Formation of Three-carbon Sugar Fragment at an Early Stage of the Browning Reaction of Sugar with Amines or Amino Acids

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Formation of a product easily converted to methylglyoxal on TLC with silica gel was observed in an early stage of the reaction mixture of sugar with an alkylamine or amino acid. NMR spectra of the ether extract of reaction mixtures indicated that methylglyoxal dialkylimine was produced mainly at an early stage of the reaction of glucose with alkylamine, and was assumed to change to methylglyoxal on the TLC. The C₃ imine production in the *t*-butylamine system was apparently little and slow compared to that in the normal alkylamine system. A large, rapid production of C₃ imine was also observed in the system of the Amadori product and *n*-butylamine. These results suggested that the C₃ formation in the system with normal alkylamine may occur mainly *via* a newly proposed mechanism, though the *t*-butylamine system may possibly produce it according to the scheme proposed by Hodge.

As previously reported,^{1,2)} we have found that a novel free radical product was formed at an early stage in the browning reactions of sugar-amino compound systems, and ESR analyses led to the assignment of an *N,N'*-disubstituted pyrazine cation radical structure. This structure reasonably led us to the assumption of the formation of an C₂ fragmentation product of the material sugar as a precursor of the radical product, and this was demonstrated by isolation and identification of glyoxal dialkylimine.³⁾ Based on various other experimental evidence^{4,5)} concerning sugar fragmentation and free radical formation, we have proposed a new pathway for browning that involves cleavage of the sugar moiety of the Schiff base at an initial stage of the amino-carbonyl reaction before the Amadori rearrangement, followed by formation of the free radical and C₂ products. Besides the formation of C₂ product, we have also recognized the presence of a C₃ compound as another fragmentation product in an early stage of the sugar-amine reaction.

In the browning reaction mechanism proposed by Hodge,⁶⁾ no consideration has been

given to sugar fragmentation producing reactive intermediates of browning, especially at an early stage, except for a postulation of a C₃ carbonyl compound as one of the active products formed from 1-deoxyhexosones in the middle stage of the reaction. This study deals with the identification of the C₃ fragmentation product of sugar at an early stage of the amino-carbonyl reaction, and a proposal of its formation pathway.

RESULTS AND DISCUSSION

When mixtures of various sugar-alkylamine systems at an early stage of the reaction were developed on silica gel TLC plates and sprayed with 2,4-DNP solution, two particular orange spots appeared instantaneously (Fig. 1). The lower one observed in every system has previously been identified as glyoxal, and was shown to be an artifact formed from glyoxal dialkylimine by hydrolysis with silica gel.³⁾ An additional spot with a higher *R_f* value which appeared in the systems with alkyl amines other than *t*-butylamine has remained to be identified.

FIG. 1. TLC of Reaction Mixtures of Sugars with Amines.

Mixtures of the materials in distilled water (1 M each) were heated for 10 min in a boiling water bath, developed on silica gel TLC plates, and sprayed with 2,4-DNP reagent. A, glucose + ethylamine; B, glucose + cyclohexylamine; C, glucose + *t*-butylamine; D, glucose + *n*-butylamine; E, xylose + *n*-butylamine; F, glyoxal; G, methylglyoxal.

FIG. 2. TLC of the Phenylhydrazones of the Reaction Products.

TLC: silica gel, benzene-*n*-hexane-ethyl acetate (5:2:1). A, ether extract of the reaction mixture of glucose and *n*-butylamine; B, authentic glyoxal phenylhydrazone; C, isolated substance; D, authentic methylglyoxal phenylhydrazone.

An ether extract of the reaction mixture of glucose and *n*-butylamine was treated with phenylhydrazine reagent. The obtained phenylhydrazones gave 5 spots on TLC (Fig. 2-A), and two of them have been identified as conformational isomers of glyoxal bisphenylhydrazone (B). The topmost spot on TLC was a new main one and was isolated by preparative TLC to give a yellow crystalline product. The product gave three spots on the same TLC corresponding to methylglyoxal phenylhydrazones even after further purification by preparative HPLC (C), and they agreed well in their *R_f* values to those observed with authentic methylglyoxal phenylhydrazone (D in Fig. 2), indicating the presence of its conformational isomers. Confirmatory evidence was that NMR data of the isolated phenyl-

FIG. 3. NMR Spectra of the Ether Extract of the Reaction Mixture of Glucose and Cyclohexylamine (A), and Authentic Methylglyoxal Dicyclohexylimine (B).

hydrazone was identical to that of the authentic one.

Methylglyoxal was also expected to be present as its dialkylimine form in the reaction mixture, as was the case with glyoxal.³⁾ Many trials to isolate it from the reaction mixture using TLC and HPLC were unsuccessful, perhaps due to its instability. However, the presence of methylglyoxal dialkylimine in the early stage of the reaction could be demonstrated by comparison of its spectral features with those of the authentic one as follows.

Methylglyoxal dicyclohexylimine was synthesized by the reaction of methylglyoxal with cyclohexylamine as unstable crystals. The MS spectrum gave M^+ of 234 corresponding to the Schiff base-type condensation product of 2 molecules of cyclohexylamine with 1 molecule of methylglyoxal. A large peak at m/z 191 can be explained by the fragment

resulting from ring fission of the cyclohexylamine derivatives.⁷⁾ The NMR spectrum (Fig. 3B) showed proton signals for two cyclohexyl groups (1.3~1.8, 3.1 and 3.4 ppm), a signal for one methyl group (2.08 ppm), and one for a proton of the imine group (7.8 ppm), indicat-

ing that the synthesized one is methylglyoxal dicyclohexylimine.

