Reactions of Monosaccharides during Heating of Sugar−Casein Systems: Building of a Reaction Network Model

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The Maillard reaction is important during the heating and processing of foods for its contribution to food quality. To control a reaction as complex as the Maillard reaction, it is necessary to study the reactions of interest quantitatively. In this paper the main reaction products in monosaccharide−casein systems, which were heated at 120 °C and pH 6.7, were identified and quantified, and the reaction pathways were established. The main reaction routes were (i) sugar isomerization, (ii) degradation of the sugar into carboxylic acids, and (iii) the Maillard reaction itself, in which not only the sugar itself but also its reaction products react with the ϵ-amino group of lysine residues of the protein. Significant differences in reaction mechanism between aldose and ketose sugars were observed. Ketoses seemed to be more reactive in the sugar degradation reactions than their aldose isomers, and whereas the Amadori product was detected as a Maillard reaction intermediate in the aldose−casein system, no such intermediate could be found in the ketose−casein system. The reaction pathways found were put together into a model, which will be evaluated by kinetic modeling in a subsequent paper.

Keywords: *Maillard reaction; isomerization; monosaccharides; glucose; fructose; galactose; tagatose; casein; reaction network model*

INTRODUCTION

Heating is a frequently used process in the food industry to obtain safe products with a prolonged shelf life. Heat is also used to improve the sensory properties of food. However, it may also cause changes that decrease food quality. Many desired but also undesired effects of heating are due to the Maillard reaction.

The Maillard reaction is a type of nonenzymatic browning which involves the reaction of carbonyl compounds, especially reducing sugars, with compounds that possess a free amino group, such as amino acids and proteins. The reaction products are significant in foods because they are responsible for flavor and color, which may be desirable or undesirable depending on the type of food. The Maillard reaction can also result in nutritional damage, in the development of components with antioxidant properties (*1*), and in the formation of mutagenic and antimutagenic compounds (*2*). The Maillard reaction is actually a complex network of chemical reactions, which is traditionally divided into three stages: the early, advanced, and final Maillard reaction. The early Maillard reaction between an aldose sugar and an amino group leads to the formation of the Amadori product as a relatively stable intermediate. The Heyns compound is supposed to be formed as the analogous compound when a ketose sugar is the starting sugar. In many foods, the ϵ-amino groups of the lysine residues of proteins are the most important source of reactive amino groups. Due to blockage in the Amadori product, these lysine residues are no longer available for digestion and consequently the nutritive value decreases. In the advanced Maillard reaction the Amadori product is broken down into numerous fission products. In this stage flavor compounds are formed. In the final stage of the Maillard reaction, reaction products condense with amino compounds and form high molecular weight compounds, the melanoidins, which are mainly responsible for the brown color (*3*). In this stage also, proteins are cross-linked to a considerable degree (*4*).

Another type of nonenzymatic browning occurring during heating is caramelization, a complex process in which sugar reaction products condense and form brown macromolecules (without nitrogen). This caramelization reaction is preceded by sugar isomerization and sugar degradation reactions. Monosaccharides in aqueous alkaline medium undergo both reversible and irreversible transformations (*5*). The reversible reactions include (i) ionization, resulting in an equilibrium of neutral and ionized monosaccharides; (ii) mutarotation, resulting in an equilibrium of the different cyclic hemiacetal structures of monosaccharides; and (iii) enolization, resulting in the transformation of interconvertible monosaccharides. The isomerization via the enolization reaction is known as the "Lobry de Bruyn-Alberda van Ekenstein transformation" (*6*) and is accompanied by (iv) irreversible transformation of the monosaccharides into carboxylic acids, generally known as the alkaline degradation reaction. According to Berg and Van Boekel (*7*), the degradation products of lactose found in heated milk appeared to be mostly the same as those mentioned in the degradation route of monosaccharides in alkaline medium. This means that the described sugar reactions not only occur in alkaline medium but also take place at neutral pH (the pH of milk is ∼6.7).

Sugar isomerization and degradation reactions were reported to be much more important from a quantitative

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point of view than the Maillard reaction (*7*, *8*). Because these sugar reactions occur simultaneously with the Maillard reaction and the sugar reaction products subsequently take part in the Maillard reaction, the Maillard reaction becomes even more intricate. To be able to control the Maillard reaction, it is necessary to study the reactions of interest quantitatively.

The objective of this paper was to identify and quantify the main reaction products in heated monosaccharide−casein systems and to establish the main reaction pathways. Glucose and galactose (aldose sugars) and fructose and tagatose (ketose sugars) were the studied monosaccharides. In this paper a model for the reaction mechanism will be proposed, amenable to quantitative purposes. A subsequent paper will deal with the kinetic analysis of the reactions.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Glucose, fructose, and galactose were supplied by Merck (Darmstadt, Germany). Fluka Chemie (Buchs, Switzerland) supplied tagatose. Sodium caseinate (a spray-dried powder) was obtained from DMV (Veghel, The Netherlands) and contained 90% protein.

Preparation of Reaction Model Systems. Sodium caseinate (3% w/w) and sugar (150 mM monosaccharide) were dissolved in a phosphate buffer (0.1 M; pH 6.8) to give a molar ratio of sugar to lysine residues of 10:1. The samples were heated for various times (0−40 min) at 120 °C in an oil bath in screw-capped glass tubes (Schott, 16 × 160 mm). The chosen heating temperature corresponds to sterilization temperatures usually used in the food industry. The reported heating times include the heating-up period of ∼2−3 min. After a given heating time, samples were cooled in ice water, prior to analysis. The reaction mixtures were heat-treated and analyzed in at least duplicate.

