Mutagenicity of Heated Sugar−Casein Systems: Effect of the Maillard Reaction†

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The formation of mutagens after the heating of sugar−casein model systems at 120 °C was examined by the Ames test, using *Salmonella typhimurium* strain TA100. Several sugars (glucose, fructose, galactose, tagatose, lactose, and lactulose) were compared in their mutagenicities. Mutagenicity could be fully ascribed to Maillard reaction products and strongly varied with the kind of sugar. The differences in mutagenicity among the sugar−casein systems were caused by a difference in reaction rate and a difference in reaction mechanism. Sugars with a comparable reaction mechanism (glucose and galactose) showed a higher mutagenic activity corresponding with a higher Maillard reactivity. Disaccharides showed no mutagenic activity (lactose) or a lower mutagenic activity (lactulose) than their corresponding monosaccharides. Ketose sugars (fructose and tagatose) showed a remarkably higher mutagenicity compared with their aldose isomers (glucose and galactose), which was due to a difference in reaction mechanism.

Keywords: *Ames test; casein; Maillard reaction; mutagenicity; sugars*

INTRODUCTION

The Maillard reaction of amino acids or proteins with reducing sugars takes place during the processing, cooking, and storage of foods. The first step of the reaction, that is, the condensation of free amino groups with carbonyl compounds, is followed by a series of other chemical reactions. These reactions cause changes in flavor and taste, formation of a brown color, and loss of nutritive value (Ames, 1992).

Another reported result of the Maillard reaction is the formation of mutagenic compounds. Maillard reaction mixtures are found to induce chromosome aberrations in Chinese hamster ovary cells (Powrie et al., 1981), to induce gene conversion in yeast (Powrie et al., 1981; Rosin et al., 1982), and to be mutagenic to *Salmonella* species (Shinohara et al., 1980, 1983; Powrie et al., 1981; Kitts et al., 1993). Shinohara et al. (1980) demonstrated that reaction products in a glucose−lysine solution heated at 100 °C for 10 h induced reverse mutations in *Salmonella typhimurium* TA100 without S9 activation. Kitts et al. (1993) observed that glucose−lysine mixtures heated for 1 h at 121 °C showed mutagenic activity in *S. typhimurium* TA98 and TA100 strains without S9 pretreatment. Mutagenicity was reduced in TA100 and eliminated totally in TA98 with added S9. Heated solutions of glucose with other amino acids also showed mutagenic activity in the *S. typhimurium* TA100 strain, although the mutagenic response was less than with lysine (Powrie et al., 1981; Shinohara et al., 1983).

Most mutagenicity research of Maillard reaction products is performed with glucose as the source of carbonyl group and amino acids as the source of amino group. Less is known about the mutagenicity of reaction mixtures consisting of other sugars and other sources of amino groups. In most foods, the ϵ-amino groups of the lysine residues of proteins are the most important source of reactive amino groups. Because proteins are well-known for their antimutagenic activity (Vis et al., 1998), it might be possible that sugar−protein systems show no mutagenicity at all.

This paper reports a study on the mutagenicity of heated sugar−protein model systems. The protein casein was used as the source of amino groups. Several sugars were compared in their mutagenicities. Glucose, an aldose sugar, is the most studied sugar in Maillard reaction research and also the most abundant sugar in nature. Fructose is the ketose isomer of glucose. During heating of glucose, substantial amounts of fructose are formed due to the Lobrey de Bruyn-Alberda van Ekenstein rearrangement (Speck, 1958). The reaction mechanism of fructose is known to be considerably different from that of glucose (Reynolds, 1965). Both glucose and fructose were selected as sugar reactants in this study under the presumption that each sugar may produce different amounts and perhaps different types of mutagens. These sugars were compared with other aldose and ketose sugars, galactose and tagatose, and the disaccharides lactose and lactulose. Mutagenicity was examined by the Ames test, using *S. typhimurium* strain TA100 (Maron and Ames, 1983).

