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Comparative Study of the Composition of Melanoidins from Glucose and Maltose

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The composition of melanoidins formed in the reactions of either glucose or maltose with glycine (70 °C, pH 5.5, [glucose] = [maltose] = [glycine] = 0.25 M) (MW > 3500) was investigated by microanalysis and the use of ^{14}C-labeled sugars and amino acid. The most reliable parameter obtained from microanalysis data is the C/N value, as it was calculated with no model assumption. The C/N value (7.6 ± 0.2 for glucose and 10.5 ± 0.2 for maltose) does not change with molecular weight (MW > 3500) as the polymers grow in size. A comparison between the radiochemically determined composition and that obtained from microanalysis suggests that the amino ketone, which is one of the products of Strecker degradation reaction, forms part of the of the melanoidin structure, together with the sugar-derived moiety and the Strecker aldehyde. Evidence is presented that glucose is formed at intermediate stages of the maltose−glycine reaction. The melanoidins are the result of the polymerization of glucose and intact, or substantially intact, maltose residues with glycine.

KEYWORDS: Nonenzymatic browning; Maillard reaction; glucose; maltose; glycine; melanoidins; Strecker degradation

INTRODUCTION

In the final stage of the Maillard reaction between sugars and compounds possessing a free amino group, brown polymers, the so-called melanonidins, are produced. They are a heterogeneous mixture of high and low molecular weight compounds (*1*), and the chemistry at this point is very complex (*2*). Despite the large number of investigations to examine the structures and compositions of melanoidins, their detail is still far from complete. The most common hypothesis for their formation is that the high molecular weight compounds are a result of the polymerization of low molecular weight intermediates, such as pyrrole derivatives (*3*). The structure of the brown polymer is greatly affected by the reaction conditions (*4*). The formation of protein oligomers acting as a colorless backbone, cross-linked by low molecular weight colored Maillard reaction products, was found to result in color formation in sugar−protein systems (*5*, *6*).

In sugar−amino acid systems, the observed changing ratio of carbon to nitrogen atoms in the polymer, the C/N value, caused by different reaction conditions, has been accounted for by a structure proposed by Cämmerer and Kroh (*7*). It is based on the reaction between dicarbonyl compounds (especially 3-deoxyhexosulose) themselves, as well as a substitution reaction with amino compounds. This is in agreement with the structure of the nitrogen-containing polymer suggested by Yaylayan and Kaminsky (*8*), formed from the Amadori compound of the reaction of glucose with glycine. Essentially these structures show the polymers to consist of a repeating unit of conjugated carbon double bonds and tertiary nitrogen atoms. Although monosaccharides have been subjected to intensive study in this respect, very little work has been carried out on disaccharides, despite these sugars being important components of food and food ingredients and often responsible for food browning.

Together with the melanoidins, Maillard flavor and color components are formed, for example, via the Strecker degradation of amino acids with carbohydrate-derived dicarbonyl compounds (*9*) to give carbon dioxide and an aldehyde with one fewer carbon atom than the original amino acid. The Strecker aldehydes are able to form brown products by condensation with sugar fragments or other dehydration products.

Whereas extensive work has been carried out on the formation of Strecker aldehydes in food and food-related model systems (*10*), little is known about how these products are involved in melanoidin formation. Feather and Huang (*11*) reported that the Strecker aldehyde of glycine is incorporated into the polymer. It was later shown by Wedzicha and Vasiliauskaite (*12*) that intact and decarboxylated glycine residues are incorporated into the melanoidins in similar amounts, in the reaction of glucose with glycine. There is no report of how the involvement of the amino acid in the structures of melanoidins from disaccharides compares with that from monosaccharides.

This investigation is part of a systematic study of the extent to which the Strecker aldehyde of glycine is incorporated into the melanoidins from different sugars. We will consider here the browning of glucose with glycine in comparison to that of maltose with glycine. This paper reports data on the composition

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of melanoidins obtained from microanalysis and from the use of selectively radiolabeled sugar and glycine to illustrate how a combination of these two methods can provide new understanding of melanoidin composition.

