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The incorporation of formaldehyde into melanoidins from the browning of glucose and glycine

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An investigation of the role of formaldehyde, produced during the Strecker degradation of glycine, in the formation of melanoidins from glucose and glycine, is described. This was carried out by adding formaldehyde to the reaction of glucose + glycine (initial pH 5·5, 55°C) and following the incorporation of glucose and C_1 and C_2 of glycine and formaldehyde into non-dialysable ($M_r > 12\,000$) melanoidins by means of ^{14}C-labelled reactants.

It is found that formaldehyde inhibits the browning of glucose + glycine, but the melanoidins formed in the presence of formaldehyde are spectrally different from those formed in its absence. Kinetic behaviour suggests that the formaldehyde reacts with intermediates in browning. Whilst added formaldehyde is incorporated into melanoidins, it also inhibits the incorporation of the Strecker degradation product of glycine into the polymer.

INTRODUCTION

Melanoidins are believed to be condensation polymers of carbohydrate degradation products and amino acids. In this respect, the most well-known intermediates derived from glucose in the Maillard reaction are 3-deoxyhexosulose and its dehydration product, 3,4-dideoxyhexosulos-3-ene (Anet, 1960, 1962); the latter is one of the most reactive intermediates known.

One of the simplest examples of the Maillard reaction is that between glucose and glycine. This has become the most widely studied of all browning reactions and as a model for browning in food, as reviewed by Wedzicha and Kaputo (1992). In its simplest form, the stoichiometric equation for the production of melanoidin,

$$aC_6H_{12}O_6 + C_2H_5NO_2 = C_{6a+2-x}H_{12a+5-2y}NO_{6a+2-2x-y} + xCO_2 + yH_2O$$

shows that, in addition to the polymer, the major products are CO_2 and H_2O. Typically, the value of y/a is 3 mol H_2O mol^{-1} glucose (Maillard, 1916; Enders & Theis, 1938; Wolfrom *et al.*, 1953; Reynolds, 1963; Olsson *et al.*, 1978; Feather & Nelson, 1984), whilst the degree of decarboxylation of glycine (which is the main source of CO_2) is somewhat variable. The value of a is usually close to 1; Wedzicha & Kaputo (1987) obtained $a = 1{\cdot}25$ for melanoidins with $M_r > 12000$, suggesting that glucose- and glycine-derived polymer subunits are combined in the ratio of 5:4 (for [glucose] = 1 M, [glycine] = 0·5 M, initial pH 5·5, 55°C). This non-integral stoichiometry has led us to consider whether or not the simple stoichiometric equation for calculating the composition of melanoidins is acceptable. In particular, it does not allow for the possible different tendencies of the Strecker aldehyde (in this case HCHO) and the nitrogen-containing moiety after Strecker degradation, to become incorporated into the melanoidin.

Despite the large amount of information available on the structures of intermediate products of Maillard browning, the detailed structure of melanoidins is still far from complete. Structures in which repeating furan or pyrrole moieties occur are considered less likely (Kato & Tsuchida, 1981) than condensation products derived from the Schiff base of 3-deoxyhexosulose and glycine, which also gives *N*-substituted pyrroles as well-known by-products (Kato, 1967). Such polymers, if formed regularly, contain a 1:1 molar ratio of glucose- to glycine-derived subunits. However, any explicit structures that have been proposed can only be regarded as speculative.

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The importance with which the glucose–glycine reaction has been regarded as a 'typical' Maillard reaction means that it is essential to review the significance of the Strecker aldehyde in melanoidin formation. Whilst the glucose–glycine reaction demonstrates the reactivity of the α-amino group in the Maillard reaction, without complications arising from side-chains on amino acid molecules, the potential formation of HCHO in Strecker degradation leads to a substance that is unusual among aldehydes; all other Strecker aldehydes have an α-carbon atom. It is possible, therefore, that if the Strecker aldehyde has a specific role in melanoidin formation, then the glucose–glycine reaction could prove somewhat atypical of Maillard reactions in general. The fact that polymers prepared from [$^{14}C_1$]glycine tend to have lower ^{14}C-activity than those prepared from [$^{14}C_2$[glycine (Feather & Huang, 1986) is good evidence for the belief that the Strecker aldehyde is incorporated into the melanoidin or that the amino acid residue is decarboxylated once it is in place in the polymer.

