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The Formation and Reactivity of Osuloses in the Sulphite-Inhibited Maillard Reaction of Glucose and Glycine

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ABSTRACT

A simple mechanism for the sulphite-inhibited Maillard reaction of glucose and glycine has been critically appraised. By spiking mixtures of glucose, glycine and hydrogen sulphite ion (S(IV)) with ^{35}S-labelled S(IV) or 3,4-dideoxy-4-sulphohexosulose (DSH), or ^{14}C-labelled 3-deoxyhexosulose (DH), it has been shown that the rate determining step is the formation of DH and that, while S(IV) is present, the major reaction product is DSH. Once S(IV) has run out, DSH shows significant reactivity. The rates of formation and loss of DH are analysed in detail.

INTRODUCTION

3,4-Dideoxy-4-sulphohexosulose (DSH) is an important reaction product in the sulphite-inhibited Maillard reaction of glucose and glycine. It is formed by addition of sulphite ion to 3,4-dideoxyhexosulos-3-ene (DDH), which is a key intermediate in the browning reaction and arises from dehydration of 3-deoxyhexosulose (DH), a product from the decomposition of ketoseamines. The formation of DSH is thought to be the main path in the inhibition of browning by converting DDH to a less reactive product.

Much of our recent information concerning the mechanism of the inhibition of the browning reaction has come from kinetic data and, in particular, the fitting of data for the loss of total sulphur(IV) oxospecies,

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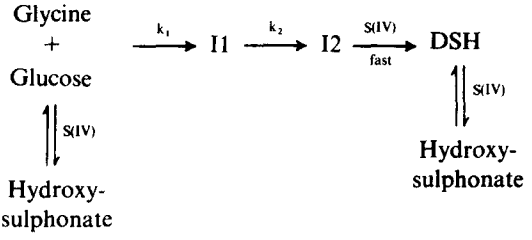

Fig. 1. Kinetic model for the sulphite-inhibited Maillard reaction of glucose + glycine (Wedzicha, 1984). I1 and I2 represent kinetically significant intermediates and k_1 and k_2 are rate constants.

S(IV), and the formation of hydroxysulphonates in the system glucose–glycine–S(IV), to the basic model shown in Fig. 1 (Wedzicha, 1984), where I1 and I2 represent kinetically significant intermediates. It is now reasonably certain from kinetic data that I1 is DH (Wedzicha & Kaban, 1986). The data also suggest that whilst DSH is much less reactive towards browning than is DH or DDH when there is S(IV) remaining, DSH could lead to the formation of colour as the concentration of inhibitor becomes small.

The main limitation of kinetic data is that they refer to the rate determining step of a reaction and the view of the mechanism tends to be simplified. It is important, therefore, to support kinetic data with analytical data on the rate at which identifiable products are formed and lost and to compare the actual distribution of reaction products with that predicted from the kinetic model. The Maillard reaction is known to be one of the greatest complexity and it is reasonable to ask, therefore, whether a simple mechanism, such as that shown in Fig. 1, could be realistic. In this investigation we approached the question by observing the distribution of reaction products after spiking mixtures of glucose, glycine and S(IV) with ^{35}S-labelled sulphite ion or DSH, or ^{14}C-labelled DH. In this paper, S(IV) will be used to denote oxospecies of sulphur in oxidation state +4 when it is not desired to specify an individual ionic form.

MATERIALS AND METHODS

All reagents were of AnalaR grade and were obtained from BDH Chemicals plc, Poole, Dorset, UK. Radiochemicals were obtained from Amersham International plc (Amersham, Buckinghamshire, UK).

Preparation of 3,4-dideoxy-4-^{35}S-sulphohexosulose

A mixture of glucose (91 mg), $Na_2S_2O_5$ (24·8 mg), Na_2SO_3 (25·6 mg) and $Na_2{}^{35}SO_3$ (6·4 mg, 52 MBq) in water (0·2 ml) was heated at 100°C for 4 h in a

sealed 5 ml capacity glass ampoule. The products mixture was mixed with Dowex 50W-X8 cation-exchange resin in the H^+ form (1 ml) to bring the pH to pH 1 and the resulting solution together with washings was diluted to 50 ml with water. Unreacted sulphite ion was removed by steam distillation which was continued until the distillate showed no reaction towards iodine. In preliminary experiments absence of sulphur dioxide in the distillate at this stage was confirmed by scintillation counting. The residue after distillation was passed through Dowex 50W-X8 cation-exchange resin in the Na^+ form to convert acids to their sodium salts and concentrated to 3·5 ml under reduced pressure at 40°C.