MATERIALS AND METHODS

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

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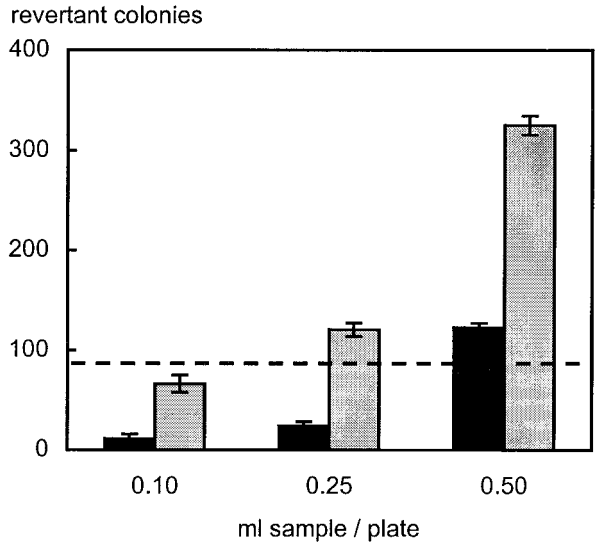

Figure 1. Mutagenicity response of glucose−casein samples (black bars) and fructose−casein samples (shaded bars) heated for 60 min, tested without S9 (dotted line: spontaneous mutagenic activity = 94 ± 2).

Preparation of Reaction Mixtures. Sodium caseinate (3% w/w) and sugar (150 mM) were dissolved in a phosphate buffer (0.1 M; pH 6.8) and heated for 20, 40, and 60 min at 120 °C in screw-capped glass tubes in an oil bath.

Browning Intensity. The browning intensity of the heated reaction mixtures was determined by measuring the absorbance at 420 nm against water with a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The samples were diluted four times with sodium dodecyl sulfate (SDS; 16% w/w) to reduce scattering due to protein aggregates. If necessary the samples were diluted once more with water.

Mutagenicity Assay. The mutagenicity was examined in the Ames assay (Maron and Ames, 1983). Histidine-requiring strains of *S.typhimurium* with mutations in the histidine operons can be reverted to histidine prototrophy by active mutagens (Ames et al., 1975). In this study, mutagenicity was investigated using strain TA100, which is susceptible to base-pair substitution. The test was performed with and without metabolic activation by S9, a liver homogenate from Aroclor 1254 pretreated rats. In the test, 0.1 mL of an overnight culture of TA100 was mixed with 0.4 mL of S9 mix or 0.4 mL of phosphate buffer (0.1 M; pH 7.4) and 0.5 mL of the reaction mixture. In the negative control (spontaneous mutations), phosphate buffer (0.1 M; pH 6.8) was used instead of the reaction mixture. After a preincubation at 37 °C for 30 min, 2.5 mL of top agar was added and the entire liquid was poured onto an agar plate. Histidine-revertant colonies were counted after a 48-h incubation at 37 °C. Each assay was performed in triplicate. As a positive control, 0.1 μg of 4-nitroquinoline-*N*-oxide (4-NQO) per plate without S9 and 5 μg of benzo[*a*]-pyrene (B[a]P) with S9 were used. The mutagenic activity was corrected for spontaneous mutations by subtracting the number of revertants in the negative control. The result of the assay was considered mutagenic if the total number of revertants per plate was at least twice as high as the number of spontaneous revertants per plate.

Statistical Analysis. All data are expressed as mean ± SEM. Different mutagenic responses observed from different sugars heated for the same time were analyzed by using the Student's *t* test. Statistical differences at $P \leq 0.05$ were considered to be significant.

RESULTS

First, heated glucose−casein and fructose−casein systems were tested for their mutagenicity. A dose response of mutagenic activity was observed in both systems heated for 60 min at 120 °C (Figure 1). The samples were preincubated for 30 min at 37 °C in the absence of S9 mix. Preincubation was a necessity to get a good response. The best response was obtained with 0.5 mL of sample per plate.