This investigation is part of a systematic study of the extent to which Strecker aldehydes are incorporated into melanoidins during the browning of glucose. The aim is to use the radiochemical approach of ^{14}C-labelled reactants to measure the stoichiometry of binding of glucose and, in the present work, decarboxylated and non-decarboxylated glycine, together with added HCHO. It was decided to adopt reaction conditions ([glucose] = 1 M, [glycine] = 0·5 M, initial pH 5·5, 55°C) which correspond to those used in major investigations of Maillard reactions under 'accelerated' food storage conditions within the authors' group. Melanoidins were classified as those products that were retained after dialysis ($M_r > 12\,000$) because this provided an unambiguous method of separation.

MATERIALS AND METHODS

Materials

All chemicals were of AnalaR grade and were supplied by Sigma Chemical Company. The radiolabelled substances were supplied by Amersham International.

Preparation of reaction mixtures

All reaction mixtures were prepared by dissolving solid reactants in water to give final concentrations of 1 M glucose and 0·5 M glycine. To enable pH adjustment and allow addition of HCHO, the reactants were dissolved in 80% of the final volume of solvent and HCHO (final concentration 0–50 mM), and NaOH (1 M) or HCl (1 M) was added as appropriate, before making up to the final volume. Reaction mixtures were dispensed into 100 ml flasks which were closed and heated at 55°C. To determine the extent of browning, samples were withdrawn at timed intervals and absorbances measured in the range 400–500 nm. For the investigation of the effect of adding HCHO to an already browned glucose + glycine mixture, a reaction containing glucose (2 M) and glycine (1 M), prepared as before, was allowed to brown for 144 h at 55°C, and an aliquot (50 ml) was made up to 100 ml with water or 0·1 M HCHO solution. The reaction was allowed to continue at 55°C and was analysed as before.

To obtain non-dialysable melanoidins, an aliquot (10 ml) of reaction mixture was dialysed against water (3×1 litre) using Visking tubing.

Radiochemical investigations

Two reaction mixtures (100 ml) containing glucose (2 M) + glycine (1 M) and glucose (2 M) + glycine (1 M) + HCHO (0·1 M) were prepared. The pH had been adjusted initially so that, after dilution to twice the volume by adding water, the pH value would be 5·5. To separate aliquots (10 ml) of each mixture, approximately 4·6 MBq of [^{14}C-U]glucose, [$^{14}C_1$]glycine and [^{14}C-U]glycine of negligible mass were added and the volume made up to 20 ml with water. An aliquot (25 ml) of the reaction solution containing HCHO was mixed with 18·5 MBq of [^{14}C]HCHO of negligible mass, and the volume made up to 50 ml with water.

Aliquots (1 ml) of each reaction mixture were withdrawn at timed intervals and were dialysed against water (3×1 litre) as before. Additionally, all samples containing [^{14}C]HCHO were dialysed against HCHO solution (50 mM). After dialysis, the contents of the tubing were washed out with water and made up to 25 ml. Aliquots (1 ml) of each sample were mixed with scintillation fluid (10 ml of Scintillator 299; Packard) in separate vials and counted using a Packard TR1500C scintillation counter. Similarly, aliquots (1 ml) of appropriately diluted (×1000) reaction mixtures were counted to obtain the total ^{14}C-activity in each reaction. Quench correction was by the channels ratio method, calibrated using chloroform-quenched standards. There was no need for colour quench correction at the concentration of melanoidin used.

RESULTS AND DISCUSSION

Effect of added formaldehyde on the browning of glucose + glycine

The pH of reaction mixtures was set initially to pH 5·5, but no other form of pH control was applied. Typically, the pH of reaction mixtures containing glucose (1 M) + glycine (0·5 M) fell to 4·6 and 4·2 after 100 and 400 h, respectively.