Analyses of Sugars and Organic Acids. After heating, sugars and organic acids were separated from the protein via Sephadex G25 disposable columns (NAP-25, Pharmacia, Uppsala, Sweden). A sample of 1 mL was brought on the column and was eluted with 10 mL of water. The last 6 mL, containing the sugars and organic acids, was analyzed by HPLC using an ion-exchange column (ION-300, Interaction Chromatography Inc., San Jose, CA). The eluent consisted of 0.0025 M sulfuric acid in water, the flow rate was 0.4 mL/min, and the column was kept at 85 °C. Sugars were detected by monitoring the refractive index and organic acids by their UV absorbance at 210 nm.

Analyses of Total Acid Formation. Titrations were performed to determine total acid formation in heat-treated samples. Samples of 15 mL of reaction mixture were titrated with 0.1 N NaOH to pH 8.3. From the difference in added NaOH between the heated and unheated sample the total amount of acid formed was calculated.

Analyses of Available Lysine Residues. Samples of 0.5 mL were diluted with 1.5 mL of sodium dodecyl sulfate (SDS; 16% w/w) and refrigerated overnight. Available lysine residues were determined after derivatization with *o*-phthaldialdehyde (*9*). A fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.) was used at emission and excitation wavelengths of 430 and 340 nm, respectively.

Analyses of Amadori Compound. The Amadori compound was determined by means of furosine, using HPLC. Furosine is one of the reaction products of the acidic hydrolysis of the protein-bound Amadori compound. A sample was mixed with hydrochloric acid (end concentration = 8 M), sparged with nitrogen for 2 min, and heated in an oven for 23 h at 110 °C. The hydrolyzed solution was centrifuged, and the supernatant was purified by means of a solid phase extraction cartridge (Waters, Milford, MA). The eluate was injected on a furosine dedicated column (Alltech, Breda, The Netherlands), and furosine was detected by its UV absorbance at 280 nm (*10*). Furosine concentration was recalculated to that of the Amadori compound using a conversion factor of 3.1 (*11*). This factor was confirmed by our own research using the periodate assay (*12*). In this method formaldehyde is released by periodate oxidation of C1-hydroxyls. The formaldehyde is converted to a chromophore (diacetyldihydrolutidine) by reaction with acetylacetone in ammonia, which can be determined spectrophotometrically at 405 nm.

Analysis of Heyns Compound. Methods developed to determine glycated protein are mainly focused on protein glycated with aldoses, the so-called Amadori compounds. Methods to detect Heyns compounds are hard to find. According to Rutkatt (*13*), carboxymethyllysine (CML) is formed after periodate oxidation of both fructosylated and glucosylated proteins and is therefore useful for detection of the Heyns compound. In the present study, heat-treated samples were oxidized and hydrolyzed as described by Badoud and coworkers (*14*). CML was detected by HPLC after derivatization with dabsyl (*15*). Dr. R. Badoud generously provided the external CML standard.

Analysis of Heterocyclic Compounds. In the protein-free fraction, obtained via the Sephadex G25 columns, the compounds 5-(hydroxymethyl)furfural (HMF), furfuryl alcohol, 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone (HHMF), and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) were determined by HPLC, using a reversed phase column (Lichrosorb RP-18, Merck). The eluent was 7.5% methanol in water, and the flow rate was 0.8 mL/min. Furfuryl alcohol was detected by its UV absorbance at 220 nm, the others by their UV absorbance at 280 nm.

Analyses of Methylglyoxal. α-Dicarbonyl compounds are highly reactive reaction intermediates but can be trapped with *o*-phenylenediamine (OPD). The then formed quinoxaline derivatives can be detected by HPLC using a C18 column (Lichrosorb RP-18, Merck) and measuring the absorbance at 320 nm (*16*). The eluents were water (A) and methanol (B) with a gradient of 20−100% in 35 min and a flow rate of 0.5 mL/min.

Analyses of Brown Compounds. The browning intensity of the heated reaction mixtures was determined by measuring the absorbance at 420 nm with a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The samples were diluted four times with SDS (16% w/w) to reduce scattering due to protein aggregates. If necessary, the samples were diluted once more with water. The browning of the protein-free fraction was measured without dilution. The browning of the protein fraction was calculated by subtracting the browning of the sugar fraction from the browning of the total mixture. The absorbance can be recalculated to the concentration of melanoidins by using the Lambert−Beer equation. The extinction coefficient needed to solve this equation is a constant, and its value depends on the kind of amino acid (*17*, *18*). The extinction coefficient of protein-bound melanoidins formed in glucose−casein and fructose−casein systems was measured to be 500 $mol^{-1} \cdot L \cdot cm^{-1}$ (unpublished results). The concentration of melanoidins is thus expressed as sugar units incorporated in the brown products.

Mass Balance. The total concentration of reactants and reaction products was calculated. To determine whether the main reaction products were identified, the mass balance was expressed as a percentage of the initial sugar concentration.

