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A new approach to study the significance of Amadori compounds in the Maillard reaction

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A new approach to study the significance of Amadori compounds in the Maillard reaction involves a comparison of their rates of reaction with sulphite species, S(IV), with the rates of reaction of S(IV) in the corresponding reducing sugar-amino acid-S(IV) reactions. The rate of formation of monofructoseglycine, MFG, in the glucose-glycine-S(IV) reaction ([glucose] = 1 M, [glycine] = 0.5 M, [S(IV)] = 0.05 M, pH 5.5, 55°C) in 0.2 M acetate buffer is 30×10^{-5} mol l^{-1} h^{-1} and the rate of loss of S(IV) of the order 8×10^{-5} mol l^{-1} h^{-1}. The concentration of MFG increases to 40 mM by the time all the S(IV) has undergone reaction. On the other hand, the reaction of MFG with S(IV) is immeasurably slow ($<1\times10^{-5}$ mol l^{-1} h^{-1} when [MFG] = 10-40 mM, [Glycine] = 0.5 M, [S(IV)] = 0.02 M), under the same conditions. The rate of browning of MFG, MFG-glucose and MFG-glycine mixtures was found to be much slower than the rate of browning in the corresponding glucose-glycine reaction. This evidence all points to the non-involvement of MFG in the glucose-glycine Maillard browning reaction during the stage where S(IV) exerts its inhibitory effect. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The accepted mechanism of Maillard browning involves the initial reaction of a reducing sugar (e.g., glucose) with an amino compound (e.g., amino acid) to give the corresponding glycosylamine, rearrangement to the corresponding ketoseamine (Amadori rearrangement product) (Hodge & Rist, 1953), and decomposition of this to a 3-deoxyosulose and a 3,4-dideoxyosulos-3-ene. The latter is regarded as a key intermediate in colour formation (McWeeny *et al.*, 1974). Sulphite species, S(IV), inhibit the Maillard reaction by combining with the 3,4-dideoxyosulos-3-ene to give the 4-sulpho-derivative.

The kinetics and mechanism of the reaction of S(IV) in the model system containing glucose, glycine and S(IV) have been studied (Wedzicha, 1984*a*). The kinetics reveal two consecutive rate-determining steps, identified as the formation of 3-deoxyhexosulose, DH, (Wedzicha & Garner, 1991) and its conversion to an intermediate which is capable of a rapid reaction with S(IV) to give 3,4-dideoxy-4-sulphohexosulose, DSH (Wedzicha, 1984*b*). The kinetics of the conversion of DH to DSH (Wedzicha & Kaban, 1986) support this mechanism and the kinetics of the individual steps are in agreement with the kinetics of the overall glucose-glycine-S(IV) reaction (McWeeny *et al.*, 1969; Wedzicha & Vakalis, 1988).

Previous studies on the significance of Amadori products in the Maillard reaction have focussed on investigation of their rates of browning (Hashiba, 1982; Pokorny *et al.*, 1988) and their chemical transformation into known Maillard intermediates. Examples include 1,2- and 2,3-enolisation, β-elimination, the migration of the carbonyl group and retroaldol reactions (Yaylayan & Huyghues-Despointes, 1994). The long sequence of reaction steps involved in Maillard browning means that it is not possible to obtain unambiguous kinetic data concerning early steps in the reaction from the rate of formation of coloured products. On the other hand, many of the products formed by transformation of Amadori compounds are found also in Maillard systems as intermediates. However, this does not necessarily imply that the Amadori compounds are the sources of these intermediates in Maillard browning. The kinetic significance of the formation and reactivity of the Amadori rearrangement product, monofructoseglycine, MFG, which is conventionally regarded as a precursor of DH in the glucose-glycine reaction, has not been assessed critically.

In this paper we report the rate of formation of MFG in the glucose-glycine-S(IV) reaction and the kinetics of the reaction of MFG with S(IV). This provides an opportunity to test critically the involvement of MFG in the sequence of events leading to the binding of S(IV)

in glucose-glycine-S(IV) mixtures. If MFG were to be relatively unreactive towards S(IV), this would provide good evidence that MFG does not give rise to the key Maillard intermediate which reacts with S(IV).

