of heterocyclic amines in heat-processed fish and meats. These compounds are the most potent mutagens known. They induce a variety of tumors in rodents and are extremely potent hepatocarcinogens when tested in primates (Adamson, 1990; Negishi et al., 1990; Weisburger, 1991).

**Chemistry.** The mechanism of formation of a key precursor for the production of heterocyclic mutagenic compounds is shown in Figure 9. The precursor then undergoes further dehydration and cyclization to form the observed pyrrole and pyridine derivatives (Figure 10). The heterocyclic pyridines and pyrazines then undergo further transformation with participation of aldehydes and creatinine to produce imidazaquinolines and imidazaquinoxalines.

Another class of heterocyclic amines, the carbolines, are formed when free or protein-bound tryptophan is exposed to heat under food-processing conditions (Friedman and Cuq, 1989). Carbolines have been found in commercial foods such as beef extracts and fried hamburger and heated milk. Some of them show significant mutagenic activity in *S. typhimurium* tester strains after metabolic activation.

**Risk Assessment.** The potential for formation of

mutagens and carcinogens in foods during processing is a major area of concern for human health and safety, as documented in the following review on risk assessment. We will briefly describe possible risks associated with consuming mutagens in foods to which heterocyclic amines make a major contribution.

Bjeldanes et al. (1982) and Krone et al. (1986) report that in foods subjected to commercial baking, canning, dehydration, and related thermal treatments, levels of bacterial mutations are 3–17 times greater than spontaneous rates. Mutagen formation was related to heating time and to processing temperature, which ranged from about 100 to 200 °C.

Zhang et al. (1993) subjected a data base of 61 heterocyclic amines formed during food preparation and their deamino analogues to structure–mutagenic activity analysis using computer modeling programs to contribute to our understanding of the chemical basis for the biological activities of these compounds. They found that the major structural determinant of activity is the aromatic amino group associated with the heterocyclic amines. Quantum mechanical calculations showed that mutagenic potency associated with the amino groups derived from their contribution to the energy of the lowest unoccupied molecular orbital (LUMO).

The authors suggest that (a) the amino group of heterocyclic amines plays an essential role in the biological activity of these compounds, presumably as a result of oxidation to the corresponding hydroxylamine, which can form reactive nitrenium ions directly or following O-acetylation; (b) the cited bioactivation pathways then lead to direct reaction with cellular DNA; (c) the LUMO method can predict the electrophilicity of the amino group to the cited modification and thus the relative potency of a compound in this series; and (d) because mutagenic potency correlates with LUMO energy, which measures electrophilicity, structural features alone can account for both the probability and the potency of mutagenicity. It should thus be possible to both predict the mutagenicity of structurally different amines and devise cooking conditions to minimize formation of the most potent ones.

In a related analysis, Bogen (1994) extrapolated results from animal data to estimate cancer potencies of heterocyclic amines found in cooked foods. He concludes that estimates are consistent with an upper-bound cancer risk between  $10^{-3}$  and  $10^{-4}$  for an average lifetime cooked-beef intake of  $3.3 \text{ g kg}^{-1} \text{ day}^{-1}$ , which corresponds to about 0.5 lb/day.

Layton et al. (1995), on the basis of analysis of the diets of 3563 individuals and using cancer potencies based on body surface area, concluded that ingestion of PhIP is responsible for 46% of the overall risk and that consumption of cooked meat and fish products was responsible for about 80% of the total risk.

PhIP injected in lactating rats can undergo a dose-dependent transfer to the milk (Brittebo et al., 1994; Jägerstad et al., 1994). This finding implies that exposure of pregnant women to PhIP might also give rise to a significant exposure in the newborn and that milk could be used to monitor risk assessment of PhIP consumption by humans.

**Metabolism.** Alexander et al. (1994) and Turesky et al. (1994) offer the following summary of the metabolism of heterocyclic amines and the relevance of metabolic studies to human risk assessment: (a) cooked meats and fish contain more than a dozen heterocyclic amines at

the low parts per billion level; (b) all amines tested are carcinogenic in rodent assays; (c) IQ is a potent carcinogen in nonhuman primates; (d) activation of the amines by human tissues is comparable to that observed in rodents; (e) the most direct evidence for genetic damage is through measurement of DNA adducts in cells and their excretion products in urine; (f) analysis of urinary metabolites can provide information on the ability of humans to metabolically activate or detoxify the procarcinogenic amines; (g) MeIQx and IQ are rapidly absorbed from the gastrointestinal tract and transformed into several detoxification products which are excreted in the urine and feces; (h) amine acetylation and  $\text{N}^2$ -glucuronidation are important routes for the detoxification of MeIQx in rodents, nonhuman primates, and humans; and (i) human liver metabolically activates MeIQx and other amines through cytochrome-P450-mediated *N*-oxidation and subsequent esterification reactions to produce the ultimate carcinogenic metabolites.