MATERIALS AND METHODS

Chemicals. All chemicals were of AnalaR grade and were supplied by Sigma Chemical Co. Ltd. or Aldrich Co. Ltd. D-[U-^{14}C]Glucose, D-[U-^{14}C]maltose, [U-^{14}C]glycine, and [1-^{14}C]glycine were supplied by Amersham Life Science Ltd., U.K. Emulsifier Scintillator Plus for scintillation counting was supplied by Packard Instruments, U.K. Dialysis cassettes (MW > 3500) were obtained from Pierce & Warriner, U.K, and Visking tubing was supplied by Medicell Ltd., U.K. The D-maltose/D-glucose and D-lactose/D-galactose analysis kits were supplied by R-Biopharm Ltd., U,K.

Composition of Melanoidins. Melanoidins were obtained from a reaction mixture containing glucose or maltose (0.25 M) and glycine (0.25 M). The reaction mixtures were prepared by dissolving solid glucose, maltose, and glycine in water and, before the volume was made up with water, an aliquot (10% of the final volume) of a 2.0 M sodium acetate/glacial acetic acid, pH 5.5, buffer was added and the pH adjusted to 5.5 with NaOH. The solutions were heated at 70.0 ± 0.1 °C in a water bath until an absorbance at 470 nm of 2 units was reached. The resulting brown solutions were either dialyzed against water (10 × 10 L) using Visking tubing (MW > 12500) or continuously against water for 10 days in dialysis cassettes (MW > 3500). The minimum time for dialysis was established by measuring either the absorbance or the ^{14}C activity of the retentate until equilibrium was reached. The retentate was vacuum-dried in a rotary evaporator at 35 °C (repeated with the addition of 2 × 3 mL ethanol) and kept in a desiccator at room temperature for up to 2 days until sent for microanalysis (Chemistry Department, University of Leeds).

Radiochemical Investigation. Reaction mixtures (25 mL) were prepared containing the same final concentrations of glucose, maltose, and glycine as above. Before makeup to the final volume, reaction mixtures containing glucose and glycine were individually spiked with ~0.925 MBq of D-[U-^{14}C]glucose, [U-^{14}C]glycine, or [1-^{14}C]glycine, and reaction mixtures containing maltose and glycine were spiked individually with 0.75 MBq of D-[U-^{14}C]maltose or 0.925 MBq of [U-^{14}C]glycine or [1-^{14}C]glycine. The reaction mixtures were placed in a water bath and heated at 70.0 ± 0.1 °C. Aliquots (2 mL) of each reaction mixture were withdrawn at timed intervals, injected into dialysis cassettes (MW > 3500) and dialyzed as above. The minimum time for dialysis was established by counting the retentate of a melanoidin mixture while it was dialyzed over a period of 8 days. The ^{14}C activity appears to have reached its minimum value after the sixth day as shown in **Figure 1**. To ensure that the dialysis is complete before the retentate is counted, all reaction mixtures were allowed to dialyze for 10 days. After dialysis, the contents of the cassettes were removed, rinsed, and transferred into 10 mL volumetric flasks. The flasks were made up to the volume with distilled water. An aliquot (1 mL) of each diluted retentate was pipetted into a scintillation vial containing 10 mL of scintillation fluid. The vials were shaken before they were placed in the counter (Packard TR1500C scintillation counter) and counted for 100 min.

At the start of the reaction, aliquots (1 mL) of each reaction mixture were withdrawn and diluted to 100 mL with water. An aliquot of this solution (1 mL) was counted as before to obtain the total ^{14}C activity in each reaction mixture. To determine the counting efficiency, an internal standard was added to all previously counted sample vials. For all vials, the differences between the ^{14}C activity of the internal standard and the increase in counts, after it was added to the samples, was negligibly small. Hence, the data obtained in this investigation could be used directly without quench correction.