MATERIALS AND METHODS

All chemicals were of AnalaR grade and were supplied by BDH Chemicals Ltd. Ion exchange resin, DOWEX-50W 1-8880 (H^+) was supplied by Sigma. An aqueous solution of uniformly ^{14}C-labelled glucose (9.25 MBq) of negligible mass was obtained from Amersham International. Emulsifier Scintillator Plus was obtained from Canberra Packard.

Preparation and purity of MFG

Monofructoseglycine was prepared according to the method of Mossine *et al.* (1994) except that Dowex-50W 1-8880 (H^+) was used in place of Amberlite IRN-77 (H^+). The product was obtained in 11% yield based on the amount of glycine used and, on microanalysis, gave C: 40.7%, H: 6.55, N: 5.6%. Calculated for $C_8H_{15}NO_7$, C: 40.51%, H: 6.33%, N: 5.91. TLC on silica gel 60 F_{254} and butan-1-ol-acetic acid-water (2:1:1, v/v) as solvent gave one spot revealed with ninhydrin: $R_{gly} = 0.66$.

Reference samples of MFG were prepared also by the methods of Xenakis *et al.* (1983) and Röper *et al.* (1983). These procedures include one highly specific synthesis (Xenakis *et al.*, 1983) from which the identity of the product is unequivocal. The method described by Mossine *et al.* (1994) was chosen because it proved to be the best to prepare gram quantities of the compound. The ^{13}C-NMR spectra were in close agreement with those reported by Mossine *et al.* (1994) and by Röper *et al.* (1983), and were identical for all the samples prepared.

Kinetics of MFG formation

Sufficient glucose, glycine and sodium metabisulphite were weighed to give final concentrations of 1 M, 0.5 M and 0.05 M S(IV), respectively, in 50 ml. The solids were dissolved together in water (20 ml) and acetate buffer (10 ml, 1 M $CH_3COONa + CH_3COOH$ to give pH 5.5) was added. This mixture was used to transfer ^{14}C-labelled glucose from the vial in which it was supplied into a volumetric flask and the volume made up to 50 ml with water. Half of this mixture was transferred into another 50 ml flask containing additional solid sodium metabisulphite, to give a final concentration of [S(IV)] = 0.2 M in that reaction. Both mixtures were placed in a water bath at 55.0 ± 0.1°C. A reaction mixture without ^{14}C-labelled glucose was prepared and allowed to react under the same conditions in order to measure the rate of loss of S(IV) as described below.

An aliquot (1 ml) of each ^{14}C-containing reaction mixture was withdrawn at timed intervals, and applied to the top of a column (10×0.5 cm) containing 1.5 ml of Dowex 50W-X8 (H^+) ion exchange resin in 50% ethanol/water. The column was washed with ethanol/water (50% v/v, 25 ml), followed by water (50 ml), to ensure that all ^{14}C-glucose had been removed from the column. The adsorbed MFG and glycine were eluted with aqueous ammonia (25 ml, 0.1 M) and made up to 25 ml. An aliquot (1 ml) of this eluent was placed into a scintillation vial, scintillation fluid (10 ml) was added and the ^{14}C-activity measured using a TR1500C Packard scintillation counter. Quench correction was determined by the channels ratio method calibrated with chloroform-quenched standards.

The capacity of the ion exchange resin was 1.7 milliequivalents per ml, i.e., a total capacity of 2.6 milliequivalents. The maximum concentration of MFG + glycine was 0.5 milliequivalents per ml of reaction mixture. This gave a sufficiently high safety factor for the sample. The efficiency of the elution with aqueous ammonia was confirmed by eluting the column with an additional 10 ml of aqueous ammonia and measuring the ^{14}C-activity of the eluent.