In critical assessments of the significance of heterocyclic amines in the human diet, Bogen (1994), Bradfield et al. (1991), Loprieno et al. (1991), Schutt (1994), Skog (1993), Starvic (1994a,b), and Starvic et al. (1995) made the following observations: (a) Heterocyclic amines represent a potential risk factor in the etiology of human cancer; heterocyclic amines are the only known animal colon carcinogens that humans (except vegetarians) consume daily. Since all mutagens tested so far in animal studies have been found to be carcinogens, it is desirable, although difficult, to control levels of mutagens in food; the higher temperatures recommended to destroy pathogens in meat may lead to the formation of greater amounts of heterocyclic amines. Data on possible adverse effects of heterocyclic amines on the reproductive system or on teratogenicity are lacking. (b) DNA adducts with MeIQx are formed in a linear dose–response manner, suggesting the lack of a threshold dose for carcinogenicity. Levels of DNA adducts, but not the Ames test, are a reliable indicator of carcinogenicity; DNA adducts are biomarkers, reflecting current exposure to the carcinogenic compound and also representing initiation of chemical carcinogenesis. In intravenously fed monkeys (20 mg/kg, orally for 35 months) that developed liver tumors, total DNA adduct levels were highest in the liver (28.6 adducts/ $10^7$  nucleotides), followed by the kidney, heart, and bladder. (c) IQ and MeIQ induce neoplasms at multiple organ sites in rodents and monkeys; doses required to produce tumors in laboratory animals for IQ and MeIQ were 10–20 mg/kg of body weight, much higher than the amounts found in human diets. PhIP was the most abundant amine in human food, followed by MeIQx; a person consuming a 250 g serving of processed meat will consume about 2500 ng of PhIP and 5 ng of IQ.

The following specific findings reinforce the cited summary: Kerdar et al. (1993) studied DNA adduct formation of IQ and PhIP by means of a  $^{32}\text{P}$ -postlabeling technique. Rats and cells expressing cytochrome P450 1A1 and/or human acetyltransferases NAT1 and NAT2 formed common metabolites and DNA adducts. Additional studies showed that (a) there appeared to be no adducts formed *in vitro* which are not formed *in vivo*, suggesting that metabolic activation of IQ and PhIP *in vivo* occurs exclusively at the amino group; and (b) metabolic activation of IQ in cultured cells expressing human cytochrome P450 and human acetyltransferase

produce intermediates and adducts indistinguishable from those observed in other systems.

Sinha et al. (1994) found that in humans heterocyclic amines are activated *in vivo* by cytochrome P450 1A2 and by acetyltransferase (NAT2) to mutagens and carcinogens. They studied the induction of P450 1A2 and NAT2 in 66 healthy humans consuming pan-fried meat. Meat pan-fried at low temperature (100 °C) had no detectable levels of the amines. Meat pan-fried at 250 °C, however, contained high levels: 9.0 ng/g MeIQx, 2.1 ng/g DiMeIQx, and 32.8 ng/g PhIP.

Evidently, (a) meat samples cooked at high temperature contain components which induce P450 1A2, suggesting that this activates the procarcinogens MeIQx, DiMeIQx, and PhIP; and (b) because of wide variability in the induction of the enzyme among the 72 individuals tested and because some individuals were not affected, genetic factors also must contribute to the relative susceptibility of individuals to enzyme induction and carcinogen activation.

*Minimizing Formation and Chemoprevention.* The cited considerations suggest a need to develop new approaches and strategies to prevent the formation during food processing of heterocyclic amines and other browning products (Rhee et al., 1987; Tanaka, 1994).

Skog (1993) and Skog et al. (1995) discuss possible approaches to minimize heterocyclic amine/mutagen/carcinogen formation during cooking. These include type of cooking procedure, cooking temperature, and the role of added food ingredients.