There is no single wavelength at which browning can be measured; to investigate the effect of HCHO on the glucose–glycine reaction, absorbances of reaction mixtures were measured in the range 400–500 nm. Absor-

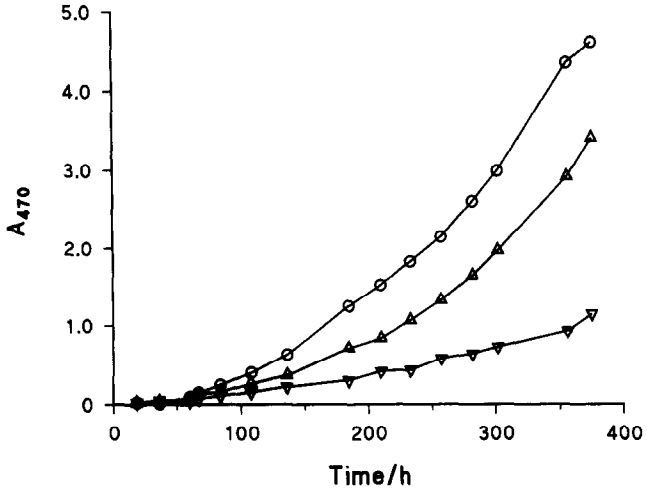

Fig. 1. Absorbance (at 470 nm) versus time curves for the reaction of glucose (1 M) + glycine (0.5 M) in the absence of HCHO (○) and in the presence of 5 mM (△) and 50 mM (▽) HCHO. Reaction conditions: initial pH 5.5, 55°C.

bance–time data for three reaction mixtures containing glucose (1 M), glycine (0·5 M) and HCHO (0, 5 and 50 mM), obtained at 470 nm, are shown in Fig. 1. These demonstrate the ability of HCHO to inhibit the browning of glucose + glycine as measured at this wavelength; the non-linear concentration behaviour of HCHO and the wavelength dependence of this effect are illustrated in Fig. 2 for a reaction that had been allowed to proceed for 375 h. We see that low concentrations of HCHO (e.g. 1 mM) are sufficient to cause a significant reduction in the absorbance due to the reaction products at all wavelengths investigated. Studies of the inhibition of the glucose–glycine reaction by sulphite species (Wedzicha, 1984; Wedzicha & Vakalis, 1988; Wedzicha & Kaputo, 1992) suggest that, over the timescale investigated here, the turnover of intermediates in browning is equivalent to a few millimoles of glucose per litre, and of the same order of magnitude as the concentration of HCHO found to have an effect on browning. High concentrations of HCHO (e.g. > 30 mM) have no further effect. These results suggest that HCHO is reacting either with an intermediate in melanoidin formation or with the melanoidins once they have formed.

These possibilities were tested simply by adding HCHO (50 mM) to an already browned ($A_{470} = 0 \cdot 646$) mixture of glucose + glycine and following the subsequent development of colour. The results, shown in Fig. 3, indicate that there is no significant rapid reaction between the melanoidins and HCHO but that the additive appears to inhibit further increase in absorbance as the glucose–glycine reaction continues. The rate of increase in absorbance is similar to that shown in Fig. 1 (lowest trace) when HCHO had been added at the start of the reaction. This behaviour is seen at all wavelengths in the range 400–500 nm. It is suggested, therefore, that HCHO reacts with intermediates in the browning reaction, either inhibiting the formation of the melanoidins or modifying their absorbance characteristics.

The data in Fig. 2 indicate that the shapes of the absorbance spectra of the melanoidins are changed when browning occurs in the presence of HCHO. There is a significant increase in the ratio of absorbance at 400 nm to absorbance at 500 nm in HCHO-containing reactions as illustrated in Table 1, and consistent with the apparent redder colour of glucose–glycine mixtures with added HCHO. The fact that this difference is seen both in whole reaction mixtures and in non-dialysable melanoidins suggests that the HCHO is either incorporated

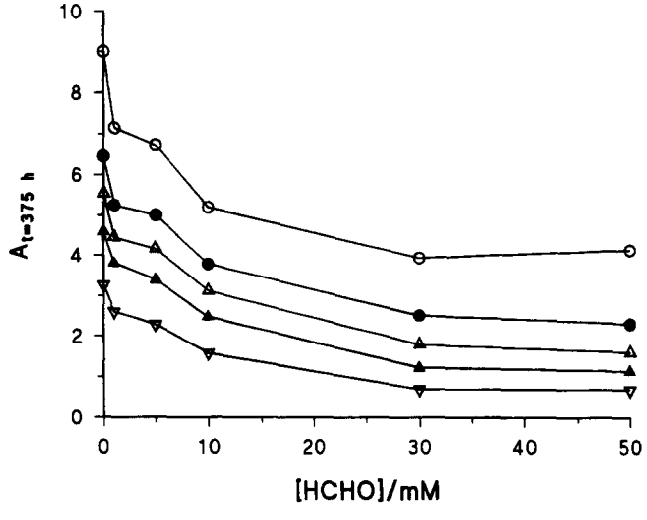

Fig. 2. Effect of formaldehyde concentration on the absorbance of glucose (1 M) + glycine (0.5 M) reaction mixtures at 400 nm (○), 430 nm (●), 450 nm (△), 470 nm (▲) and 500 nm (▽), after 375 h of reaction. Reaction conditions: initial pH 5.5, 55°C.