Kinetics of reactions involving S(IV)

Monofructoseglycine (0.592 g, 0.125 M) was dissolved in water (20 ml). Aliquots of this solution (1-8 ml) were placed into 25 ml volumetric flasks, S(IV) solution (10 ml, 0.05 M) and acetate buffer (5 ml, 1 M $CH_3COONa + CH_3COOH$ to give pH 5.5) were added to each flask, and the volume made up with water. The flasks were placed in a water-bath at 55.0 ± 0.1°C. When required, glycine (0.937 g, 0.5 M) or glucose (4.5 g, 1.0 M) was weighed into the flask before making up to the mark.

Aliquots of each reaction mixture were withdrawn at time zero and at timed intervals. These were diluted to 50 ml with water (containing 1% v/v ethanol). In order to measure their S(IV)-contents, aliquots (5 ml) of these solutions were mixed with DTNB solution (10 ml, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.02 M phosphate buffer, pH 7) and the volume made up to 25 ml with the phosphate buffer. The absorbance of the resulting solution was measured at 412 nm in 1 cm cells against a reagent blank.

Investigation of the rate of browning

Aliquots (4 ml) of an aqueous solution of MFG (0.125 M) were placed into 3×25 ml volumetric flasks. To one was added glucose (4.5 g), to another glycine (0.935 g). A fourth reaction mixture contained glucose (4.5 g) and glycine (0.935 g) but no MFG. Acetate buffer (5 ml, 1 M $CH_3COONa + CH_3COOH$ to give pH 5.5) was added to all four reaction mixtures and the volume made up with water. The stoppered flasks were placed in a water-bath

at 55.0±0.1°C. At time zero and at timed intervals the absorbance of each mixture was measured at 470 nm, using water as the reference.

RESULTS AND DISCUSSION

Formation of MFG in glucose-glycine mixtures

The kinetics of the formation of MFG were studied in order to obtain the concentration of this intermediate in the model glucose-glycine-S(IV) reaction and to compare its rate of formation with the rate of reaction of S(IV) in the model system. Chromatographic analysis by hplc (with post-column derivatisation) or by gc of derivatised samples is effective and sensitive (Eichner *et al.*, 1990) but the methods are not well suited to kinetic investigations where a number of analyses need to be carried out rapidly and simultaneously. These techniques also require regular calibration. In the present investigation, a new procedure, based on simple ion-exchange separation, was developed. This is a radiochemical technique which measures the concentration of glucose carbon atoms which have been converted to basic products or intermediates. The measured specific activity of glucose (0.2 MBq $mmol^{-1}$) in the reaction mixtures was used to obtain the yield of MFG in conventional concentration units. Thus, the analysis required no further calibration. Whilst, in principle, the procedure was nonspecific, TLC revealed that the only ninhydrin-positive substance analysed was MFG, provided that the reaction mixture had not become brown. The extent of conversion of glucose to MFG is illustrated in Fig. 1. This shows the maximum concentration of MFG in a glucose-glycine-S(IV) reaction ([glucose] = 1 M, [glycine] = 0.5 M, [S(IV)] = 0.05 and 0.2 M, 55°C, pH 5.5) as *c.* 40 mM and the concentration increased to this value, over a period of 130–150 hours. After 150 h the mixture containing 0.05 M S(IV) was showing signs of browning and further measurements were not meaningful.

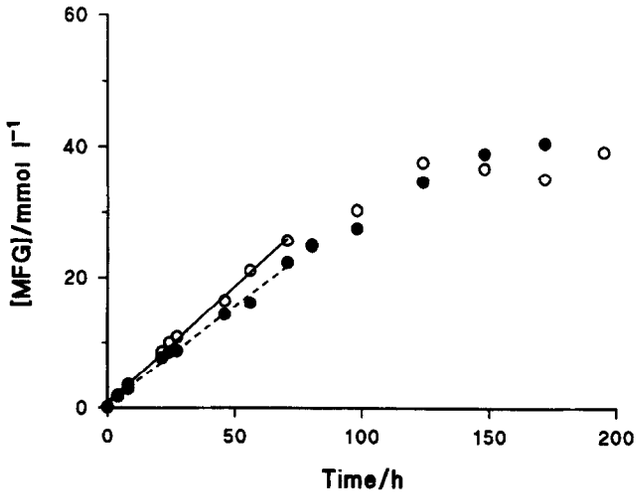

Fig. 1. Formation of MFG in the glucose-glycine-S(IV) reaction. Reaction conditions: [glucose] = 1 M; [glycine] = 0.5 M; [S(IV)] = 0.05 M ●; [S(IV)] = 0.2 M ○; 55°C; pH 5.5 (0.2 M acetate buffer).