Analysis of Glucose and Maltose. The measurements of maltose and D-glucose were carried out using an enzyme assay kit containing α-glucosidase, hexokinase, and glucose-6-phosphate dehydrogenase. The advantage of using the enzyme assay for the quantitative analysis of maltose and glucose is its specificity without the need for separation of the analyte. Reaction mixtures containing sugar (0.25 M) and glycine (0.25 M) were prepared as above. To follow the concentrations of maltose and glucose during the reaction, aliquots (1 mL) of the reaction mixture were removed at timed intervals and allowed to cool to room temperature. After appropriate dilution, the sequence of events in the enzyme assay was followed as described by the kit supplier.

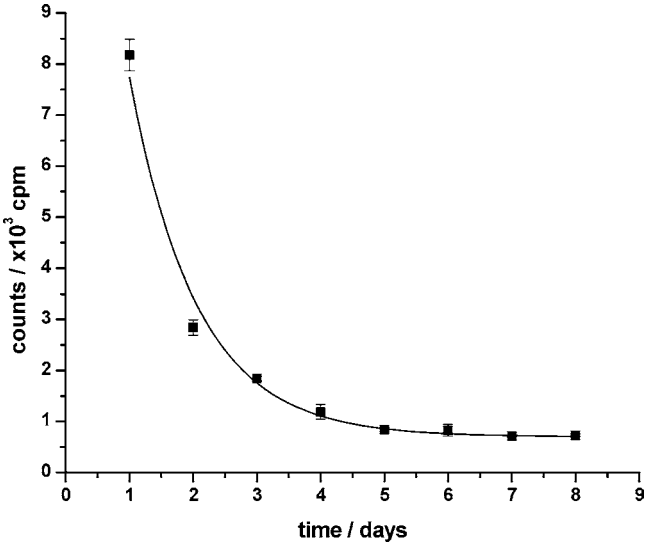

Figure 1. Radioactivity of melanoidins from browned reaction mixtures after dialysis for 1–8 days.

Table 1. Elemental Composition of Nondialyzable Melanoidins Prepared from Sugar (0.25 M)–Glycine (0.25 M) Reaction Mixtures in Acetate Buffer (0.2 M), pH 5.5, Heated at 70.0 ± 0.1 °C[a]

reaction mixture	C%	H%	N%	C/N
Melanoidins MW >12500				
glucose–glycine	42.9 ± 1.4	5.4 ± 0.2	6.6 ± 0.4	7.64 ± 0.21
maltose–glycine	43.6 ± 0.8	5.7 ± 0.1	4.84 ± 0.1	10.55 ± 0.3
Melanoidins MW >3500				
glucose–glycine	42.25	5.2	6.25	7.88
maltose–glycine	43.55	5.65	4.75	10.7

[a] Errors are standard deviations (n = 4). Data with no errors shown were single measurements.

RESULTS AND DISCUSSION

Elemental Composition of Melanoidins. The compositions of the high molecular weight melanoidins produced by the reactions of glucose or maltose with glycine were calculated from microanalysis data. Microanalysis of the products with a molecular weight >12500 were repeated four times to check reproducibility and error in calculation. The compositions are given in **Table 1**.

The C/N values for the glucose−glycine and the maltose−glycine melanoidins with a MW > 12500 were found to be the same as for the corresponding melanoidins with a MW > 3500, indicating that the C/N value does not change with molecular weight in this range for the systems investigated.

The C/N value for the glucose−glycine melanoidins found here (C/N = 7.64 ± 0.21) is similar to that obtained by Cämmerer and Kroh (*7*) (C/N = 7.22) for melanoidins prepared under similar conditions. Comparison of the C/N value for the melanoidins prepared from glucose−glycine here with those prepared at different temperatures and pH reported by other workers (*4*, *8*, *13*) supports the findings of Cämmerer and Kroh

(*7*) that the amount of amino acid incorporated into the polymer increases with decreasing temperature, resulting in a lower C/N value. This is also consistent with the findings by Martins and van Boekel (*14*), who reported a lower value for C/N when the temperature or pH was decreased.

Cämmerer and Kroh (*7*) found that Maillard reactions of disaccharides can be characterized into those forming melanoidins with a high nitrogen content (lactose and sucrose) and those forming melanoidins with a lower nitrogen content (maltose). Different reactivities of the sugars with the amino acid were used as an explanation.