Well-done meat contains more than 10 times the concentration of mutagens than rare-cooked meat, so that keeping the temperature as low as possible should minimize mutagen formation. Discarding the pan residue of pan-fried meat, and substituting commercial bouillon or gravy, will also lower mutagenic potential. Bovine kidney and liver contained low levels of creatinine needed for mutagen formation and consequently showed low mutagenicity after frying compared to other parts of the animal subjected to the same type of frying.

Although a certain amount of glucose is needed for mutagen formation, excess glucose, lactose, and milk powder inhibit the formation of mutagens in fried beef patties.

Roasting at 180 °C produced few mutagens in cooked meat compared to other methods of preparation. Microwave cooking of beef patties, and discarding the dripping before frying, resulted in production of low levels of mutagen, presumably because the water-soluble precursors for mutagen formation (amino acids, glucose, and creatinine) were discarded. Soaking or marinating beef steaks before frying may achieve the same objective. Similarly, coating beef steaks with bread crumbs before frying may also reduce mutagen formation since the coat may act as an insulating layer, reducing the outer meat temperature.

Weisburger (1991) reports that mutagen formation was reduced when the amino acid tryptophan was added to a meat sauce which was then applied to the surface of meat before frying. Tryptophan and other amino acids such as proline that sometimes inhibit mutagen formation may compete with creatinine for the reactive intermediates involved in mutagen formation. The reported inhibition of mutagen formation by soy protein concentrates may be due to a higher water content of the fried minced beef (Wang et al., 1982).

Arimoto et al. (1993) and Hayatsu et al. (1993) report that porphyrins such as hemin, chlorophyll, chlorophyl-

lin, and phthalocyanine inhibit the mutagenicity of heterocyclic amines and other mutagens. Edenharder et al. (1995) report that chlorophyll in green beans, broccoli, and spinach reduces considerably the mutagenicity of IQ and MeIQx. The metal ions in the center of the porphyrin molecule are not required for the inhibition. The inhibitory activities were related to the adsorption capability of structurally different porphyrin moieties.

Guo and Dashwood (1994) and Breinholt et al. (1995) found that chlorophyllin produced dose-related inhibition of IQ-DNA binding in the small and large intestines and livers of rats. Simultaneous ingestion of chlorophyll and IQ appears to offer the best protection against tumorigenesis. Although chlorophyll is considered to be relatively nontoxic, the possibility that high doses may promote carcinogenesis cannot be ruled out.

Heme-containing proteins such as hemoglobin and myoglobin but not globin, which lacks the heme part of hemoglobin molecule, also inhibited activated forms of the heterocyclic amines such as Trp-P-2, presumably by blocking DNA alkylation. Preliminary *in vivo* studies with humans showed that consumption of cooked beef together with chlorophyllin diminished the urinary mutagenicity induced by meat ingestion (Hayatsu et al., 1993).

Since the desmutagenic and anticarcinogenic effects of chlorophyll appear to be due to formation of face-to-face  $\pi$ - $\pi$  complexes between the planar aromatic rings of the amines and chlorophyll, highly aromatic tetra- and octaarylporphyrins (Friedman, 1965) should also bind strongly to heterocyclic amines.

Johansson et al. (1993) found that the addition of corn oil, olive oil, or linoleic and/or linolenic acid to a model system increased the amount of MeIQx formed compared to the amount observed without fatty acid addition. This finding suggests that the formation of food mutagens can increase when frying is prolonged in the presence of certain fats. This is often the case during deep-fat frying of meat and fish with oils heated for several hours.

In contrast to the cited observation that lipids promote the production of IQ in model systems, Johansson et al. (1993) and Schutt (1994) found that menhaden oil, a fish oil high in  $\omega$  fatty acids such as eicosapentaenoic and docosahexaenoic acids, inhibits hepatic and small intestine IQ-DNA adduct formation in rats. Since menhaden oil may also inhibit tumorigenesis at the promotional initiation stages, dietary  $\omega$  fatty acids may prevent carcinogenesis through multiple mechanisms.

Johansson and Jägerstad (1994) and Skog et al. (1995) investigated the formation of heterocyclic amines in meat and fish prepared under domestic conditions. They report that in most products the amount was low, except in pan residues, especially from frying sausage, which contained 18.5 ng of amines/g; fried bacon and well-done bacon contained 10.5 ng/g. Smoked fish prepared by a hot smoking process for 406 h at 80–85 °C contained 0.8 ng of MeIQx/g. In contrast, fried salmon contained 5.7 ng of amines/g. Possible inhibiting effects of antioxidants were also investigated.