The initial rate of formation of MFG was found to be similar, 30×10^{-5} and 36×10^{-5} mol l^{-1} h^{-1} for initial concentrations of S(IV), 0.05 and 0.2 M, respectively. Two different S(IV) concentrations were used in this study to check whether or not the species exerted any catalytic effect on the formation of MFG. It had been suggested that sulphite ion could assist in the conversion of glucosylglycine to MFG by removing a hydrogen ion at position 2 of the protonated glycosylglycine molecule (Wedzicha & Vakalis, 1988). This mechanism had been inferred from the behaviour of other weak bases, e.g., carboxylate or phosphate, that are known to catalyse the Maillard reaction. The results obtained here indicate that any effect of S(IV) on the rate of formation of MFG in this buffered system is very small.

Kinetics of the MFG-S(IV) reaction

The concentration of MFG in the glucose-glycine-S(IV) reaction reaches a value of *c.* 40 mM after 130-150 hours. The aim of this investigation was to study the kinetic behaviour of the reaction of S(IV) with MFG at a realistic concentration. Thus, it was proposed to vary the concentration of MFG in the range 5–40 mM.

Preliminary experiments showed the rate of any reaction between MFG and S(IV) to be very slow and of similar rate to the background rate of autoxidation of S(IV) found previously in the investigators' laboratory. Experience shows that autoxidation of S(IV) by dissolved oxygen is also the greatest source of irreproducibility in kinetic measurements. In practice, the rate of autoxidation can be reduced by the addition of ethanol (1 vol%) (Wedzicha, 1984*a*), which was used throughout this investigation.

The effect of the concentration of MFG (5-25 mM) on the rate of loss of S(IV) (initial concentration 20 mM) is illustrated in Fig. 2. Despite the presence of ethanol, it is seen that MFG tends to reduce the rate of loss of S(IV). If MFG were to react with S(IV), the

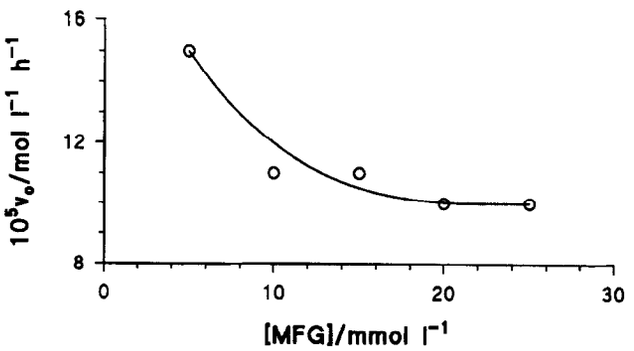

Fig. 2. Effect of the concentration of MFG on the initial rate, v_o, of loss of S(IV) in the MFG-S(IV) reaction. Reaction conditions: [S(IV)] = 0.02 M; 55°C; pH 5.5 (0.2 M acetate buffer).

Table 1. The effect of the concentration of MFG on the initial rate, v_o, of loss of S(IV) in the MFG-glycine-S(IV) reaction. Reaction conditions: [glycine] = 0.5 M; [S(IV)] = 0.02 M; 55°C; pH 5.5 (0.2 M acetate buffer)

[MFG]/mM	$10^5 v_o$/mol l^{-1} h^{-1}
10	8.87
15	9.39
20	8.83
25	8.27
30	8.86
35	8.92
40	8.79

concentration of intermediates reactive towards S(IV) would depend on the concentration of MFG. This reactant would be expected to exert a kinetic effect on the reaction. Thus, the order n of the reaction with respect to MFG should not equal zero, and common sense dictates that $n = 1$. It is inferred that MFG probably further inhibits the autoxidation of S(IV) and there is no evidence here that MFG reacts with S(IV).