Comparison of the maltose−glycine melanoidins with those from the glucose−glycine reaction (**Table 1**) shows that a significantly lower amount of nitrogen is incorporated into the polymer from maltose. To us, this suggests that differently sized subunits are derived from the reactions of the two sugars with glycine. A possibility is that the maltose-derived residues from the reaction of maltose with glycine consist of more than six carbon atoms, whereas only glucose-derived residues are possible from the glucose−glycine reaction. Alternatively, whole maltose molecules could be incorporated into melanoidins by direct binding of maltose with the polymer, for example, as an Amadori product, whereas the polymer itself grows by the polymerization of smaller residues, for example, those formed by cleavage of maltose into its monosaccharides. It has been reported by Cämmerer et al. (*15*) that, in the Maillard reaction under "water free" conditions, significant amounts of dimeric and oligomeric carbohydrates were incorporated into the melanoidin skeleton as complete oligomers with intact glycosidic bonds. They are said to form side chains to the melanoidin skeleton, those side chains being responsible for the different C/N ratios under different reaction conditions but not affecting the chromophore (*7*, *8*). It was also found that, with increasing water content, the formation of intact carbohydrate side chains was decreased. Thus, according to these authors, our aqueous systems should not contain oligomeric residues. Nevertheless, we advocate the presence of intact maltose residues, which we will aim to demonstrate through radiochemical studies.

Radiochemical Investigation. A radiochemical approach was used to determine the composition of the melanoidins. It has been shown by Wedzicha and Leong (*16*) that the concentration of melanoidins can be expressed in terms of the concentrations of sugar "residues" incorporated into the melanoidins using U-^{14}C-labeled sugar as the reactant and measuring the ^{14}C activity of the melanoidins, provided that the unreacted material can be separated from the products.

The use of sugar and amino acid labeled in different positions allows one to obtain information about the incorporation of labeled fragments of these reactants into the melanoidins (*16*). To identify the incorporation of the Strecker product of glycine into the melanoidins, the reaction mixtures were individually spiked with either [U-^{14}C]glycine or [1-^{14}C]glycine. **Figure 2** shows the incorporation of either [U-^{14}C]glucose, [U-^{14}C]-glycine, or [1-^{14}C]glycine in the glucose−glycine reaction into the nondialyzable melanoidins (MW > 3500) as their absorbances increase.

Since the absorbance of the reaction mixtures is an indicator of the progress of browning and is common to all of the experiments reported, the slope of each graph gives the relative incorporation of each labeled reactant into the products. It is striking that all data sets are excellent straight-line graphs, indicating that the relationship between the absorbance and concentration is constant throughout the browning reaction. Therefore, the incorporation of glucose, [U-^{14}C]glycine, or

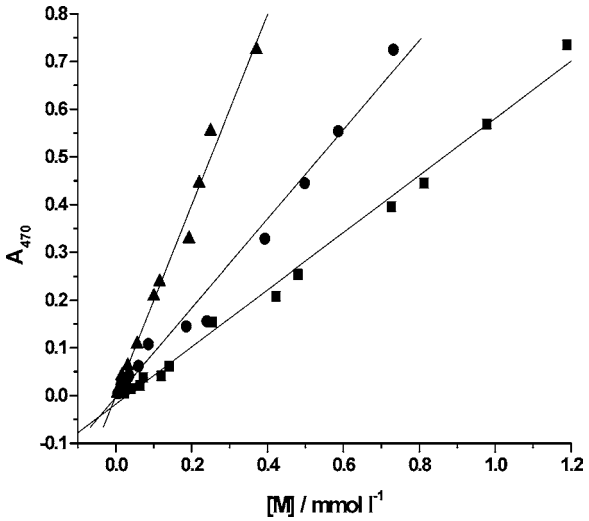

Figure 2. Graphs of absorbance versus [U-^{14}Cglc] (■), [U-^{14}Cgly] (●), and [1-^{14}Cgly] (▲) of melanoidins (MW > 3500) from the glucose–glycine reaction. Reaction conditions: [glc] = 0.25 M, [gly] = 0.25 M, acetate buffer 0.2 M, pH 5.5, heated at 70.0 ± 0.1 °C.