Since antioxidants such as vitamin C,  $\beta$ -carotene, tocopherols, *N*-propylgallate, and *tert*-butylhydroquinone decreased the formation of IQ compounds, free radical reaction may be involved in the formation of heterocyclic amines. One possibility is that the hydroxylamine IQ derivative can alkylate DNA both as a



nitrenium ion or as a free radical. Little is known about possible synergism of combinations of amines.

Edenharder et al. (1995) report that lignin reduces the mutagenic activity induced by IQ.

Hirose et al. (1995) found that antioxidants can prevent PhIP-induced mammary carcinogenesis in rats.

Kanazawa et al. (1995) and Samejima et al. (1995) suggest that the antimutagenic action of flavone and flavonoid phytochemicals against Trp-P-2 found in dried and grilled sardines is due to their ability to act as antioxidants, adsorbents, and inhibitors of cytochrome P450 enzymes.

Apostolides and Weisburger (1995), Kinae et al. (1994), and Yen and Chen (1994) showed that tea extracts inhibited mutagenic activities of heterocyclic amines.

Oral consumption of caffeine led to a 47% reduction in the number of mutants induced by MeIQx (Alldrick et al., 1995).

Conjugated linoleic acids (CLAs) protected rats against IQ-induced colon cancer (Liew et al., 1995; Pariza, 1991).

Consideration of the cited studies suggests that sufficient information is now available to devise processing conditions and diets to minimize adverse effects of heterocyclic amines.

#### PREVENTION

Additional conditions could be devised to minimize or prevent the formation of browning products during food processing or to prevent their deleterious effects in animals. The following approaches could be used to prevent or minimize food browning and the consequent antinutritional and toxicological manifestations.

**Sulfhydryl Compounds.** Sulfur-containing amino acids such as cysteine, *N*-acetylcysteine, and the tripeptide glutathione play key roles in the biotransformation of toxic compounds by actively participating in their detoxification. These antioxidant and antitoxic effects are due to a multiplicity of mechanisms including their ability to act as (a) reducing agents, (b) scavengers of reactive oxygen (free radical species), (c) destroyers of fatty acid hydroperoxides, (d) strong nucleophiles that can trap electrophilic compounds and intermediates, (e) precursors for intracellular reduced glutathione, and (f) inducers of cellular detoxification (Davis and Snyderwine, 1995; Friedman, 1994; Kroh et al., 1989; Stevens et al., 1995).

Thus, positive results were expected from an evaluation of the effectiveness of sulfur amino acids and sulfur-rich proteins to (a) prevent the formation of toxic browning products by trapping intermediates and (b) reduce the toxicity of browning products in animals by preventing activation of such compounds to biologically active forms. These expectations were fulfilled, as evidenced by the following observations on the prevention of both enzymatic and nonenzymatic browning by sulfur amino acids (DeFlora et al., 1989; Friedman, 1994a,b; Friedman and Bautista, 1994; Friedman and Molnar-Perl, 1990; Friedman et al., 1982b, 1992; Molnar-Perl and Friedman, 1990a,b).

Reflectance measurements were used to compare the relative effectiveness of a series of compounds in inhibiting browning in freshly prepared and commercial fruit juices including apple, grape, grapefruit, orange, and pineapple juices. For comparison, related studies were carried out with several amino acids and protein-containing foods such as casein, barley flour, soy flour, and nonfat dry milk and a commercial infant formula.

**Table 4. Prevention of Browning in Protein-Containing Foods by Sodium Bisulfite and *N*-Acetyl-L-cysteine (Molnar-Perl and Friedman, 1990a)**

protein source	inhibition (%)								
	sodium bisulfite (mM)				<i>N</i> -acetyl-L-cysteine (mM)				
	25	50	100	200	2.5	6.2	125	250	500
casein	12	44	82	100	0	25	42	101	101
barley flour	43	61	98	95	36	42	79	96	104
soy flour	27	80	98	102	19	38	84	99	101
nonfat dry milk	23	44	94	104	19	43	78	98	101
Isomil	29	72	88	100	7	43	65	93	109

The results show that SH compounds do indeed inhibit browning reactions (Table 4). They merit study for their ability to prevent browning in potato chips (Khanbari and Thompson, 1993) and by oxidized lipids (Gardner et al., 1985; Gavish and Breslow, 1991).