RESULTS AND DISCUSSION

Identification of Reaction Products. During heating of the glucose−casein and fructose−casein systems the concentration of reactants decreased and reaction products were formed (Figures 1 and 2). The main reaction product detected in a heated glucose−casein system was fructose. In the fructose−casein system glucose was formed in considerable amounts. No other sugars were detected. In both sugar−casein systems, formic acid and acetic acid were determined. In the glucose−casein system protein-bound fructosyllysine

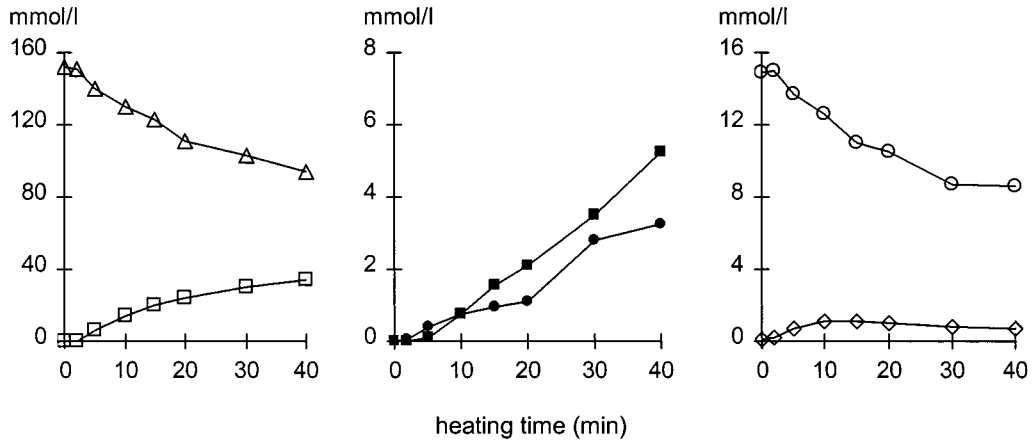

Figure 1. Glucose–casein solutions heated at 120 °C: glucose (△); fructose (□); formic acid (●); acetic acid (■); lysine residues (○); Amadori compound (◇).

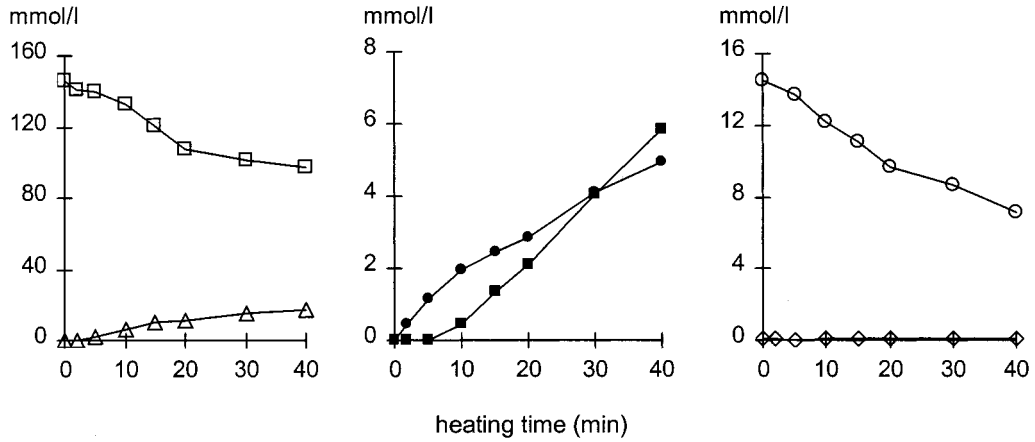

Figure 2. Fructose–casein solutions heated at 120 °C: fructose (□); glucose (△); formic acid (●); acetic acid (■); lysine residues (○); Amadori compound (◇).

was detected as the Amadori product. The Heyns compound glucosyllysine was not detected in the heated fructose–casein system, and only a very small amount of Amadori compound was found.

The formation of organic acids caused pH decreases of 0.3 pH unit in a glucose–casein system and 0.4 pH unit in a fructose–casein system after heating for 40 min at 120 °C (Figure 3). The formation of acid, as determined by titration, was in both sugar–casein systems considerably higher than the total amount of acetic and formic acid found by HPLC (Figure 3).

Other identified compounds were HMF, furfuryl alcohol, HHMF, and DDMP. However, HMF and furfuryl alcohol were formed in very low amounts (0–40 μM). HHMF and DDMP could not be quantified because no reference material was available, but they could be identified via their spectra, which were generously provided by Dr. M. Pischetsrieder. These compounds were presumably also formed in low amounts (as judged using the response factor of HMF).

The concentration of protein-bound brown compounds (measured as the amount of sugar incorporated) was calculated from the absorbance data (Figure 4). More browning was observed in the fructose–casein system than in the glucose–casein system.

The results of the mass balance calculations (Figure 5) showed an almost negligible amount of missing compounds after 40 min of heating at 120 °C when all reaction products including brown compounds and unidentified acids were calculated. Of course, many more reaction products were formed, but the fact that we come to an almost 100% recovery indicates that the acids formed are stable end products of scission reactions leading to numerous C1–C5 reaction products. Between 10 and 30 min more compounds were missing.

The reaction products that were detected in the heated galactose– and tagatose–casein systems were comparable with those in the glucose– and fructose–casein systems (results not shown). The decrease of the reactants and the increase of reaction products were more rapid for the galactose– and tagatose–casein systems compared to the glucose– and fructose–casein systems, respectively. The more rapid reaction of galactose compared to glucose is in line with the literature (*19*).