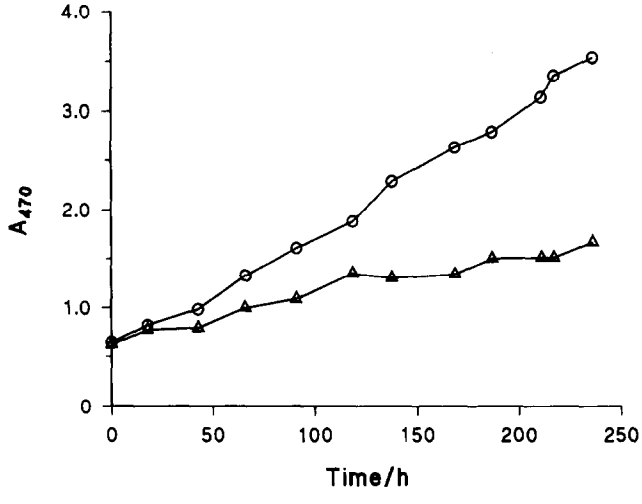

Fig. 3. Effect of adding HCHO to an already browned mixture of glucose + glycine on the subsequent absorbance of the mixture at 470 nm. Time is measured from the time of addition of HCHO. Reaction conditions for initial browning of glucose + glycine: [glucose] = 2 M, [glycine] = 1 M, initial pH 5.5, 55°C, reaction time = 144 h. Reaction conditions for subsequent browning experiment: [glucose] = 1 M, [glycine] = 0.5 M, [HCHO] = 0 mM (○) or 50 mM (△), 55°C.

Table 1. Ratio of absorbance at 400 nm to absorbance at 500 nm of whole glucose + glycine reaction mixtures without and with 50 mM HCHO, and of the melanoidins ($M_r > 12000$) separated from these reactions

Sample	A_{400}/A_{500}
Whole reaction, no HCHO	2·65
Melanoidins, no HCHO	2·25
Whole reaction, 50 mM HCHO	5·83
Melanoidins, 50 mM HCHO	3·94

Reaction conditions: [glucose] = 1 M, [glycine] = 0·5 M, initial pH 5·5, 55°C, reaction time 280 h.

into the melanoidins, changing their chromphore or, by binding to specific intermediates in browning, HCHO changes the way in which other intermediates can polymerise to form the melanoidin chromophore. It is evident that HCHO also changes the spectra of the fraction with $M_r < 12000$. Whilst the spectra of the lower and higher molecular weight components of reactions without HCHO are similar, the lower molecular weight fraction of the HCHO-containing reactions is more red than the melanoidins. Similar behaviour is seen for melanoidins formed after HCHO is added to an already browned mixture, as in the experiment shown in Fig. 3.

Radiochemical investigation

The rationale behind the radiochemical investigations described here was to measure the number of HCHO molecules incorporated into the melanoidin per molecule of glucose. As has been the practice with the use of microanalysis data to establish the composition of melanoidins with regard to glucose- and glycine-derived subunits, it was assumed that all six carbon atoms of glucose become incorporated into the polymer. The source of HCHO is either that which is added to reaction mixtures or that formed by the decarboxylation of glycine. The specific incorporation of C_2 and $C_1 + C_2$ of glycine into the polymer can be measured through the use of selectively labelled (C_1) and uniformly labelled glycine, and measurement of the relative amounts of [^{14}C]glucose and [^{14}C]glycine incorporated into the non-dialysable melanoidins. [^{14}C]HCHO is available for measurement of the incorporation of added HCHO into the melanoidin. The approach involved the setting up of identical reaction mixtures in which only one component was labelled. The amount of ^{14}C in the retentates after dialysis was measured, as a function of reaction time to identify the time-dependent changes in composition as well as to establish consistency of the data.