The cited studies show that under certain conditions, SH-containing compounds may be as effective as sodium sulfite in preventing both enzymatic and nonenzymatic browning. Studies on the effects of concentration of inhibitors, storage conditions, and pH revealed that *N*-acetyl-L-cysteine, cysteine ethyl and methyl esters, and reduced glutathione were nearly as effective on a molar basis as sodium sulfite in preventing browning of both apples and potatoes.

Although mechanisms of enzymatic browning prevention are not well understood, any event that can disrupt PPO-catalyzed oxidation of phenolic compounds should reduce browning. Inhibition may occur directly or indirectly. Direct inhibition changes the copper-containing active site of the enzyme. Indirect inhibition reacts with intermediates, preventing further transformation of the quinone to brown pigments (Figure 8).

The reported antibrowning effect of ascorbic acid (Sapers and Miller, 1992) may be due to its ability to reduce quinones to hydroxyphenols.

**Acetylation of Amino Groups.** Modifications of amino groups prevent them from participating in browning reactions. For example, treatment of foods with the enzyme transglutaminase will transform lysine amino groups to amide groups. The former initiate browning, whereas the latter do not (Friedman, 1978; Friedman and Finot, 1990).

**Antioxidants.** Our studies of browning in simulated bread crust (Ziderman and Friedman, 1985) revealed that oxygen seems to be required for nonenzymatic browning. We showed that baking in a vacuum oven caused less damage. These results imply that antioxidants should suppress browning in foods. A number of antioxidants that are on the GRAS (generally accepted as safe) need to be evaluated for their potential to minimize nonenzymatic browning during baking and storage.

These considerations suggest that trapping or preventing the formation of intermediates in the Maillard and related reactions (Figures 1–9) may prevent the formation of undesirable compounds during food processing.

**Deglycation.** Watanabe et al. (1987) discovered that an extract from soil microorganisms catalyzed the deglycation of  $\alpha$ - and  $\epsilon$ -fructosyllysines to lysine. This finding suggests that these purified enzymes could be used to prevent or reverse Maillard reactions in foods and *in vivo* provided they are safe in other regards.

Gerhardinger et al. (1995) describe deglycation of Amadori compounds by a bacterial enzyme, fructosyl-*N*-alkyl oxidase (EC 1.5.3), which they isolated from a

*Pseudomonas* strain. The usefulness of such "Ama-doriases" to reverse browning reactions in foods and *in vivo* also merits study (Jiang et al., 1989).

Finally, Vertommen et al. (1994) found that feeding the flavonoid diosmin to diabetic rats reduced formation of glycated collagen and hemoglobin compared to control animals. Diosmin also decreased the formation of malondialdehyde, a product of lipid peroxidation.

## CONCLUSIONS

Future studies should emphasize the prevention of browning and the consequent antinutritional and toxicological manifestations of browning products in whole foods as consumed. Many of the safety concerns cited, especially those of genotoxic potential, are based on *in vitro* data which may not always be relevant to *in vivo* effects following the consumption of whole food products containing the browning-derived constituents. The presence of other dietary constituents in the food and the process of digestion and metabolism can be expected to decrease or increase the adverse manifestations of browning products.

The proposed approaches to preventing adverse consequences of food browning need to be coordinated with analytical, chemical, and appropriate animal studies to identify which food ingredients have the greatest antinutritional or toxicological potential. In cases for which only *in vitro* effects have been reported, appropriate *in vivo* genotoxicity assays should be used to assess the potential for DNA and chromosome damage. Such information will lead to the development of a ranking scale of relative toxicity of browning products in specific foods. This, in turn, will permit our efforts to be directed toward minimizing or preventing the formation of the most deleterious food ingredients. Most urgent is the need to develop food-processing conditions to prevent the formation of carcinogenic heterocyclic amines and to define the safety of amino acid oxidation products that may form *in vivo*, especially following excess protein intake (Friedman, 1996).

Understanding the chemical, nutritional, and toxicological consequences of browning reactions and related transformations *in vitro* and *in vivo* can lead to better and safer foods and feeds and improved human health.

I conclude with a word of caution about the general value of antioxidants in disease prevention, in view of apparent conflicting findings about beneficial effects from clinical and epidemiological studies (Rautalahti and Huttunen, 1994; Tanaka, 1994).

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