[1-^{14}C]glycine can be expressed as

$$\frac{A_{470}}{[\text{U-}^{14}\text{Cglc}]} = 0.575; \frac{A_{470}}{[\text{U-}^{14}\text{Cgly}]} = 0.927$$

and

$$\frac{A_{470}}{[\text{1-}^{14}\text{Cgly}]} = 1.993$$

respectively, where all concentrations are millimolar. The ^{14}C activity incorporated into the melanoidins obtained from [1-^{14}C]-glycine gives a direct measurement of the non-decarboxylated glycine relative to the concentration of glucose incorporated. This is given by

$$\frac{[\text{1-}^{14}\text{Cgly}]}{[\text{U-}^{14}\text{Cglc}]} = 0.289 \text{ mol/mol of glucose}$$

In the experiment with uniformly labeled glycine, the amount of the amino acid that is decarboxylated remains as 0.289 mol/mol of glucose. Thus, the contribution of the total ^{14}C activity in the melanoidins prepared from uniformly labeled glycine can be calculated by difference. This calculation reduces to the following equation, where the factor of $^1/_2$ is the result of the specific activity of the decarboxylated uniformly labeled glycine being half that of the whole glycine molecule as the radioactivity in the decarboxylated glycine is equally distributed between both C atoms. Thus, it can be written that

$$0.62 = \frac{[\text{U-}^{14}\text{Cgly}]}{[\text{U-}^{14}\text{Cglc}]} = \frac{[\text{1-}^{14}\text{Cgly}]}{[\text{U-}^{14}\text{Cglc}]} + \frac{^1/_2[\text{decarbox.gly}]}{[\text{U-}^{14}\text{Cglc}]} = 0.289 + \frac{^1/_2[\text{decarbox.gly}]}{[\text{U-}^{14}\text{Cglc}]}$$

$$\frac{[\text{decarbox.gly}]}{[\text{U-}^{14}\text{Cglc}]} = 2x(0.62 - 0.289) = 0.662 \text{ mol/mol of glucose}$$

The ratio of glucose- to glycine-derived subunits in the

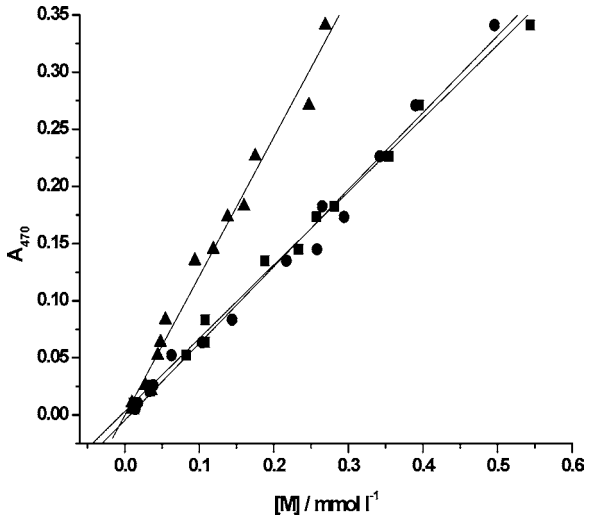

Figure 3. Graphs of absorbance versus [U-^{14}Cmal] (■), [U-^{14}Cgly] (●), and [1-^{14}Cgly] (▲) of melanoidins (MW > 3500) from the maltose–glycine reaction. Reaction conditions: [mal] = 0.25 M, [gly] = 0.25 M, acetate buffer 0.2 M, pH 5.5, heated at 70.0 ± 0.1 °C.

melanoidins is, therefore, 1:0.95 (i.e., 1:0.289 + 0.662) and is in remarkably good agreement with the previously reported value of 1:0.92 (*12*). The results also show that both intact and decarboxylated glycine are incorporated with a fixed stoichiometry with respect to glucose-derived residues, where approximately twice as much decarboxylated glycine is incorporated compared to intact glycine.