The specific activities of glucose, glycine and HCHO in reaction mixtures were 238–244, 496–536 and 5660 MBq mol^{-1}, respectively. These were used to convert ^{14}C-activities to the amounts, in mole per litre, of each reactant incorporated into the melanoidins. These concentrations were expressed as those in the reaction mixtures from which the melanoidins were isolated. In the case of the uniformly labelled glycine, the ^{14}C-activity is distributed over both carbon atoms, and the activity associated with each carbon atom is, therefore, half that associated with C_1 of [$^{14}C_1$]glycine. It was established, by counting the diffusate, that the dialysis procedure was exhaustive, and the ^{14}C-activity in the retentate could be attributed to the non-dialysable melanoidin fraction. The amounts of each reactant incorporated into the melanoidins are given in Table 2. Measurements at 15 days represent the time limit of the experiments shown in Figs 1–3; it was not possible to obtain reliable ^{14}C-activity data at times shorter than 10 days because the amounts of non-dialysable melanoidins were too small to be counted accurately.

We see from Table 2 that the amounts of glucose and glycine incorporated into the polymer increase progressively with time. On the other hand, the amount of HCHO incorporated appears to increase for the first 15–18 days and subsequently no further increase occurs. The concentration of HCHO in the reaction mixture was 50 mM, and approximately 8% (mol mol^{-1}) of the HCHO became incorporated into the melanoidin. Since the conversion of HCHO to high molecular weight products does not appear to continue beyond 8%, it is likely that the remainder is present in the form of

Table 2. Amount of glucose, glycine and formaldehyde incorporated (mmol litre^{-1}) into melanoidins ($M_r > 12000$) analysed after given reaction times

Labelled reactant	[HCHO](mM)	Reaction time (days)						
		10	12	15	18	20	23	25
Glucose	0	1·05	2·08	2·12	2·95	5·22	6·01	7·30
Glycine-C_1	0	0·30	0·81	1·00	1·53	2·00	2·56	2·92
Glycine-U	0	0·42	1·47	1·65	2·24	3·38	3·77	5·04
Glucose	50	0·59	1·13	1·13	1·50	2·59	3·88	4·74
Glycine-C_1	50	0·96	1·51	1·81	2·82	2·99	3·80	4·20
Glycine-U	50	1·63	1·05	2·24	3·03	3·15	3·66	4·55
HCHO	50	2·61	2·63	3·97	4·17	3·38	4·09	3·90
HCHO[a]	50	2·19	2·14	2·13	2·86	2·53	3·02	3·09

Reaction conditions: [glucose] = 1 M, [glycine] = 0·5 M, initial pH 5·5, 55°C.
[a]These results were obtained when melanoidins were dialysed against 50 mM HCHO solution instead of water.

dialysable, lower molecular weight products, which are much less reactive towards polymer formation. It is suggested, therefore, that HCHO reacts to form both melanoidins with $M_r > 12\,000$ and reaction products that are of lower molecular weight.

It has long been known that HCHO exerts a marked effect on the pH-titration curves of amino acids (formol titration). Levy (1933) explained this behaviour in terms of two parallel reactions involving the unprotonated amino group, as follows:

$$RNH_2 + HCHO \rightleftharpoons RNHCH_2OH$$

$$RNH_2 + 2HCHO \rightleftharpoons RN(CH_2OH)_2$$

The equilibrium constants, K_1 and K_2, for the formation of mono- and di-adduct, respectively, are given by:

$$K_1 = x/g(f - x - 2y)$$

$$K_2 = y/g(f - x - 2y)^2$$

where x and y are the concentrations of the two products at equilibrium, respectively, f and g are the initial concentrations of HCHO and unprotonated amino acid (glycine in the present study), respectively, and the equations have been derived assuming that $g \gg f$. Simultaneous solution gives a quadratic equation which may be solved for x:

$$2x^2K_2/K_1 + x(1 + K_1g) - K_1fg = 0$$

and the relationship between x and y is given by:

$$y = x^2K_2/K_1^2g$$

Consider a glucose–glycine–HCHO reaction with a total glycine concentration of 0·5 M and $f = 10$ mM, at which concentration it shows a marked kinetic effect on browning. Levy (1933) gives $K_1 = 60\ M^{-1}$ and $K_2 = 290\ M^{-2}$ at 30°C. These data were obtained with $f \gg g$ (as in the formol titration) and no results are available at 55°C. Since the effects of temperature and concentration on these values are not known, any calculation of x and y can only give an order of magnitude estimate of the concentrations that are to be expected in the reaction mixtures.