An aliquot (2 ml, total ^{35}S-activity 3 MBq) of crude products was applied to a TSK DEAE 3SW HPLC column (7·5 × 150 mm) and eluted with a linear ammonium acetate gradient (0–0·5M, 1 ml min^{-1}). Column effluent was split so that 3% passed to a Ramona LS flow-through radioactivity monitor where the stream was mixed (1:40) with Monofluor (National Diagnostic) scintillation fluid before counting in a 0·5 ml flow cell. The ratemeter was operated with a time constant of 1 s. The remainder of the column effluent was fractionated into 0·5 ml samples. Aliquots of fractions corresponding to each of three chromatographic peaks (eluted at 38, 49 and 57 min) were acidified with $HClO_4$ and mixed with an equal volume of a solution of 2,4-dinitrophenylhydrazine hydrochloride (2% w/v in 30% $HClO_4$). Only the peak running at 38 min showed any sign of reaction. The product which formed was analysed by TLC on silica ($60F_{254}$, butanol:0·880 ammonia solution) when it was found to run as a single component at R_f 0·38. This corresponded to the 2,4-dinitrophenylhydrazone of authentic DSH (Wedzicha & Kaban, 1986). Fractions corresponding to this peak were bulked (yield 1·35 ml, total ^{35}S-activity 0·68 MBq).

The product was diluted to 10 ml and passed through Dowex 50W-X8 ion-exchange resin in the H^+ form (1 ml) to remove ammonium ions. Remaining acetic acid was removed by subsequent extraction with diethyl ether (5 × 50 ml). The resulting product was analysed by HPLC using a TSK DEAE 3SW (7·5 × 150 mm) column eluted with linear ammonium acetate gradient (0–0·5M, 1 ml min^{-1}). All the column effluent was passed to the radioactivity monitor and counted as described above using a ratio of effluent: scintillation fluid of 1:1.

Preparation of ^{14}C-3-deoxyhexosulose

A mixture of glucose (0·2 g), *p*-toluidine (0·11 g), ethanol (95%, 4·5 ml), acetic acid (0·22 ml) and ^{14}C-glucose (0·18 mg in 3% v/v ethanol, 1·25 ml, 9·25 MBq) was heated under reflux for 30 min. Benzoylhydrazine (0·33 g) in ethanol (1 ml) was added and the mixture heated under reflux for 5 h. The

product was filtered and washed with methanol (3 × 1 ml), diethyl ether (3 × 1 ml) and finally again with methanol (3 × 1 ml). The dry product was dissolved in a mixture of ethanol (3·6 ml), water (6 ml), acetic acid (0·037 ml) and benzaldehyde (0·19 ml), and boiled under reflux for 2·5 h. Water (5 ml) was added and excess benzaldehyde removed by distillation (volume of distillate 3 ml, distillation time 12 min). The mixture was filtered through a glass wool plug and Dowex 1-X8 cation-exchange resin in the CO_3^{2-} form added. When neutral, the mixture was filtered and its volume reduced to 5 ml under reduced pressure at 40°C. The product was washed with diethyl ether (6 × 3 ml), and treated with acid-washed activated charcoal (200 mg). To the resulting solution was added a mixture of Dowex 50W-X8 (H^+ form) and Dowex 1-X8 (CO_3^{2-} form) ion-exchangers (total volume 0·5 ml) and filtered.

The product was analysed by TLC on silica ($60F_{254}$, ethanol:0·880 ammonia solution:water, 4:2:1) and using *p*-anisidine to reveal spots. The ^{14}C-activity in each spot was measured by scraping into a liquid scintillation vial, suspending in 0·5 ml water and adding 4 ml scintillation fluid (Triton X100 + toluene, 1 + 2, containing 4 g PPO per litre). Vials were counted in a Beckmann LS100C liquid scintillation counter.

Kinetic experiments

The standard sulphite-inhibited browning reaction mixture consisted of glucose (1 M), glycine (0·5 M) and hydrogen sulphite ion (0·04–0·05 M) whose pH had been adjusted to pH 5·5 with NaOH. All experiments were carried out at 55°C. Mixtures containing ^{35}S-sulphite were spiked with $Na_2{}^{35}SO_3$ (0·72 mg, 5·01 MBq). The radiolabelled compound was found to contain 0·82 MBq SO_4^{2-}. In other experiments the standard reaction mixture containing unlabelled reactants (10 ml) was spiked either with ^{35}S-DSH (5 ml, 0·05–0·08 MBq ml^{-1}) or ^{14}C-DH (1 ml, 0·047 MBq ml^{-1}) at zero reaction time and after the standard reaction had proceeded for 288 h. In each case the volume of the mixture after spiking was reduced to slightly less than 10 ml by concentration under reduced pressure at 40°C and making up with water. For all kinetic runs involving labelled reactants aliquots of reaction mixture (150 μl) were placed in capillary tubes which were sealed. Capillary tubes were opened at timed intervals for analysis.