Identification of Reaction Pathways. Sugar isomerization, in which aldoses and ketoses can isomerize into each other, is one of the main reaction pathways in the sugar–casein systems. Another important reaction route was the Maillard reaction, in which sugars react with the lysine residues. Loss of lysine residues was about equal or somewhat higher in the heated ketose–casein system than in the aldose–casein system. In the aldose–casein system the loss of lysine could partly be explained by the formation of Amadori compound, partly, because the concentration of Amadori compound did not equal the loss of available lysine. The Amadori compound was subject to substantial breakdown after an initial buildup phase, whereas increasing loss of lysine must have been due to formation of

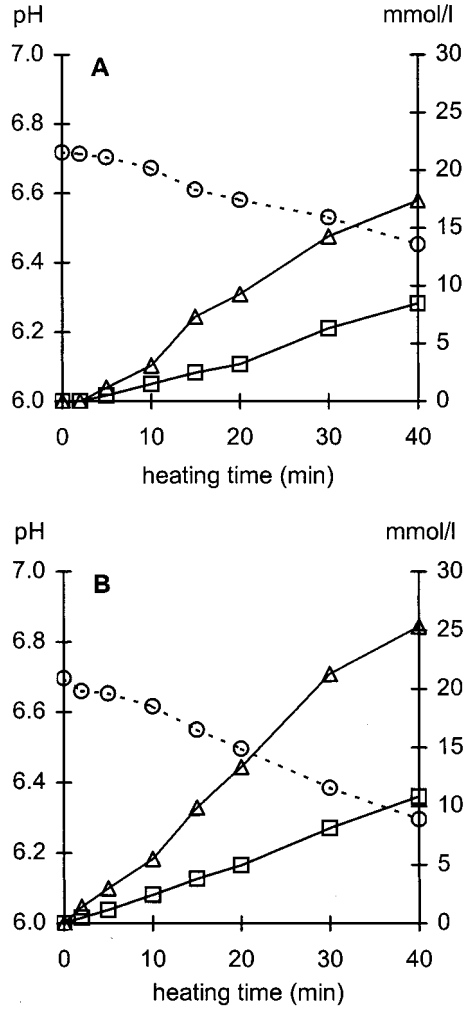

Figure 3. pH (○) and total amount of acids as found by titration (△) and HPLC (□) in heated glucose−casein (A) and fructose−casein (B) systems.

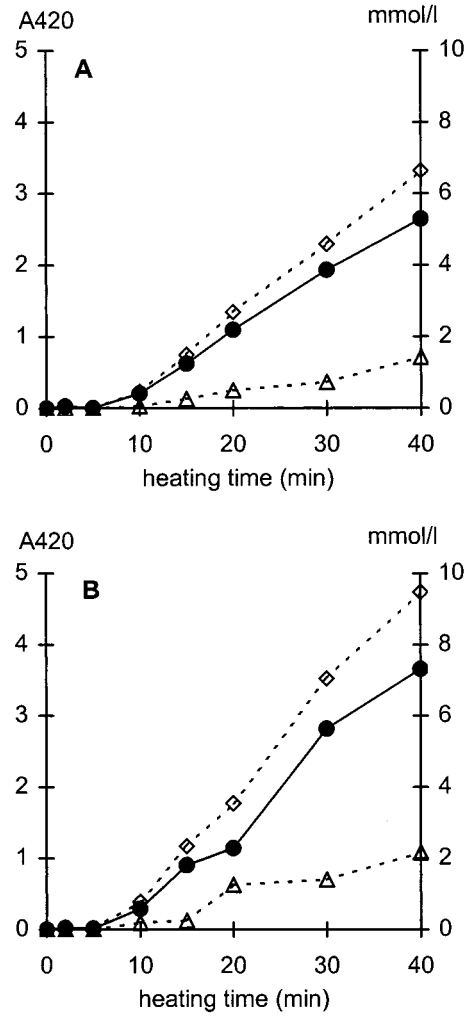

Figure 4. Browning of total system (◇), protein fraction (●), and sugar fraction (△) expressed in absorbance units measured at 420 nm and concentration of protein-bound melanoidins (●) in heated glucose−casein (A) and fructose−casein (B) systems.

advanced and final Maillard reaction products. It should be noted that advanced Maillard products are to a large extent bound to protein, presumably via lysine. In the heated ketose−casein system no product of the early stage of the Maillard reaction was found. This may mean that it was not formed at all or that it was quickly degraded again after formation and that lysine residues were incorporated in advanced and final Maillard reaction products.

An important group of reaction products was the carboxylic acids. It was, however, not clear whether these acids were formed via the sugar degradation pathway or in the Maillard reaction due to breakdown of the Amadori or Heyns product. To get a better insight in the reaction pathways, two additional experiments were performed.

In the first experiment, glucose and fructose solutions were heated in the absence of protein (remaining conditions were kept unchanged) and reaction products were determined. The results are shown in Figure 6. Besides a decrease of original sugar and an increase of isomer sugar, both formic acid and acetic acid were formed. The amounts of formic and acetic acid formed in the fructose system and in the fructose−casein system were about equal. In the glucose system, on the contrary, the amount of organic acids was much lower than in the glucose−casein system, especially that of acetic acid. In both systems a lag time was observed for the formation of acetic acid, which was evidently longer for the glucose system than for the fructose system. Apparently, an intermediate was formed in the degradation reaction of fructose into acetic acid and an extra intermediate, supposedly fructose, was involved in the degradation reaction of glucose into acetic acid. It was also observed that the glucose system browned only slightly, whereas the fructose system browned much more, although not as intensely as the fructose−casein system. This observation is in line with the literature. Pilar Buera and co-workers (*20*) noticed that caramelization browning contributed noticeably to total browning in a fructose−glycine solution, whereas it could be neglected in glucose−glycine solutions.