It was decided to extend the kinetic data to the highest concentrations of MFG (40 mM) found in the Maillard system and to add glucose and glycine separately to reaction mixtures, because those reactants accompany MFG in the Maillard reaction. Initial rates were obtained from nearly linear concentration-time plots.

The effect of MFG-concentration on the initial rate of loss of S(IV) in the system MFG-glycine-S(IV) is given in Table 1 for [MFG] > 10 mM. These data indicate that the initial rate of the MFG-S(IV) reaction is independent of MFG concentration in the range 10-40 mM. Similar results are obtained in the absence or presence of glycine (0.5 M), 11×10^{-5} and 9×10^{-5} mol l^{-1} h^{-1}, respectively. It is proposed here that, in comparison to the background rate of autoxidation of S(IV), the rate of the MFG-S(IV) reaction is immeasurably slow, i.e., perhaps an order of magnitude slower. Thus, if the background rate of autoxidation of S(IV) in these experiments is in the region of 10×10^{-5} mol l^{-1} h^{-1} it is suggested that the rate of the MFG-S(IV) reaction does not exceed 1×10^{-5} mol l^{-1} h^{-1} at the highest concentration of MFG where the greatest kinetic effect would be seen.

The effect of MFG concentration on the rate of loss of S(IV) in the presence of glucose is illustrated in Fig. 3. Glucose reduces further the overall rate of loss of S(IV). If the autoxidation of S(IV) contributes to the background rate of loss in these experiments, the antioxidant activity of glucose serves to reduce further the rate of oxidation. Extrapolation of rate-concentration data in Fig. 3 to zero concentration suggests that the background rate of autoxidation of S(IV) in this experiment is no greater than 5×10^{-5} mol l^{-1} h^{-1}, and this rate would apply to autoxidation in a glucose-glycine-S(IV) reaction under the same conditions.

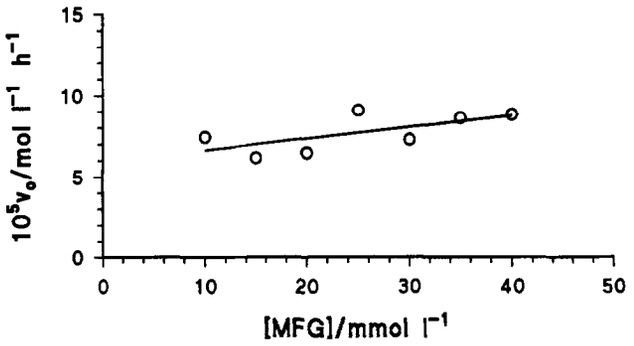

Fig. 3. Effect of the concentration of MFG on the initial rate, v_o, of loss of S(IV) in the MFG-glucose-S(IV) reaction. Reaction conditions: [glucose] = 1 M; [S(IV)] = 0.02 M; 55°C; pH 5.5 (0.2 M acetate buffer).

It is seen that MFG exerts a kinetic effect in the presence of glucose. The reason for this can only be speculative. The Amadori compound could react with glucose to give, ultimately, difructoseglycine which may be more reactive in decomposing to DH. On the other hand, MFG could act as an acid-base catalyst in some reaction for converting glucose to DH, by-passing MFG.