Unreacted S(IV) was determined by adding reaction mixture (10 μl) to a solution of 5,5′-dithiobis(2-nitrobenzoic acid) (2 mM) in phosphate buffer (10 ml, pH 8·0, 32 mM Na_2HPO_4 + 1·8 mM NaH_2PO_4, containing 10% v/v ethanol) and the absorbance of the resulting solution measured at 412 nm. The method was calibrated using iodimetrically standardised solutions of sodium metabisulphite containing ethanol (1% v/v) to prevent autoxidation.

Reaction mixtures were analysed for the formation of labelled products by HPLC using a TSK DEAE 3PW column (7·5 × 75 mm) eluting with an ammonium acetate gradient (0–1 M, linear gradient for 1 h then isocratic for 20 min, 1 ml min^{-1}). At long reaction times when the residual sulphite ion concentration had fallen to below 1 mM, aliquots of reaction mixture were applied directly to the column for analysis. In order to remove unreacted sulphite ion and decompose any hydroxysulphonates present, which could complicate the apparent distribution of reaction products, reaction mixtures were treated with iodine after raising the pH with NaOH. An aliquot (100 μl) of reaction mixture was made alkaline with NaOH (150 μl, 50 mM) and iodine solution (50 mM) added until the total volume added was equivalent to reaction of all residual sulphite ion present in the mixture (as determined spectrophotometrically).

RESULTS AND DISCUSSION

When ^{35}S-labelled DSH was analysed on the DEAE column it ran as a single peak which accounted for 93·4% of the activity applied. The identity of DSH was confirmed by TLC of the 2,4-dinitrophenylhydrazone of the appropriate fraction on silica when a single purple spot running in the same position as authentic DSH was observed. The yield of DSH was 0·55 MBq with a specific activity of 101 MBq $mmol^{-1}$ and the total weight of radiolabelled product was, therefore, 1·2 mg.

The ^{14}C-labelled DH ran as three spots at R_f 0·47, 0·61 and 0·80. It was found that 85·3% of the total activity was associated with the component running at R_f 0·61. An authentic sample of DH prepared by the method of Madson and Feather (1981) and available from previous work (Wedzicha & Kaban, 1986) was found to run in this position. Anet (1964) also reported the presence of three components and assigned the slower and faster running impurities to *cis*- and *trans*- 3,4-dideoxyhexosulos-3-enes. It has recently (Wedzicha & Tian, unpublished) been found that these impurities are formed slowly when aqueous solutions of DH are allowed to stand and their presence is unavoidable when the final product is handled as a solution. The yield of DH was 1·88 MBq with a specific activity of 8·3 MBq $mmol^{-1}$ and the total weight of DH was, therefore, 37 mg. In view of the small weights of the DH and DSH samples it was considered inadvisable to recover the corresponding solids, and solutions of the compounds were used to spike reaction mixtures.

Typical chromatograms showing the formation of ^{35}S-labelled sulphite-derived reaction products in the S(IV)-inhibited Maillard reaction of glucose and glycine are shown in Fig. 2 where reaction mixtures were sampled at

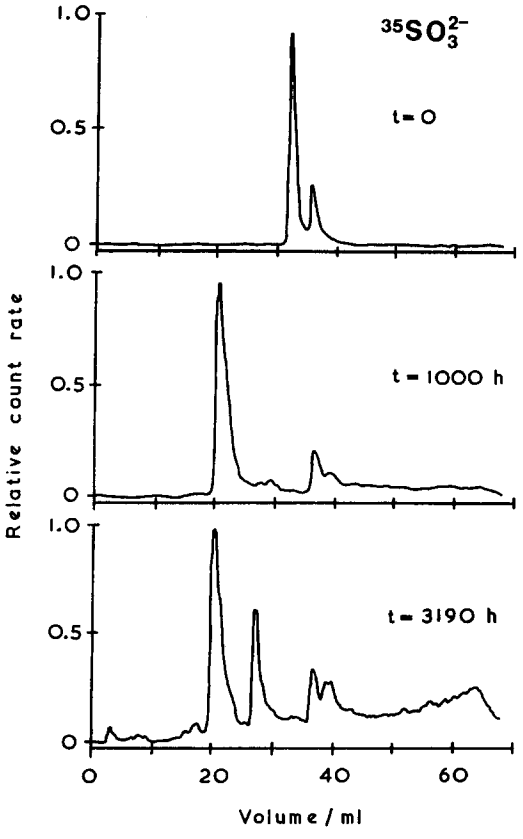

Fig. 2. Chromatograms of ^{35}S-labelled reaction products formed when a mixture of glucose (1 M), glycine (0·5 M) and ^{35}S(IV) (0·05 M), initial pH 5·5, is allowed to react at 55°C. The chromatograms shown are for reaction mixtures at times zero, 1000 h and 3190 h.