In a subsequent experiment the Amadori compound was isolated and heated. Formation of protein-bound Amadori product was induced by incubating 150 mM glucose with 3% casein at 65 °C for 15 h. After cooling to room temperature, the glycated protein was separated from sugars and reaction products via the Sephadex G25 columns as described under Materials and Methods. The solution containing protein-bound Amadori product (it was checked that no unbound sugar was present anymore) was heated at 120 °C, and reaction products were determined (Figure 7). Both acetic acid

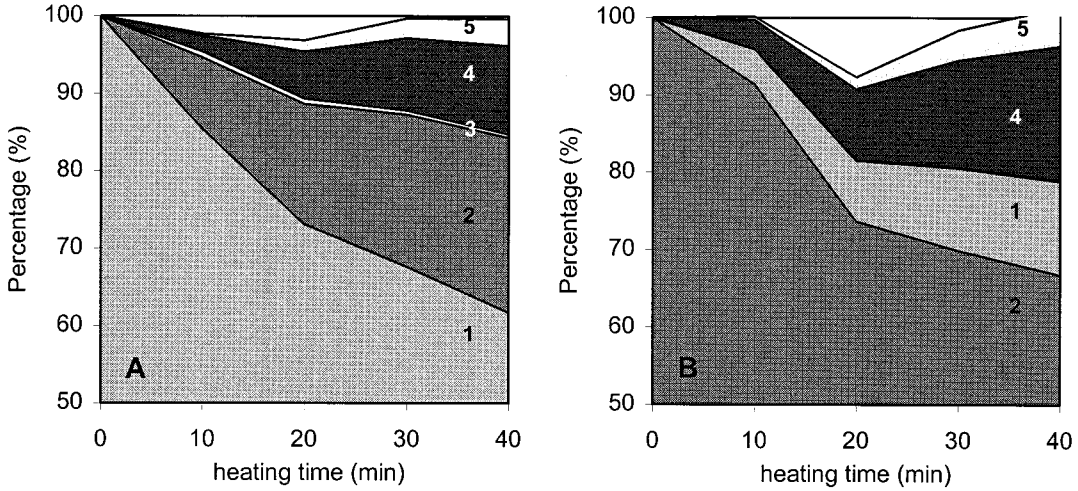

Figure 5. Mass balance of reactants and reaction products in heated glucose–casein (A) and fructose–casein (B) systems: glucose (1); fructose (2); Amadori compound (3); total acids (4); brown compounds (5).

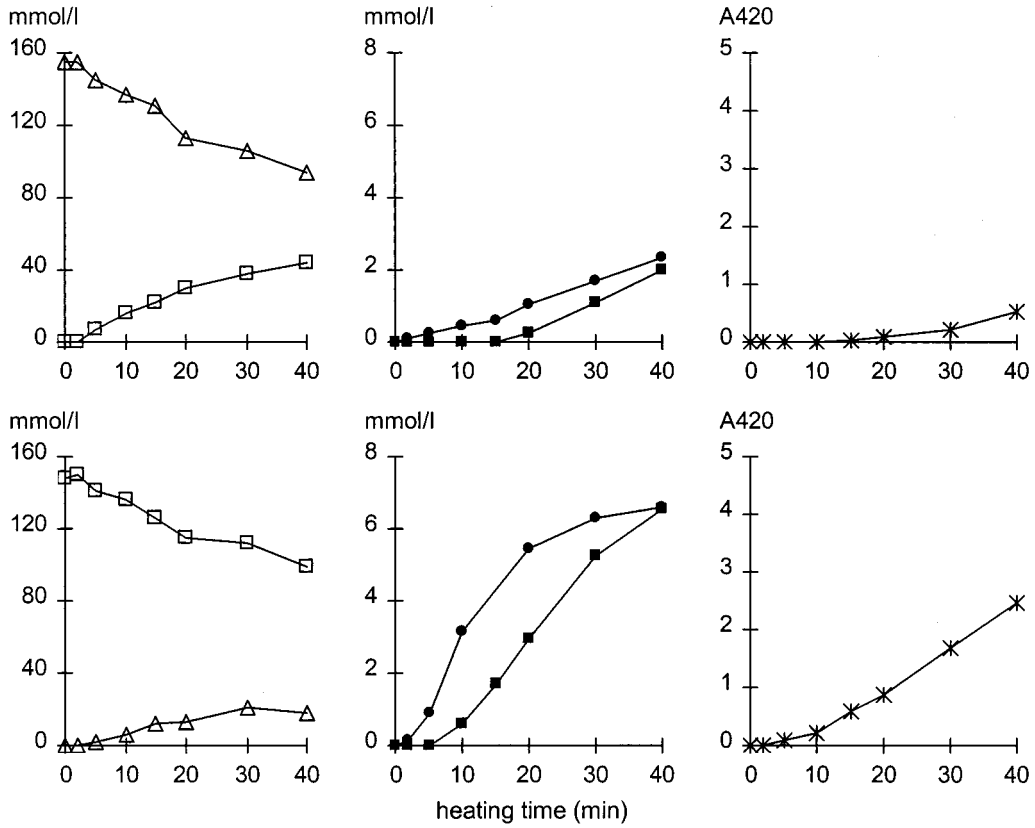

Figure 6. Glucose solutions (top) and fructose solutions (bottom) heated without casein at 120 °C: glucose (△); fructose (□); formic acid (●); acetic acid (■); absorbance at 420 nm (∗).

and formic acid were formed, but acetic acid was formed in ~1.5 times higher amounts. No sugars were formed. We tried to isolate protein-bound Heyns compound according to the same procedure (incubating fructose with casein at 65 °C), but subsequent heating of the incubated protein did not result in the formation of organic acids. This confirmed our observation mentioned above that protein-bound Heyns product could not be detected.