A critical consideration of the importance of MFG in the glucose-glycine-S(IV) reaction requires that the estimate of the maximum rate of the MFG-S(IV) reaction be compared with the rate of reaction of S(IV) in the glucose-glycine-S(IV) reaction. The latter was found to be 13×10^{-5} and 17×10^{-5} mol l^{-1} h^{-1} at initial S(IV) concentrations of 0.05 and 0.2 M, respectively. The rate equation for an acid-base catalysed reaction consists of a number of terms describing the contributions of the individual catalysts. For the glucose-glycine-S(IV) reaction in an unbuffered system, it has been shown (Wedzicha & Vakalis, 1988) that,

$$-\frac{d[S(IV)]}{dt} = k_1 + k_2[S(IV)]$$

where k_1 and k_2 are rate constants. The combined effects of all the reaction variables, except S(IV)-concentration, are taken account of in k_1. In the buffered system, k_1 includes the additional contribution from the acid-base catalytic behaviour of the components of the buffer. In the absence of buffer, Wedzicha & Vakalis (1988) found that $k_2 = 3.51\times10^{-4}$ h^{-1} at 55°C, which means that there is an expected increase in rate of 5.3×10^{-5} mol l^{-1} h^{-1} as the S(IV)-concentration is increased from 0.05 to 0.2 M. The increase in the rate of formation of MFG in the glucose-glycine-S(IV) reaction and in the rate of loss of S(IV) measured in the present work, 6×10^{-5} and 4×10^{-5} mol l^{-1} h^{-1}, respectively, are both of magnitudes similar to this value.

Given that the background rate of autoxidation of S(IV) was in the region of 5×10^{-5} mol l^{-1} h^{-1}, the actual rate of the reaction of S(IV) with intermediates derived

from glucose was of the order of 8×10^{-5} mol l^{-1} h^{-1}, at the lowest concentration of S(IV). Thus, the rate of the glucose-glycine-S(IV) reaction is greater by nearly an order of magnitude, than the maximum rate of the MFG-S(IV) reaction which could have been occurring at the highest concentration of MFG used (40 mM). In practice the S(IV) in the glucose-glycine-S(IV) system reacts completely before the concentration of MFG reaches this value, i.e., the estimated maximum rate of the MFG-S(IV) reaction is an over-estimate, making the distinction between the two processes all the more meaningful.

According to the kinetic model of the glucose-glycine-S(IV) reaction (Wedzicha, 1984*b*; Wedzicha & Garner, 1991; Wedzicha & Kaban, 1986), DH is the result of the first slow step in the reaction. The actual slow step could have taken place at any point in the sequence of events leading to DH. According to the classical mechanism of the Maillard reaction, the combination of slow and fast steps in the sequence could occur in two distinct ways as follows:

$$\text{glucose} + \text{glycine} \xrightarrow[\text{slow}]{} \text{MFG} \xrightarrow[\text{fast}]{} \text{DH} \qquad (1)$$

$$\text{glucose} + \text{glycine} \xrightarrow[\text{fast}]{} \text{MFG} \xrightarrow[\text{slow}]{} \text{DH} \qquad (2)$$

From radiochemical measurement, the initial rate of formation of MFG in the glucose-glycine-S(IV) reaction is substantially faster than the rate of loss of S(IV). It is unlikely, therefore, that mechanism (1) prevails.

The concentrations of glucose and glycine are an order of magnitude greater than the amount of MFG formed during the period over which the glucose-glycine-S(IV) reaction was observed. It has been shown that the rate of reaction of S(IV) in the glucose-glycine-S(IV) reaction is of first order with respect to both glucose and glycine; according to the kinetic model being tested, it is expected that the rate of formation of MFG should be substantially constant over this period of time because one can assume that the concentrations of glucose and glycine are approximately constant. On the other hand, it is seen that the data in Fig. 1 represent non-linear behaviour which could be indicative of a stepwise mechanism in which MFG is a reactive intermediate whose concentration increases from zero at the start of the reaction and reaches a steady-state concentration when the rate of its formation equals the rate if its loss. By way of hypothesis, assume that the rate of loss of MFG is of first order with respect to MFG, i.e.,

$$-\frac{d[MFG]}{dt} = k[MFG]$$

where k is a rate constant for the slow step in mechanism 2. The steady-state concentration of MFG, $[MFG]_{SS}$, is obtained by making the rate of formation of MFG equal to the rate of loss, i.e.,