time zero, at a time (1000 h) close to the exhaustion of S(IV) (free + reversibly bound) and after considerable browning had taken place (3190 h). The principal component at zero time is S(IV) whilst the minor component is sulphate ion. This is seen to be present on all chromatograms. The component running at 21 ml is DSH verified by co-chromatography with an authentic ^{35}S-labelled sample. It is seen that DSH is the principal reaction product while S(IV) is present in the mixture, though a small but significant presence of components along the base line in the 1000 h chromatogram is indicative of the later formation of further reaction products. The substance which runs at *c.* 28 ml may be obtained by treating DSH with limewater and is probably the corresponding metasaccharinic acid. The identity of the product running at 39 ml is unknown. It is possible that the new products include high molecular weight pigments produced in Maillard browning and are seen to incorporate ^{35}S, presumably through reaction of DSH with itself

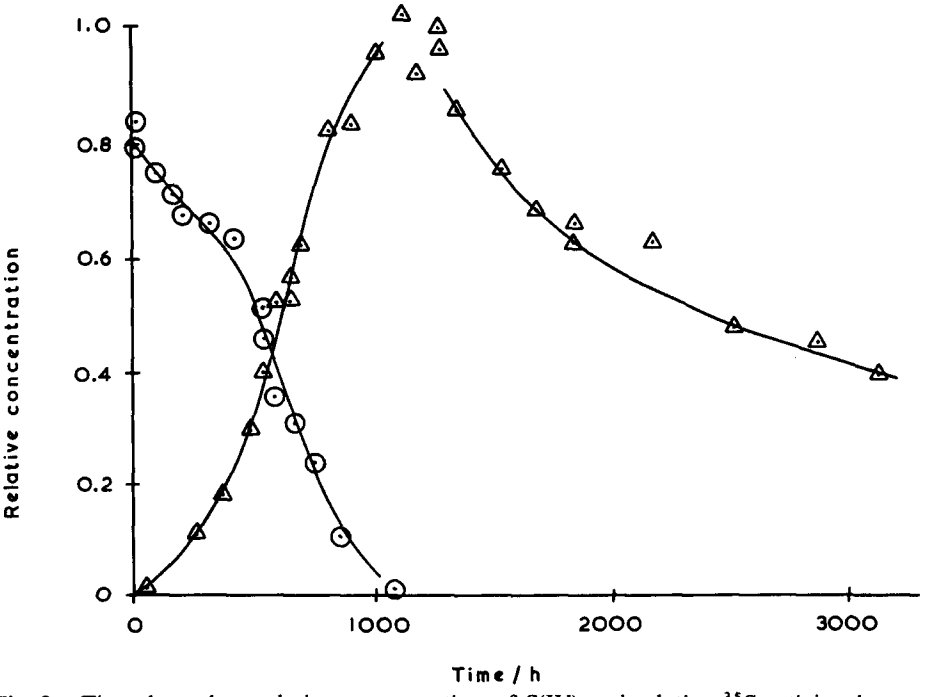

Fig. 3. Time-dependent relative concentration of S(IV) and relative ^{35}S-activity due to DSH when a mixture of glucose (1M), glycine (0·5M) and ^{35}S(IV) (0·05M), initial pH 5·5, is allowed to react at 55°C. The concentration of DSH is proportional to its ^{35}S-activity. ⊙, S(IV); △, ^{35}S-DSH.

or with intermediates in browning. The suggestion that this might take place had been made, in a previous paper (Wedzicha, 1984). This was based on identification of a kinetic relationship between the onset of colour formation and the concentrations of DSH and its hydroxysulphonate, though at that stage the connection was tentative. Quantitative data showing the time-dependent concentration of S(IV) (free + reversibly bound) and the relative area under the DSH peak are shown in Fig. 3. Thus, the concentration of S(IV) is mirrored by that of DSH and a decline in DSH concentration is very marked after S(IV) has been exhausted. It is not possible to infer from this result the rate of loss of DSH during the course of the reaction in the presence of S(IV), because a proportion of the sulphonate is in the form of a hydroxysulphonate which is likely to be less reactive than free DSH. Also, once browning commences, there will exist numerous intermediates in colour formation which are not present at significant concentrations in the presence of S(IV) but which may react with DSH.