The amount of decarboxylated and non-decarboxylated glycine per maltose residue in the melanoidins, from the maltose−glycine reaction, can be obtained in the same way. The relationship between absorbance and the amounts of maltose, [U-^{14}C]glycine or [1-^{14}C]glycine incorporated into the non-dialyzable melanoidins, with time, is shown in **Figure 3**. Again, excellent linear plots are obtained. From the slopes of the graphs, the number of decarboxylated and non-decarboxylated glycine residues per maltose residue can be obtained following the calculations described above. Hence, the amount of whole glycine residue per maltose-derived residue is given by

$$\frac{[1\text{-}^{14}\text{Cgly}]}{[\text{U-}^{14}\text{Cmal}]} = 0.538 \text{ mol/mol of maltose}$$

The amount of decarboxylated glycine residue per maltose residue is given by

$$\frac{[\text{decarbox.gly}]}{[\text{U-}^{14}\text{Cmal}]} = 2x(0.991 - 0.538) = 0.906 \text{ mol/mol of maltose}$$

This gives a total number of 1:1.44 maltose- to glycine-derived subunits in the melanoidin. As found in the glucose−glycine reaction, both intact and decarboxylated glycine are incorporated in a fixed stoichiometry with respect to the maltose-derived residues and approximately twice as much decarboxylated glycine in comparison to intact glycine is incorporated into the polymer. Hence, we show that, although the melanoidins from maltose contain less nitrogen than those from glucose, maltose combines with more glycine molecules than does glucose. In drawing any conclusion from such data, we should be mindful of the fact that 1 mol of glucose or maltose corresponds to 6 or 12 carbon atoms from the sugar, respectively, regardless of whether those sugar residues remain intact throughout the reaction or whether the carbon atoms are assembled into the melanoidins from sugar fragments.

Comparison of Microanalysis and Radiochemical Data. A way of comparing the radiochemical data here with those obtained from microanalysis is to compare the C/N values of the high molecular weight fraction calculated by using the two methods. The C/N value can be estimated from radiochemical data using the fact that there are 6 C atoms in glucose, 12 C atoms in maltose, 2 C atoms in whole glycine, and 1 C atom in the decarboxylated glycine. For example, the ratio of whole glycine/decarboxylated glycine/glucose in the glucose−glycine melanoidins is 0.289:0.662:1. This is multiplied by the number of C atoms contributed from each molecule and, when summed, gives 7.24 C atoms to 0.289 N atoms from the whole glycine and 0.662 N atoms from the decarboxylated glycine. This gives, in total, 7.24 C atoms for 0.951 N atoms. Hence, for every N atom, the number of corresponding C atoms for the glucose−glycine melanoidins is 7.61. For the glucose−glycine-derived melanoidins the radiochemical investigation gives a result that is identical to the C/N value of the melanoidins obtained from microanalysis data (C/N for glucose−glycine melanoidins: 7.64 ± 0.21). The C/N value for melanoidins from the maltose−glycine reaction calculated in the same way is found to be 9.69. This is slightly lower than the value obtained by microanalysis (C/N: 10.53 ± 0.22). A feature of the calculation of the composition from radiochemical data is that, to obtain a result comparable to that from microanalysis, it is necessary to include in the melanoidins carbon and nitrogen atoms from the amino carbonyl formed in the Strecker degradation reaction as well as the Strecker aldehyde itself. This is a sensitive measure of the need to use the amino carbonyl in this way because, in both reactions, the decarboxylated glycine comprises approximately two-thirds of the amino acid that is incorporated into the melanoidins. The result suggests that the amino acid is incorporated into melanoidins completely except for carbon dioxide, when only the decarboxylated portion of the amino acid is incorporated.