Table 1. Mutagenicity of Heated Sugar−Casein Systems[a]

	heating time		
sugar	20 min	40 min	60 min
glucose	16 ± 5	62 ± 7	123 ± 4
fructose	128 ± 11	272 ± 10	325 ± 10
galactose	63 ± 9	139 ± 14	198 ± 10
tagatose	135 ± 11	360 ± 18	406 ± 4
lactose	21 ± 3	63 ± 19	93 ± 13
lactulose	67 ± 11	111 ± 12	134 ± 11

[a] Mutagenic activity is corrected for spontaneous mutations (= 94 ± 2 revertants/plate); positive control = 862 ± 45 revertants/plate.

In Table 1 the heating time response of mutagenicity of the glucose−casein and fructose−casein systems tested without S9 treatment is shown. Both sugar−casein systems showed a significant increase of revertants in time. The glucose−casein samples were mutagenic after 60 min of heating, whereas the fructose−casein samples were already mutagenic after 20 min. For the three heating times tested, the mutagenic activity of the fructose−casein samples was significantly higher than that of the glucose−casein samples. Mutagenic activity was absent when the systems were incubated with S9 mix (Figure 2).

When either casein or any of the sugars in solution were heated separately under the same conditions as the sugar−casein systems, no mutagenic activity was noted (Figure 3). Consequently, mutagenicity can be fully ascribed to Maillard reaction products.

Another aldose, galactose, and its ketose isomer, tagatose, were also tested in this study (Table 1). Both sugar−casein systems showed an increase of revertants in time. The tagatose−casein system was mutagenic after 20 min of heating and had a significantly higher mutagenic activity than the galactose−casein system at the tested heating times. The galactose−casein system was mutagenic after 40 min of heating.

The disaccharides lactose and lactulose were also studied. Lactose consists of a glucose and a galactose unit. The glucose unit contains the reactive carbonyl group. Lactulose is the ketose isomer of lactose consisting of fructose and galactose with the reactive group on the fructose moiety. After 60 min of heating, no mutagenic activity could be found in the lactose−casein system and only slight mutagenic activity was observed in the lactulose−casein system (Table 1).

To study the possible antimutagenic effect of the protein, glucose and fructose were heated with glycine. Glycine is the simplest amino acid and behaves similarly to lysine in the Ames test using tester strain TA100 (Shinohara et al., 1983). The glycine concentration was equal to the concentration of ϵ-lysine residues of the protein in the sugar−casein systems (15 mM). Comparison of the heated sugar−glycine systems with the sugar−casein systems (Figure 4) showed a significant increase of mutagenicity in the glucose−glycine system with regard to the glucose−casein system. Only a slight significant difference between the fructose systems was observed. Addition of 0.4 mL of 3% sodium caseinate dispersion (instead of 0.4 mL of phosphate buffer) to the sugar−amino acid mixture, just before preincubation, decreased the mutagenicity of the glucose−glycine