The reactivity of DSH is more critically appraised by examining the chromatograms shown in Fig. 4. These illustrate the effect of adding the ^{35}S-labelled DSH to a mixture of glucose, glycine and S(IV) at zero time. Even

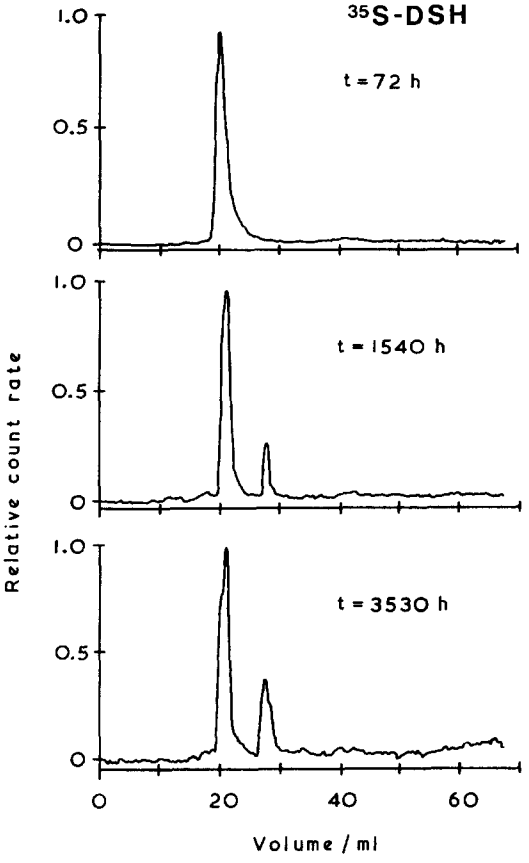

Fig. 4. Chromatograms of ^{35}S-labelled reaction products formed when a mixture of glucose (1 M), glycine (0·5 M) and S(IV) (0·05 M) is spiked with ^{35}S-labelled DSH at time zero. Initial pH 5·5, 55°C. Chromatograms shown are for reaction mixtures at times 72 h, 1540 h and 3530 h.

after 3530 h, by which time reaction mixtures had browned considerably, there is little evidence of the formation of discrete reaction products with the exception of the suspected metasaccharinic acid. To reinforce this finding, the DSH pool formed after 288 h of reaction, when the S(IV) concentration had decreased by approximately 40% of its initial value (50 mM), was spiked with ^{35}S-labelled product. Over the next 720 h the relative ^{35}S-activity in the DSH peak was 1·00 ± 0·05 (mean of 11 data points ± standard deviation). At the end of this period the S(IV) concentration was <1 mM and the ^{35}S-activity associated with DSH began to decline to a relative value of 0·46 after a further 1460 h of reaction. This result is seen to be consistent with the data shown in Fig. 3.

Particularly interesting kinetic data were obtained when glucose–glycine–S(IV) reaction mixtures were spiked with ^{14}C-labelled DH. The appearance

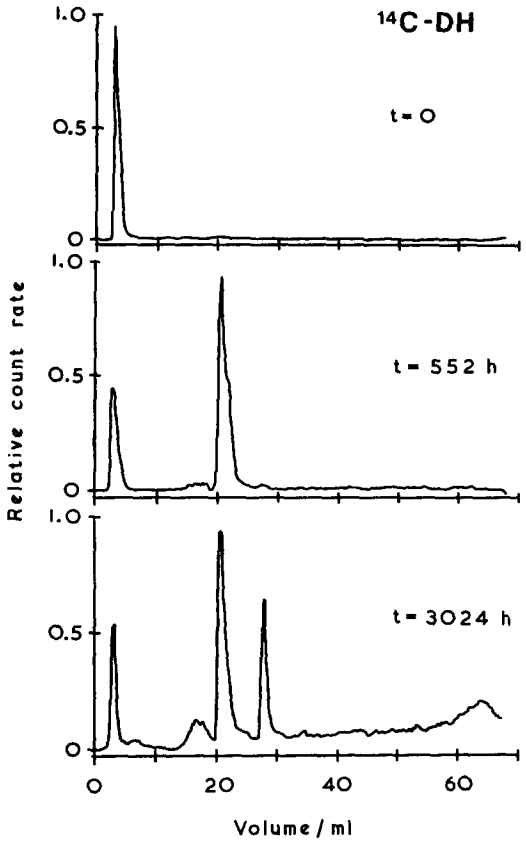

Fig. 5. Chromatograms of ^{14}C-labelled reaction products formed when a mixture of glucose (1M), glycine (0·5M) and S(IV) (0·05M) is spiked with ^{14}C-labelled DH at time zero. Initial pH 5·5, 55°C. Chromatograms shown are for reaction mixtures at times zero, 552 h and 3024 h.

of chromatograms of labelled reaction products is shown in Fig. 5. It was found that DH was eluted with the solvent front and its conversion to DSH as the main reaction product, whilst S(IV) was present, is clear from the chromatogram run after 522 h of reaction. This is typical of chromatograms obtained up to reaction times of *c.* 800 h. A quantitative recovery of ^{35}S-activity was obtained. At long reaction times (e.g. 3024 h) the chromatogram may be compared with that obtained after 3190 h using ^{35}S-labelled S(IV) as reactant (Fig. 2). Thus, with the exception of the ^{35}S-labelled product running at 38 ml and, of course, sulphate ion, all other S(IV)-derived reaction products are also obtained from DH. Thus, DH appears to be the important intermediate in S(IV) loss and the primary reaction product is DSH.