$$30 \times 10^{-5} = k[MFG]_{SS} \text{ mol } l^{-1} h^{-1}$$

where the rate of formation of MFG is assumed to be the measured initial rate, which is then maintained throughout the reaction. In the present experiments, the value of $[MFG]_{SS}$ is probably *c.* 40 mM which gives $k = 7.5\times10^{-3}$ h^{-1}. In order to achieve a turnover of MFG at a rate of 8×10^{-5} mol l^{-1} h^{-1}, i.e., the rate of loss of S(IV) in the glucose-glycine-S(IV) reaction, a MFG concentration of *c.* 11 mM is required, which is within the range of concentration measured. Thus, of the two mechanisms, the second is the most plausible. However, the rate of reaction of MFG with S(IV) is so small in comparison with the rate of reaction of S(IV) in the glucose-glycine-S(IV) reaction, that it is safe to assume that MFG does not provide the intermediate with which S(IV) reacts irreversibly.

Investigation of the rate of browning

Hashiba (1982) found that Amadori compounds prepared from glycine and various sugars contributed to browning in model systems, and it has been suggested that they are precursors of brown polymers in soy sauce (Hashiba, 1978).

Figure 4 shows that the rate of browning, at 470 nm, of MFG (20 mM) is much slower than the rate of browning of the glucose-glycine reaction. A 20 mM concentration of MFG in glucose-glycine-S(IV) reactions was reached after 60-70 hours, at which time the glucose-glycine reaction is already browning rapidly. The fact that, when the concentration of MFG is realistic, the rate of browning of MFG is much slower than in the corresponding Maillard reaction, confirms the result of the MFG-S(IV) reaction that this intermediate is relatively unreactive. Both glucose and

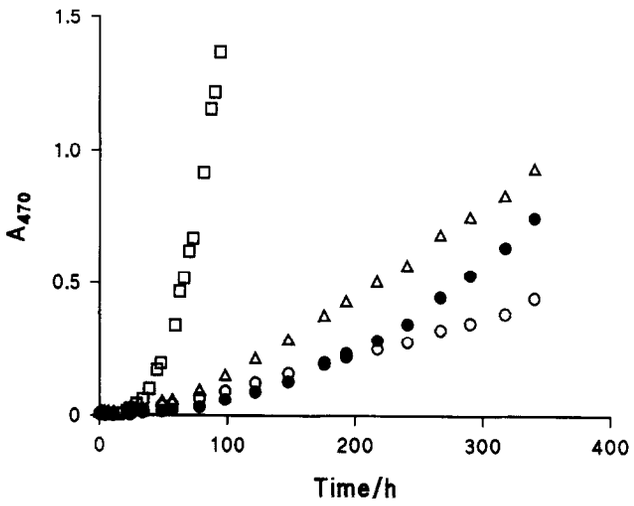

Fig. 4. Browning of model systems containing MFG (20 mM) alone ○ and with glucose (1 M) ●, or glycine (0.5 M) △. Reaction conditions: 55°C; pH 5.5 (0.2 M acetate buffer). The rate of browning of a mixture of glucose (1 M) and glycine (0.5 M) under the same conditions is shown for comparison □.

glycine accelerate the browning of MFG but, even in their presence, the increase in absorbance at 470 nm is much slower than in a glucose-glycine reaction.

These experiments do not address the possibility that MFG or degradation products from MFG require other intermediates of the glucose-glycine reaction in order to form the melanoidins. In this situation, MFG could be essential for the Maillard reaction to proceed but some other intermediate is required to form, say, a copolymer. The fact that, in the presence of S(IV), the concentration of MFG increases rapidly with time but levels off as S(IV) concentration becomes low, is consistent with the later intermediates in browning, which are allowed to accumulate in the absence of S(IV), reacting with MFG. This possibility is currently being investigated.

CONCLUSIONS

Strong evidence is presented to demonstrate that MFG is not an intermediate in the conversion of glucose to DH in the glucose-glycine reaction at pH 5.5 (0.2 M acetate buffer) and 55°C. This is supported by the relatively low tendency for MFG to brown under the same conditions.

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