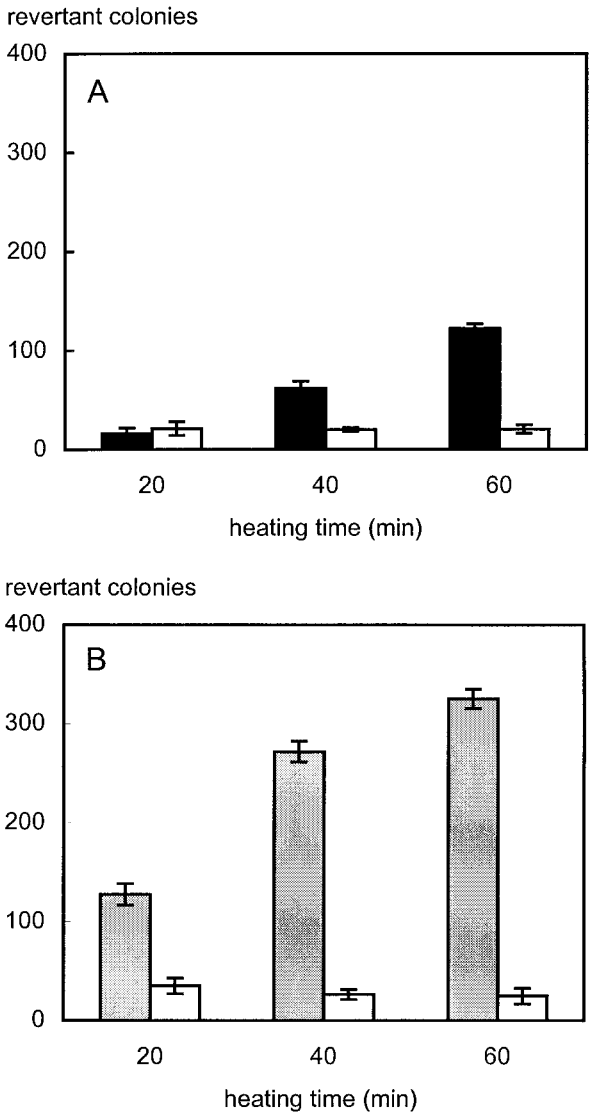

Figure 2. Mutagenic activity of 0.5 mL of glucose–casein (A) and fructose–casein samples (B): (black/shaded bars) tested without S9 (spontaneous mutagenic activity = 94 ± 2); (open bars) tested with S9 (spontaneous mutagenic activity = 118 ± 7).

system (Figure 4A). In the fructose system no significant effect of addition of casein was observed (Figure 4B).

The browning of all the sugar–casein systems investigated in this study is shown in Table 2. The color development of aldose sugars was in the following descending order: galactose > glucose > lactose. The ketose sugars browned more quickly than their aldose isomers.

DISCUSSION

This study showed that mutagenic substances can be formed in the Maillard reaction between sugars and casein heated at 120 °C. The mutagenicity of the model systems strongly varied with the kind of sugar. After 60 min of heating, the mutagenic activity was in the descending order tagatose > fructose > galactose > lactulose > glucose > lactose. Mutagenicity could be fully ascribed to Maillard reaction products, a result that is in line with the observations of Shinohara et al. (1980) and Powrie et al. (1981). On this basis, it is to be expected that sugars which are more reactive in the

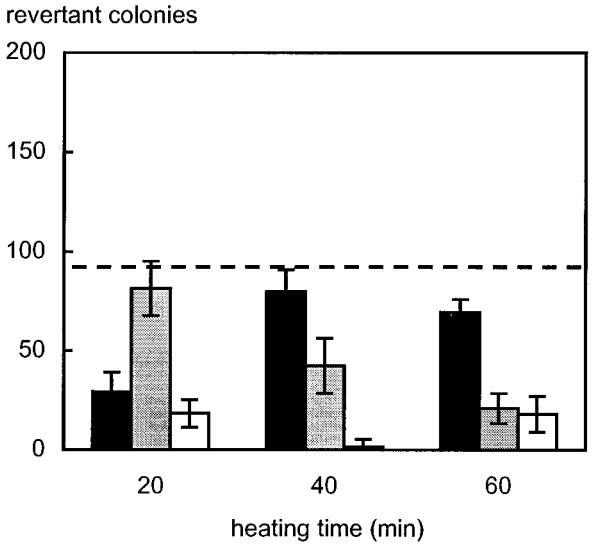

Figure 3. Mutagenic activity of the separate heated reactants: (black bars) glucose; (shaded bars) fructose; (open bars) casein (dotted line: spontaneous mutagenic activity = 94 ± 2).

Maillard reaction form higher amounts of mutagenic compounds. An indicator that is often used for Maillard reactivity is browning.

A faster browning (Table 2) was observed in the galactose–casein system as compared to the glucose–casein system, indicating that galactose was more reactive in the Maillard reaction. The mutagenic activity of the galactose–casein system was higher in the same order of magnitude (~50%) than that of the glucose–casein system. The increased reactivity of galactose can be explained by its higher amount present in the acyclic form (Hayward and Angyal, 1977). The acyclic form of the sugar is the form in which the sugar reacts with the lysine residues in the Maillard reaction.