At pH 5·5, $g = 40\ \mu M$ and calculation gives $x = 24\ \mu M$, $y = 1 \cdot 2\ \mu M$. Thus, the concentration of free HCHO is 9·97 mM, demonstrating that, under the conditions adopted here, very little HCHO is associated with the amino acid.

One mechanism whereby HCHO could bind to melanoidins is this reversible association with amino groups, if the association constant is some orders of magnitude greater than that given for glycine above. Dialysis of melanoidins prepared with [^{14}C]HCHO reveals that some of the ^{14}C-activity is exchangeable and that the 'irreversibly' bound HCHO probably represents some 6% (mol mol^{-1}) of that which was originally present in reaction mixtures.

The stoichiometry of glucose:HCHO in the non-dialysable melanoidins, from added HCHO, is understandably variable. At relatively short reaction times it is seen that more than two molecules of HCHO bind for every glucose-derived residue in the melanoidin, but this ratio falls with time. This observation could arise through the subsequent formation of a polymer that does not contain [^{14}C]HCHO, i.e. as would be normal in the glucose + glycine reaction with no added HCHO. A particularly significant observation is that added HCHO does, indeed, reduce the amount of glucose converted to melanoidin after a given time. If added HCHO does not affect the rate of *production* of intermediates in browning, then its conversion of these intermediates to low molecular weight ($M_r < 12\,000$) products that do not polymerize would explain the reduced yield of melanoidins. The low conversion of added HCHO to high molecular weight products, after all the added HCHO appears to have undergone reaction, is also consistent with this idea.

When [$^{14}C_1$]glycine is incorporated into the melanoidin, the high molecular weight product may or may not become labelled with ^{14}C, depending on whether the binding of glycine has involved the whole molecule (including C_1) or the Strecker aldehyde (after losing C_1 as CO_2). One could, therefore, write a general equation:

$$NH_2CH_2^*COOH \rightarrow x(M)NH_2CH_2^*COOH + y(M)NH_2CH_3 + y^*CO_2$$

where x and y represent the fractions of non-decarboxylated and decarboxylated glycine, respectively, and (M) draws attention to the fact that these molecules are part of a melanoidin molecule. Consider now the situation when [^{14}C-U]glycine is used, and the corresponding equation becomes:

$$NH_2^*CH_2^*COOH \rightarrow x(M)NH_2^*CH_2^*COOH + y(M)NH_2^*CH_3 + y^*CO_2$$

The ^{14}C-activity incorporated into the melanoidin from [$^{14}C_1$]glycine gives a direct measure of x. Since the same value of x is expected when both series of experiments are carried out, the ^{14}C-activity in the form of the doubly-labelled glycine in the melanoidin structure can be calculated. Thus, the ^{14}C-activity attributable to the melanoidin moiety with only C_2 from the glycine can be obtained by difference.

The relationship between the amounts of glucose and glycine incorporated into the growing melanoidin in the absence of added HCHO is shown in Fig. 4, for intact glycine residues and for decarboxylated glycine residues.

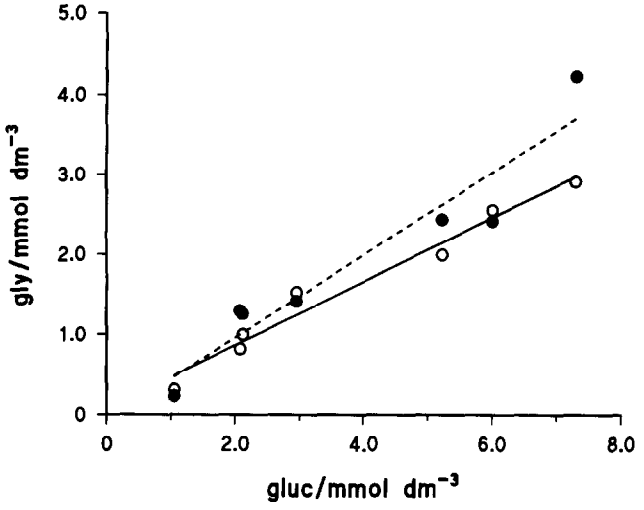

Fig. 4. Relationship between the amount of glycine- and glucose-derived subunits incorporated into melanoidins in the absence of added HCHO. ●, decarboxylated glycine; ○, non-decarboxylated glycine. Reaction conditions: [glucose] = 1 M, [glycine] = 0.5 M, initial pH 5.5, 55°C. The glucose concentrations are those given in Table 2, from which reaction times may also be deduced.