Reaction Mechanism. Glucose and fructose isomerize into one another via the Lobry de Bruyn-Alberda van Ekenstein transformation (*6*). The 1,2-enediol anion is the key intermediate in this isomerization reaction in which also mannose is involved. Mannose was, however, not detected in the heated model systems. In addition to the favorable isomerization of fructose via the 1,2-enediol anion, fructose can also epimerize to psicose via the formation of a 2,3-enediol as intermediate species. However, psicose was not detected in this study. This Lobry de Bruyn-Alberda van Ekenstein transformation is also applicable to the isomerization of other aldose and ketose sugars, among which are galactose and tagatose.

As well as being the key intermediate in the isomerization reactions, the enediol anion species are also considered to be the starting intermediates in degradation reactions (*5*). Via several pathways they lead to carboxylic acids as the final stable degradation products

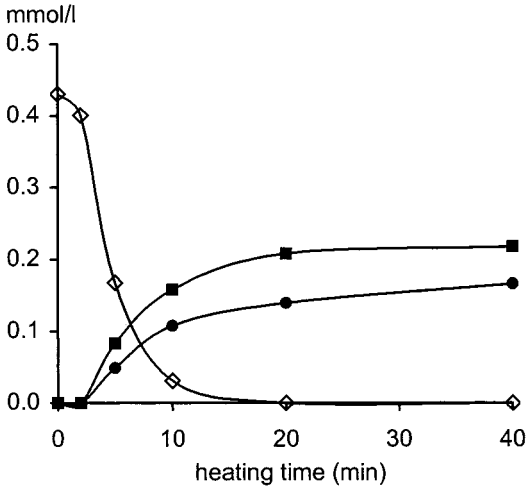

Figure 7. Isolated Amadori compound heated at 120 °C: Amadori compound (◇); formic acid (●); acetic acid (■).

(Figure 8). The 1,2-enediol anion can undergo β-elimination to yield 3-deoxyaldoketose. In the same way the dicarbonyl compounds 1-deoxy-2,3-diketose and 4-deoxy-2,3-diketose are formed from the 2,3-enediol anion. These α-dicarbonyl compounds are unstable and undergo either a benzilic acid rearrangement yielding saccharinic acids or a cleavage reaction (cleavage of the C−C bond between the carbonyl groups) toward a carboxylic acid and an aldehyde. Formic acid was determined as one of the organic acids and was likely formed via a C1−C2 cleavage of the 3-deoxyaldoketose (Figure 8A). At the same time a C5 compound should be formed. A C5 compound described in the literature is 3-deoxypentulose. However, this compound was detected in disaccharide systems, not in monosaccharide systems (*16*, *21*). Another C5 compound is 2-deoxyribose. This compound was not detected, although furfuryl alcohol, a cyclization product of 2-deoxyribose (*22*), was identified. The concentration of furfuryl alcohol did, however, not equal the amount of formic acid. Possibly, 2-deoxyribose also reacts to other compounds. Berg (*23*) observed that it rapidly degraded in similar conditions as in the present study, whereas furfuryl alcohol was quite stable during heating.

The other identified acid was acetic acid, which was formed either by a C2−C3 cleavage reaction of the 1-deoxy-2,3-diketose or via a cleavage reaction of triose intermediates (Figure 8B). According to De Bruijn (*24*), ketoses may undergo substantial retro-aldolization toward the important triose intermediates glyceraldehyde and 1,3-dihydroxyacetone. These compounds can react further to the α-dicarbonyl methylglyoxal, which can undergo a cleavage reaction and form acetic acid. Experiments in which we trapped reactive α-dicarbonyl compounds with *o*-phenylenediamine showed that methylglyoxal was indeed formed. Additional evidence for the formation of an intermediate was the observation of a lag time for the formation of acetic acid.

The titration experiment showed that acid formation was higher than the total amount of acetic and formic acid. Hence, other organic acids were formed but were not identified. Possible acids are lactic acid, which can be formed via benzilic acid rearrangement of methylglyoxal, glycolic acid, which can be formed via α-dicarbonyl cleavage of the 4-deoxy-2,3-diketose, and the already mentioned saccharinic acids (*24*).