The kinetic models of the S(IV)-inhibited Maillard reaction proposed so far (Wedzicha, 1984; Wedzicha & Vakalis, 1988) assume that the

concentration of DH reaches a steady state once the reaction has been established. Let the steady state concentration of the DH pool be c. If no DH were being removed then, during a time interval δt, the concentration of DH in this pool would increase by δc, given by:

$$\delta c = (\mathrm{d[DH]}/\mathrm{d}t)\delta t \tag{1}$$

where d[DH]/dt is the rate of formation of DH. According to previous models, the rate of formation (and hence rate of loss) of DH is equal to the rate of combination of S(IV) in the constant rate part of the reaction (where the [S(IV)]–time graph is linear). To restore the concentration of DH to its steady state value, δc mol litre^{-1} of it is converted to products.

If once the steady state has been established, ^{14}C-labelled DH, of total activity a, is added to a given volume of reaction mixture, the actual activity remaining after an interval δt is given by:

$$a_1 = ac/(c + 0{\cdot}5\delta c) \tag{2}$$

and after n intervals the activity remaining is, therefore:

$$a_n = a\{c/(c + 0{\cdot}5\,\delta c)\}^n \tag{3}$$

Predicted ^{14}C-activity–time curves may be obtained if one adopts the modelling technique of using small finite increments δt. A simpler solution is to take logarithms of both sides of eqn (3), which gives the following expression:

$$\ln a_n = \ln a + n \ln \{c/(c + 0{\cdot}5\,\delta c)\} \tag{4}$$

from which it can be seen that a graph of the logarithm of the ^{14}C-activity at a time equivalent to n intervals from the initial measurement, versus n should be linear and the slope may be used to calculate the steady state concentration of DH.

Figure 6 illustrates ^{14}C-activity–time data for the peak eluted at the void volume of the column in chromatograms of which those at zero time and 552 h reaction time, shown in Fig. 5, are typical. A limitation of using a component running at the void volume is that other ^{14}C-labelled components, derived from DH, which do not bind to the column could contribute to the observed activity. No assumptions regarding the presence or absence of such components will be made but their possible contribution to the apparent activity of DH will be allowed for. The two sets of data shown in Fig. 6 are for mixtures of glucose, glycine and S(IV) which had been allowed to react for zero and 288 h before being spiked with ^{14}C-labelled DH. In the case of the 288 h reaction mixture, the rate of S(IV) loss was constant during the remainder of the reaction; the observed reaction rate was $4{\cdot}88 \times 10^{-5}$ mol litre^{-1} h^{-1}. A linear plot based on eqn (4), but with

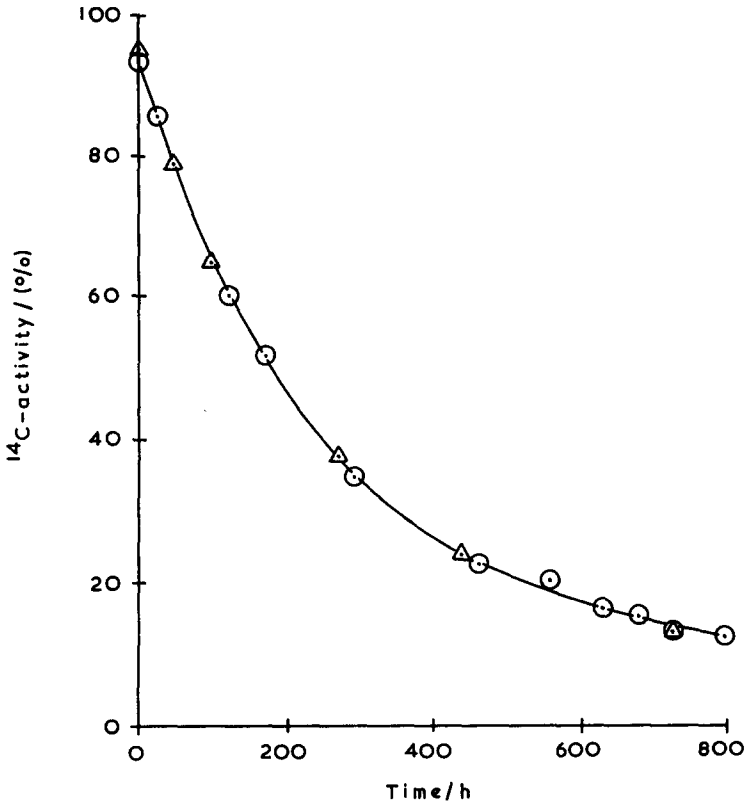

Fig. 6. Time-dependent ^{14}C-activity in the peak eluted at the void volume when reaction mixtures initially containing glucose (1 M), glycine (0·5 M) and S(IV) (0·0432 M) are spiked with ^{14}C-labelled DH at time zero (△) and after 288 h (⊙) and analysed by chromatography on a DEAE column. The time is measured from the moment the mixture was spiked. Initial pH 5·5, 55°C.