Lactose showed a lower reactivity in the Maillard reaction than glucose; that is, a slower browning was observed (Table 2). This lower reactivity was in agreement with the lower number of revertants after 60 min of heating. Although the number of revertants increased in time, no mutagenic activity could be observed in the lactose–casein system. This observation is a confirmation of the results of Rogers and Shibamoto (1982) and Berg et al. (1990), who could not detect mutagenic activity in milk and model systems containing lactose and casein. Disaccharides are considered to have a reaction mechanism different from that of monosaccharides due to the glycosidically linked sugar. The bound sugar prevents typical monosaccharide degradation products from being formed and gives rise to typical disaccharide products (Kramhöller et al., 1993). These compounds might have less or no mutagenic activity.

The ketose fructose browned somewhat more quickly than its aldose isomer glucose (Table 2). The degree of browning is, however, not a good indicator to compare ketoses with aldoses in Maillard reactivity. Besides the Maillard reaction, another reaction mechanism leads to browning, namely, caramelization of the sugar. Ketoses contribute noticeably to browning via caramelization reactions, whereas caramelization in aldose systems may be neglected as a significant browning mechanism (Buera et al., 1987). Loss of available lysine residues is another indicator that can be used for comparing sugars in their Maillard reactivities (Kato et al., 1986; Naranjo

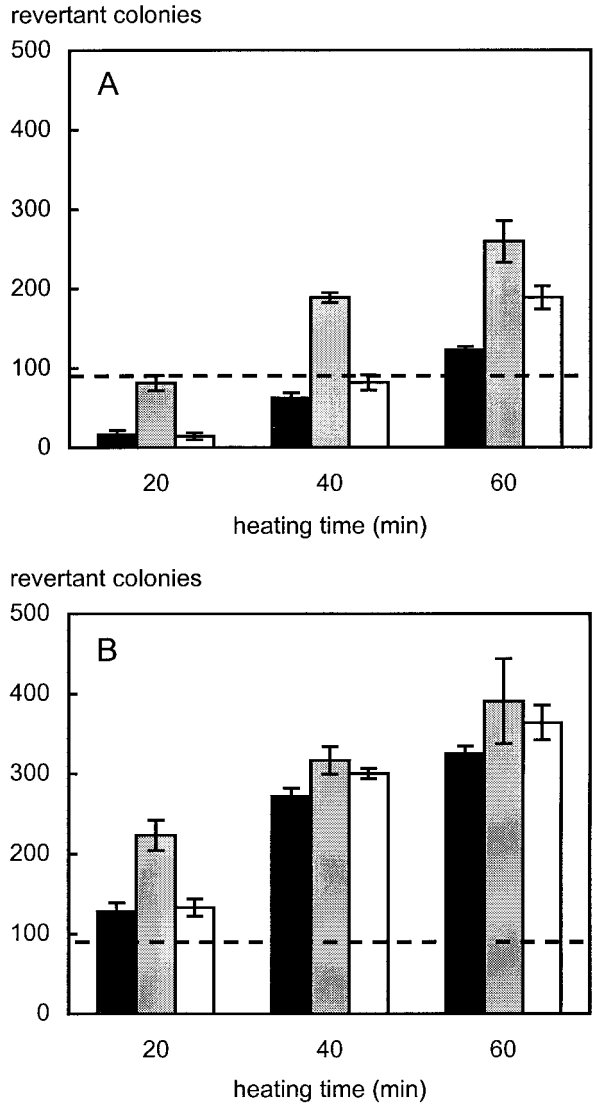

Figure 4. Mutagenic activity of glucose (A) and fructose (B) heated with (black bars) casein, (shaded bars) glycine, or (open bars) glycine and afterward the addition of casein (dotted line: spontaneous mutagenic activity = 94 ± 2).