Besides being formed via sugar degradation, organic acids were also formed in the Maillard reaction. In the early stage of the Maillard reaction between free amino groups of lysine residues of the protein and carbonyl

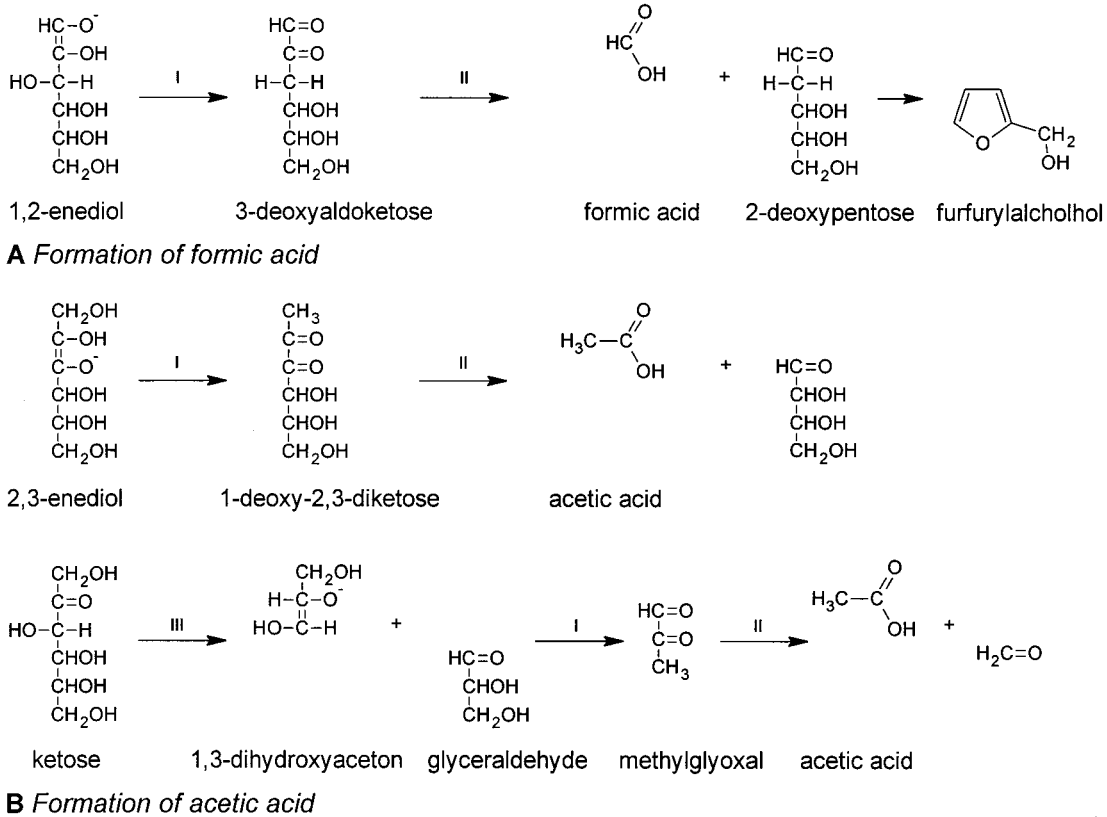

Figure 8. Degradation pathways of sugars into carboxylic acids (I = β-elimination, II = α-dicarbonyl cleavage, III = retro-aldolization) (after ref *24*).

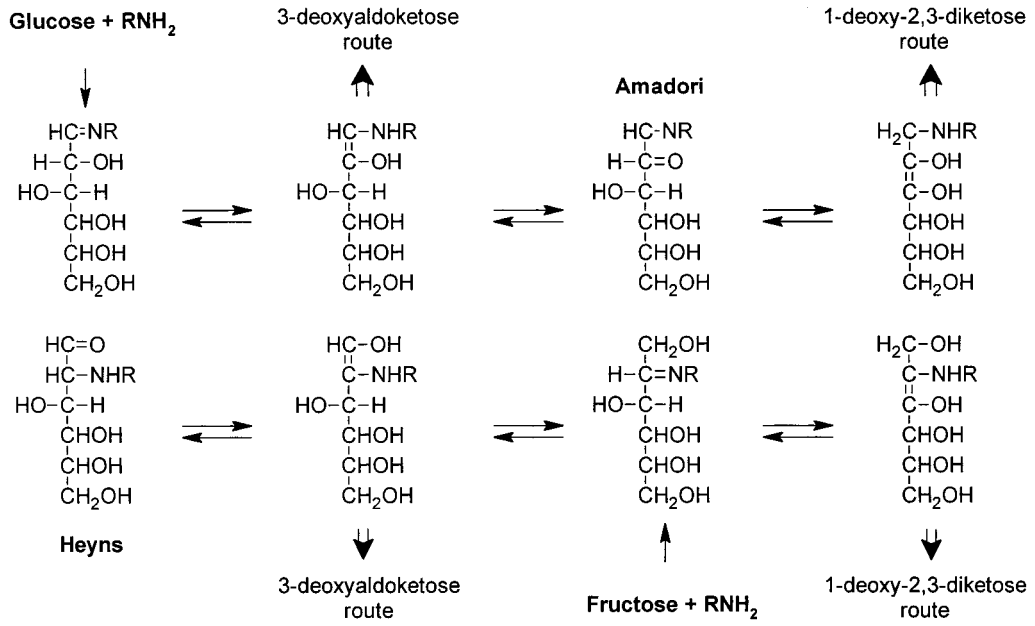

Figure 9. Early stage of the Maillard reaction of glucose and fructose (after ref *22*).