time from when mixtures were spiked as the abscissa, is shown in Fig. 7. The fact that the graph is linear over at least 90% of the change being followed is encouraging. It is seen that data from the zero-time mixture follow the same ^{14}C-activity–time profile and lie on the line in Fig. 7. This is surprising because in the latter experiment ^{14}C-labelled DH was added before steady state conditions had been reached. It is possible that near steady state conditions are established relatively quickly and deviations in the activity–time profile are not noticeable. It was found that the ^{14}C-activity versus time curve reached an asymptote at a value of 10% at long reaction times and the function plotted as the ordinate in Fig. 7 was, in fact, $\ln(a - 10)$ to allow for the presence of this contribution. It is normal practice to take account of the 'infinity value' in this way when analysing any simple first order kinetic process. The slope of the line in Fig. 7 is $-4{\cdot}06 \times 10^{-3}\,h^{-1}$ and is equal to $\ln\{c/(c + 0{\cdot}5\,\delta c)\}/\delta t$. Using the rate of loss of S(IV) to obtain δc with, say,

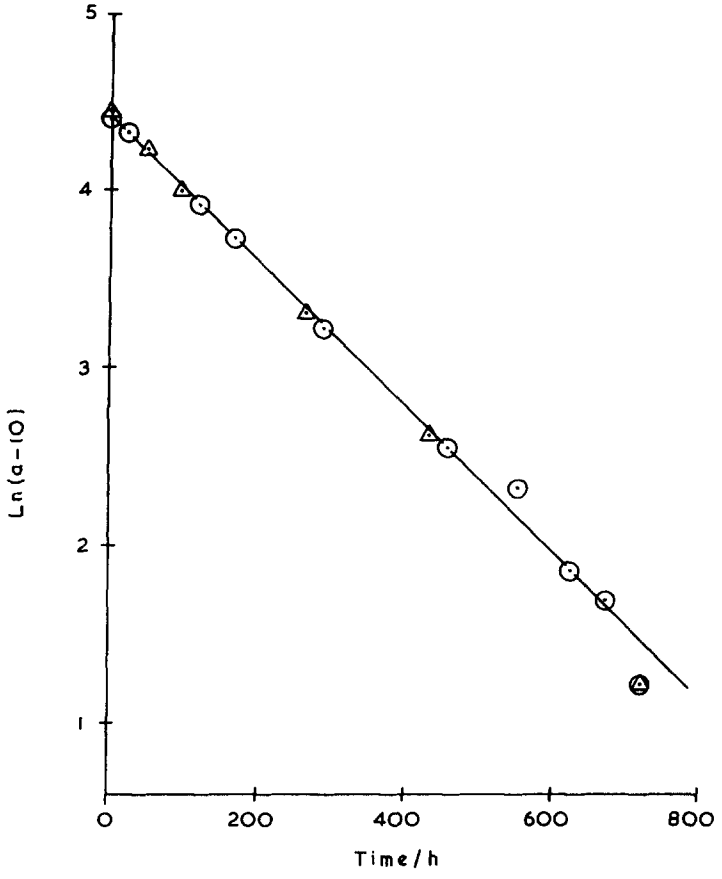

Fig. 7. Graph of $\ln(a - 10)$ versus time where a is the percent activity associated with the peak eluted at the void volume when reaction mixtures of glucose (1 M), glycine (0·5 M) and S(IV) (0·0432 M) were spiked with ^{14}C-labelled DH at time zero (△) and after 288 h (⊙), and subjected to chromatography on a DEAE column. The time is measured from the moment the mixture was spiked. Initial pH 5·5, 55°C. ^{14}C-activity versus time data are shown in Fig. 6.