Table 2. Absorbance (420 nm) of Sugar−Casein Systems

	heating time		
sugar	20 min	40 min	60 min
glucose	1.76	3.95	5.84
fructose	2.75	5.07	7.66
galactose	3.27	6.51	8.86
tagatose	4.84	9.08	12.24
lactose	1.01	2.67	3.84
lactulose	2.98	5.52	7.34

et al., 1998; Van Boekel and Brands, 1998). However, an almost 3 times higher mutagenicity was observed in the fructose−casein system as compared to the glucose−casein system (Table 1), whereas only a minor difference in lysine loss was observed (Van Boekel and Brands, 1998). Apparently, the difference in mutagenicity between the ketose and aldose−casein systems was not caused by a difference in Maillard reactivity but was due to a difference in reaction mechanism. Other compounds with higher mutagenic activity might be formed in the ketose−casein systems compared with the aldose−casein system, or, alternatively, the amount of antimutagenic compounds formed in the ketose-casein system was much lower than in the aldose−casein system. Numerous compounds are formed in the Maillard reaction. Some may be mutagenic, whereas others may be antimutagenic. In our study, we could not differentiate between those compounds. The test provides information on the net influence of combined mutagens and antimutagens.

Mutagenic activity was found to be much higher in the tagatose−casein system than in the galactose−casein system (Table 1). This observation confirmed our hypothesis that the difference in mutagenicity was caused by a difference in reaction mechanism between ketoses and aldoses.

Although we found differences between monoaldoses and monoketoses, Powrie et al. (1981) and Omura et al. (1983) did not find any difference in mutagenicity between glucose and fructose in their studies. The fact that they used amino acids as the source of amino groups might explain this difference. Heating glycine instead of casein decreased the difference in mutagenicity between the glucose and fructose systems (Figure 4). Casein apparently suppresses the mutagenic activity of some specific Maillard compounds in the glucose system. This assumption was confirmed when casein was added to the glucose−glycine system (Figure 4). It is interesting to see that this antimutagenic activity of casein was not effective in fructose−casein systems, which is yet another indication of different reaction products in the case of fructose.

Only a slight significant difference in mutagenicity could be observed between the ketose and aldose disaccharides lactulose and lactose (Table 1), in contrast with the ketose and aldose monosaccharides. Evidently, fewer or less mutagenic compounds (in amount or in activity) are formed in the disaccharide systems, due to a change in reaction mechanism.

It should be stressed that the mutagenic activity of the sugar−casein systems was weak compared to chemical mutagens such as NQO. Moreover, mutagenic activity of the sugar−casein systems incubated with S9 was absent, which means that no indirect active mutagens were formed and, even more importantly, that direct mutagens were detoxified by S9. This result was also observed by Kitts et al. (1993).

It is not yet known which substances are responsible for the slight mutagenic activity. Possible mutagens, which can be formed in the model systems, are, according to literature, methylglyoxal (Nagao et al., 1979), 5-(hydroxymethyl)furfural (HMF), ϵ-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]norleucine (LPA) (Omura et al., 1983), 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone (HHMF) (Hiramoto et al., 1995), and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) (Hiramoto et al., 1997). In the model systems of the present study, HMF as mutagen can be omitted because it is an indirect mutagen that does not show mutagenicity in the absence of S9. Like our sugar−casein systems, methylglyoxal, HHMF, and DDMP showed mutagenicity with TA100, which was significantly decreased in the presence of S9 (Nagao et al., 1979; Hiramoto et al., 1995, 1997). HHMF and DDMP are formed in monosaccharide systems and not in disaccharide systems (Kramhöller et al., 1993), which might be an explanation for the lower mutagenicity of disaccharide systems. Omura et al. (1983) suggested that LPA is one of the main mutagens formed by the Maillard reaction between glucose and lysine. In glucose−casein systems, LPA is formed in higher amounts than in

lactose–casein systems (Morales and Van Boekel, 1996), which might be another explanation for the lower mutagenicity of disaccharide systems. However, LPA is bound to protein and it is not known whether LPA is also mutagenic if bound. No explanation from the literature could be found for the higher mutagenicity of ketose–casein systems. Most likely, compounds other than those mentioned above are responsible for this.

The results obtained in this study show remarkable differences in mutagenicity, due to the Maillard reaction, between ketoses and aldoses, and mono- and disaccharides, under conditions that correspond to sterilization conditions in the food industry. The differences found may offer possibilities to optimize food quality with respect to mutagenicity due to the Maillard reaction.

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