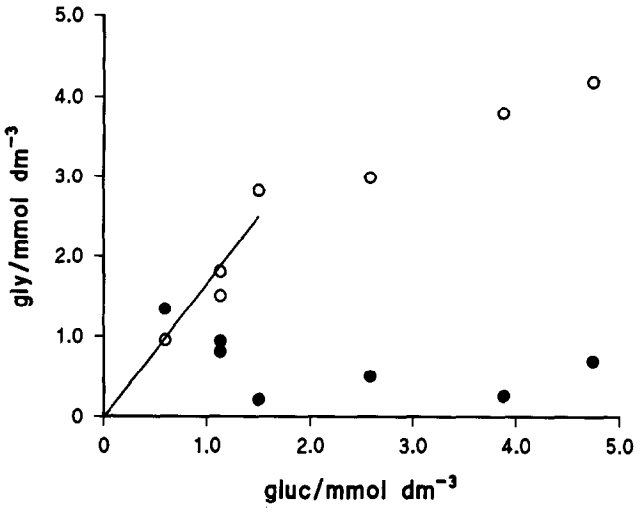

Fig. 5. Relationship between the amount of glycine- and glucose-derived subunits incorporated into melanoidins in the presence of added HCHO (50 mM). ●, decarboxylated glycine; ○, non-decarboxylated glycine. Reaction conditions: [glucose] = 1 M, [glycine] = 0.5 M, initial pH 5.5, 55°C. The glucose concentrations are those given in Table 2, for HCHO-containing experiments, from which reaction times may also be deduced. The line has been drawn through the data for non-decarboxylated glycine up to a glucose residue concentration of 1.5 mM, and the regression line forced to pass through the origin.

The two graphs are drawn as straight lines and the regression lines are found to have intercepts close to the origin. This suggests that the glycine:glucose stoichiometry is constant over the whole observation period of 25 days. The slopes of the two lines are 0·52 and 0·40 for decarboxylated and non-decarboxylated glycine residues, respectively, suggesting that these residues are incorporated in similar numbers into the growing melanoidin. The total numbers of glucose- to glycine-derived sub-units in the melanoidin is, therefore, 1:0·92 and is comparable with our previously reported value of 1:0·8 for this reaction under the same conditions, but obtained from microanalysis data (Wedzicha & Kaputo, 1987). However, the radiochemical approach is much more rigorous and we obtain here the additional information that both intact and decarboxylated glycine are incorporated with a fixed stoichiometry with respect to glucose-derived residues.

The corresponding results obtained in the presence of added HCHO (50 mM) are shown in Fig. 5 and are found to be somewhat different. Whilst examining these relationships, it should be borne in mind that the amount of HCHO that becomes incorporated into the melanoidin reaches an almost constant value by the time the concentration of glucose-derived subunits in the melanoidin is 1·1–1·5 mM (Table 2). The slope of the line drawn through the data up to this concentration shows that some 1·7 molecules of intact glycine are incorporated for every glucose-derived residue; above this concentration the stoichiometry is lower. On the other hand, the initial presence of HCHO appears to have inhibited the subsequent incorporation of decarboxylated glycine into the melanoidin. One reason for this behaviour is the possibility that the relatively high concentration of unlabelled HCHO causes this substance to compete with the [^{14}C]HCHO released from [^{14}C$_1$]glycine on decarboxylation and give a polymer which contains the unlabelled rather than the labelled HCHO.

CONCLUSION

Low concentrations of HCHO inhibit the browning of glucose + glycine; this inhibitor reacts with intermediates in melanoidin formation, reducing the amount of high molecular weight product formed and changing the absorbance spectrum of the chromophore. In the absence of added HCHO, intact and decarboxylated glycine molecules are incorporated into melanoidins in similar amounts, and the overall stoichiometry, from radiochemical measurements, of the polymer with respect to glucose- and glycine-derived subunits is approximately 1:0·9 (glucose:glycine). Added HCHO inhibits the incorporation of decarboxylated glycine into the melanoidin, suggesting that it is the Strecker aldehyde that is the reactive species.

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