Formation of Glucose during the Browning of Maltose. We consider now two distinct routes by which maltose can form melanonidins with glycine. First, the maltose residue can remain intact to be incorporated whole as the melanoidins increase in size. Second, the glycosidic bond in maltose could become broken in a step that is analogous to the dehydration of 3-deoxyhexosulose in the browning of glucose. In such a reaction, 3-deoxy-4-glycosylhexosulose could eliminate a glucose residue at the stage in the Maillard reaction when otherwise 3-deoxyhexosulose eliminates water. As a consequence, both sugars would form 3,4-dideoxyhexosulos-3-ene, a known precursor of melanoidins (*2*). **Figure 4** shows that the concentration of glucose increases to ∼13 mM over the observation period (120 h). Over this time period we find that the concentration of maltose falls by a similar amount (**Figure 4**). This implies a relatively high conversion of maltose to glucose in the reactions being observed. The fact that the formation of glucose is subject to an "induction" phase suggests that it is the result of a sequence of rate-limiting reaction steps. Had glucose been formed simply as a result of the hydrolysis of maltose, the rate of formation of glucose would have been a maximum at time zero. The third graph shows how the concentration of melanoidins increases with reaction time. The three graphs plotted in **Figure 4** have been scaled so as to reveal their relative shapes. The much more concave shape of the [melanoidins]−time plot could be indicative of the fact that there is a longer sequence of rate-limiting

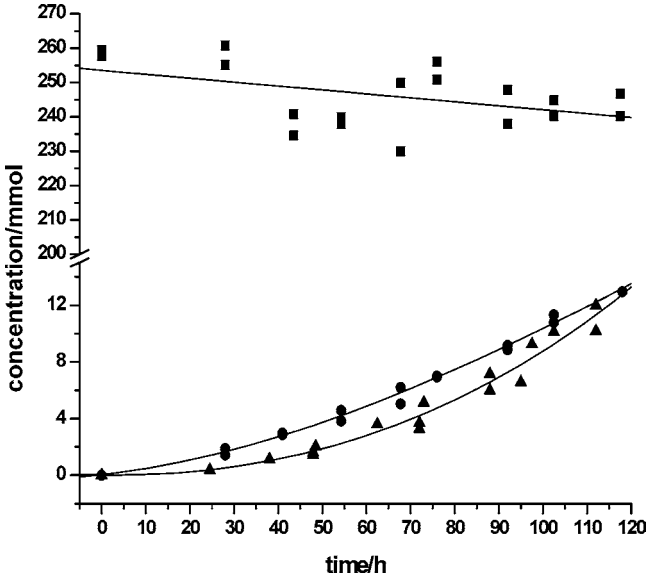

Figure 4. Release of glucose (●), loss of maltose (■), and formation of melanoidins (▲) with time in a maltose–glycine reaction ([mal] = 0.25 M, [gly] = 0.25 M, acetate buffer 0.2 M, pH 5.5, heated at 70.0 ± 0.1 °C). The concentration scale (*y*-axis) represents the actual molar concentration of glucose and maltose, whereas all values for melanoidin concentration have been multiplied by five to illustrate the difference in shape between the graphs. The trend line has been drawn as a power function with exponents 1.44 and 2.21 for glucose and melanoidins, respectively.

steps to the formation of melanoidins than to the release of glucose; this is entirely consistent with our view of glycosidic bond cleavage of maltose at an intermediate stage in melanoidin formation. Because most of the maltose that is lost is converted to products where the glycosidic bond is broken, we suggest that melanoidins from maltose at least include structures which would otherwise have been formed from glucose. The actual concentration of melanoidins (based on maltose subunits) formed during the observation period is ~2 mM. For the C/N value of melanoidins from maltose to be higher than that for melanoidins from glucose, the concentration of whole maltose residues incorporated into the melanoidins would only need to be at the level of 1−2 mM, which is below our limit of detection of concentration changes of maltose (present at an initial concentration of 250 mM) during the reaction.

In summary, melanoidins from glucose−glycine are formed with the conservation of all carbon and nitrogen atoms from glucose and glycine except for carbon dioxide. There are approximately twice as many decarboxylated glycine residues as whole glycine residues in melanoidins from both glucose and maltose. Both glucose and maltose residues are likely to be involved in the building of melanoidins in the maltose−glycine reaction.

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