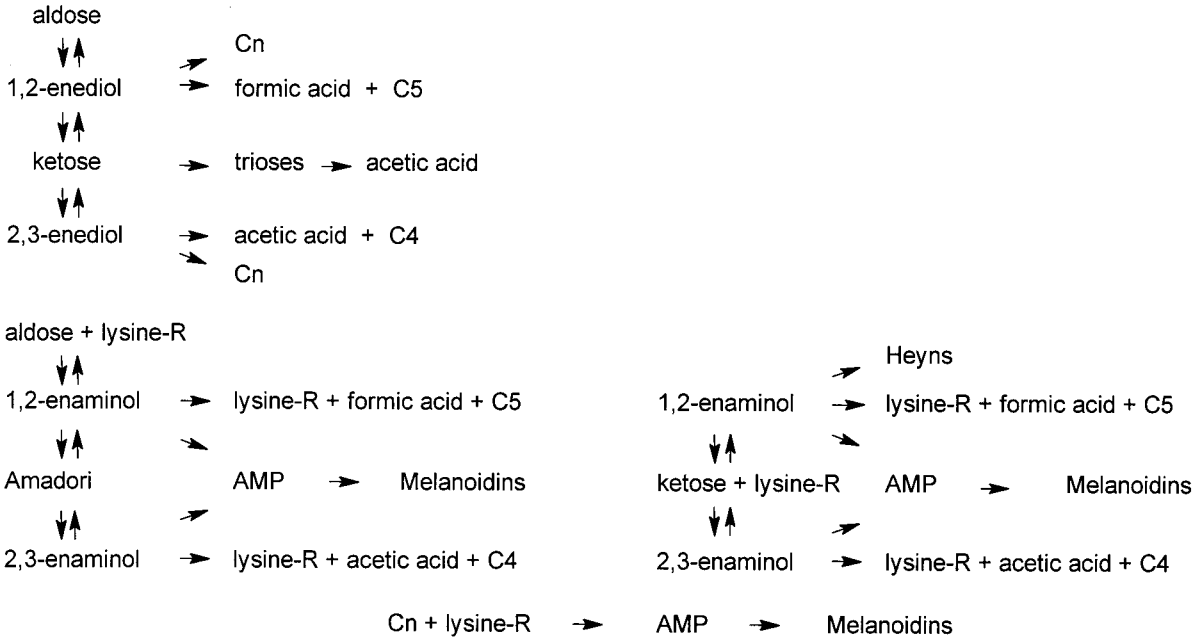

Figure 10. Reaction network model for sugar–casein reactions [Cn, unidentified sugar reaction compounds with *n* carbon atoms ($1 \leq n \leq 6$); AMP, advanced Maillard reaction products; lysine-R, protein-bound lysine residues].

groups of a sugar, an N-substituted glycosylamine is formed (Figure 9). When the sugar is an aldose, the N-substituted aldosylamine undergoes a rearrangement via a 1,2-enaminol to yield the Amadori compound (1-amino-1-deoxy-2-ketose). Instead of reacting to the Amadori compound, this 1,2-enaminol can react to a 3-deoxyaldoketose. A cleavage reaction of this compound leads subsequently to the formation of formic acid (Figure 8A). The Amadori compound can react via 2,3-enolization to a 1-deoxy-2,3-diketose. Subsequent cleavage of this compound results in the formation of acetic acid (Figure 8B). When the sugar is a ketose instead of an aldose, an N-substituted ketosylamine is formed in the early stage of the Maillard reaction and can react via a 1,2-enaminol to a 3-deoxyaldoketose or via a 2,3-enaminol to a 1-deoxy-2,3-diketose (Figure 9). Via these deoxyosones formic and acetic acid can be formed (Figure 8). Because acid formation in the ketose system was about the same in the absence or presence of lysine residues, acid formation via the Maillard reaction is apparently not significant. Via the 1,2-enaminol the Heyns compound (2-amino-2-deoxy-1-aldose) can be formed. If this reaction route is only a side reaction, the Heyns compound is only formed as a byproduct of the Maillard reaction. This would explain why we could not detect any Heyns. In foods, the Heyns compound has never been detected (*13*); it has been found only in systems that were heated at physiological temperatures (*25*).

Apart from the organic acids, several compounds can be formed in the advanced Maillard reaction (*22*, *26*). These compounds were not analyzed in the present study. Because of their significance in the advanced Maillard reaction, they were grouped among the ad-

vanced Maillard reaction products (AMP). In contrast with the Amadori compound, the Heyns compound is not necessarily involved in these reaction routes (*22*). AMP can also be formed via reaction of sugar reaction products (Cn) with lysine residues. The AMP eventually lead to the brown melanoidins in the final stage of the Maillard reaction.

The reactions described in this paper are summarized in Figure 10. The model is divided into two parts, one containing the sugar reactions (isomerization and degradation) and the other describing the sugar−casein reactions (Maillard reaction) for both the aldose and ketose sugars. In a subsequent paper the proposed reaction network model will be analyzed in kinetic terms.

Conclusion. In this paper reaction mechanisms were proposed to explain the observed reaction products for the reactions of the aldose sugars glucose and galactose and the ketose sugars fructose and tagatose in the presence of the protein casein at neutral pH and 120 °C. Ketoses seemed to be more reactive in the sugar degradation reactions than their aldose isomers and also the reaction of ketoses and aldoses in the Maillard reaction differed. Due to this difference in reaction mechanism both sugars contribute to a different extent to quality factors such as nutritional damage, color, and flavor. In line with this, remarkable differences in mutagenicity were observed for aldoses and ketoses heated under circumstances corresponding to those in the present study (*2*).

In a subsequent paper, the proposed model will be kinetically analyzed and tested by varying the temperature, pH, and concentration of reactants.

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