$\delta t = 0{\cdot}001$ h gives $c = 6$ mM. It is encouraging to compare this with a value of 5·8 mM calculated previously from kinetic data on the binding of S(IV) (Wedzicha, 1984). It has been shown that the time-dependent concentration of DSH in a reaction described by the mechanism in Fig. 1 is given by:

$$[\mathrm{DSH}] = k_1 t - (k_1/k_2)\{1 - \exp(-k_2 t)\} \qquad (5)$$

where k_1 and k_2 are the rate constants referred to in Fig. 1, and the steady-state concentration of DH is given by k_1/k_2. The value of k_2 was obtained previously (Wedzicha, 1984) by fitting concentration–time data to eqn (5), having obtained k_1 as the constant rate of loss of S(IV). During the course of the present investigation a simpler way of determining the steady state

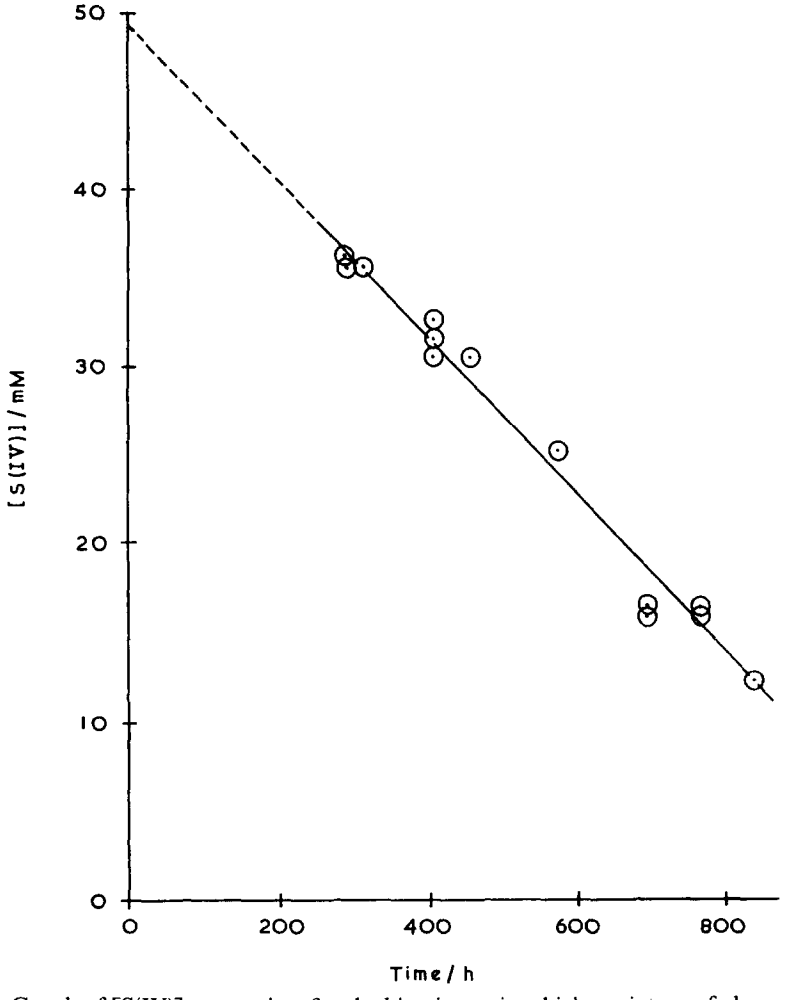

Fig. 8. Graph of [S(IV)] versus time for the kinetic run in which a mixture of glucose (1M), glycine (0·5M) and S(IV) (0·0432M) was spiked with ^{14}C-labelled DH after 288 h.

concentration of DH from [S(IV)]–time data was identified. At long reaction times (i.e. in the constant rate phase), k_2t is large and the concentration of reaction product at time t is given by:

$$[\mathrm{DSH}]_t = k_1 t - [\mathrm{DH}]_{\text{steady state}} \tag{6}$$

The model assumes that S(IV), which is irreversibly bound, is converted to DSH and the concentration of reaction product is, therefore:

$$[\mathrm{DSH}]_t = [\mathrm{S(IV)}]_0 - [\mathrm{S(IV)}]_t \tag{7}$$

where $[\mathrm{S(IV)}]_0$ is the concentration at zero time. If one extrapolates the

linear portion of the graph of [S(IV)] versus time to zero time, the value of [S(IV)] at the intercept will be:

$$[S(IV)]_{int} = [S(IV)]_0 + [DH]_{steady\text{-}state} \quad (8)$$

The available [S(IV)]–time data for one of the runs (that spiked with ^{14}C-labelled DH after 288 h) shown in Figs 6 and 7 are given in Fig. 8. The initial S(IV) concentration was 43·2 mM and the intercept is estimated to be 49·2 mM, giving a steady state DH concentration of 6 mM. This is in good agreement with that calculated previously (Wedzicha, 1984) from published data as well as the value calculated from ^{14}C-activity–time data, using mixtures which had reached the steady state before being spiked with ^{14}C-labelled DH.

CONCLUSION

The results presented here confirm the basis of the mechanistic model of the sulphite inhibited Maillard reaction. In particular, the rate determining step involves formation of DH which is converted to DSH as the primary product. DSH is relatively unreactive whilst S(IV) is present.

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