The NMR spectrum of the ether extract of the reaction mixture of cyclohexylamine with glucose (Fig. 3A) showed signals identical to those of synthetic methylglyoxal dicyclohexylimine in addition to those due to glyoxal dicyclohexylimine (7.9 ppm, imine protons), though it also contained the signals due to cyclohexylamine (1~2.7 ppm). The MS spectrum of the ether extract also gave M^+ of 234 corresponding to methylglyoxal dicyclohexylimine. When the authentic methylglyoxal dicyclohexylimine was developed on a silica gel plate as was done for the reaction mixture, only one spot was detected by 2,4-DNP at the same R_f value as that of methylglyoxal. Moreover, methylglyoxal dicyclohexylimine treated with phenylhydrazine reagent gave three spots identical to those found in C and D in Fig. 2. These results indicated that methylglyoxal dicyclohexylimine is one of the main products in an early stage of the reaction of glucose with cyclohexylamine, and that most of the methylglyoxal detected on TLC does not exist as such in the reaction mixture.

Formation of methylglyoxal di-*n*-butylimine by the reaction of glucose with *n*-butylamine was also observed by NMR. NMR of $CDCl_3$ extract from the reaction mixture gave signals corresponding to those of the ether extract of the reaction mixture of methylglyoxal with *n*-butylamine. All the signals due to methyl and imine groups were identical to those of the glucose-cyclohexylamine system.

It was shown by the experiment using GC of the reaction mixture of glucose with β -alanine, treated with *o*-phenylenediamine, that methylglyoxal and glyoxal were also detected as methylquinoxaline and quinoxaline, respectively (Fig. 4). There might be exchange reactions of amino compounds attached to carbonyl groups between the amino acid and *o*-phenylenediamine. These results indicated that methylglyoxal diimine may be produced by the reaction of sugar with amino compounds. Participation of alkaline degradation of sugar alone in C_3 formation is considered to be

FIG. 4. Capillary GC of Products from the Reaction of Glucose with β -Alanine.

A, mixture of glyoxal (2) and methylglyoxal (3); B, reaction mixture in water (2 M each, initial pH 9.29) heated for 20 min in a boiling water bath. Each sample contains naphthalene (1) as an internal standard.

Fig. 5. Formation of C_2 Imine, C_3 Imine, and Other Intermediate Products by the Reaction of Glucose with *n*-Butylamine (1 M each) heated at 80°C.

—▽—, C_2 imine; —○—, C_3 imine; —□—, glucosylamine; —▲—, Amadori compound; ---●---, browning.

negligibly small because methylglyoxal was not detected by GC in the alkaline solution of glucose (2 M, pH 9.3) heated at 95°C for 10 min.

Quantitative studies of the course of formation of the C_3 imine in the glucose-*n*-butylamine system indicated that C_3 compound production occurred at a very early stage, increased with heating time giving a maximum

at about 20 min and then gradually decreased (Fig. 5). The course of the production of C_3 imine was closely followed by that of C_2 imine, and well correlated to the decrease in the amount of glucosylamine and the increase in the formation of Amadori product. Reaction of Amadori product with *n*-butylamine rapidly produced the C_3 compound in a manner similar to that of the glucose-*n*-butylamine system (Fig. 6). These results indicate the possibility that the Amadori product participates in the C_3 formation.

As mentioned in Fig. 1, a spot corresponding to methylglyoxal was hardly detected on TLC in the glucose-*t*-butylamine system. Production of the C_2 and C_3 compound and the rate of browning in this system were compared with those of the *n*-butylamine system during the course of the reaction. As shown in Fig. 7, the formation of C_2 imine in the *t*-butylamine system was very rapid and

similar in its course to that in the system of *n*-butylamine though the yield was relatively low. However, in the case of C_3 imine formation, it was significantly suppressed and delayed in the *t*-butylamine system compared to that in the *n*-butylamine system. This apparent difference is probably due to the effect of the bulkier structure of *t*-butylamine on the reactivity in amino-carbonyl reaction with sugar. If we suppose a pathway of sugar fragmentation to yield C_3 imine by a reverse-alcohol condensation type reaction of the further aminated Amadori product (Fig. 8), there is a significant difference in the rate of formation of condensation product due to disturbance by the bulky *t*-butyl group, especially in the second amination process, resulting in different pathways in the C_3 formation. Then, in the *t*-butylamine system the C_3 product may possibly be formed according to the scheme

FIG. 6. Formation of C_3 Imine by the Reaction of *n*-Butylamine with Amadori Compound or Glucose.

Each mixture in water was adjusted to pH 10.8. Glucose (0.5 M) + *n*-butylamine (1 M): —○—, C_3 imine; ---●---, browning. Amadori compd. (0.5 M) + *n*-butylamine (0.5 M): —△—, C_3 imine; ---▲---, browning.

FIG. 7. Formation of C_2 and C_3 Imine and Browning during the Reaction of Glucose (0.5 M) with *t*-Butylamine or *n*-Butylamine (1 M) Heated at 80°C.

—△—, C_2 imine; —○—, C_3 imine; ---●---, browning.

FIG. 8. Proposed Pathway for C_3 Imine Formation.

proposed by Hodge, which is followed by deoxy-osone formation from the Amadori product at the middle stage of the amino-carbonyl reaction. On the other hand, the C_3 formation in the systems of *n*-alkylamines may occur mainly *via* a newly proposed mechanism from the doubly aminated Amadori product. Two possible pathways of the C_3 formation arising from these considerations are presented in Fig. 8. Further studies on the mechanism of this new pathway are underway.

EXPERIMENTAL

1. *Materials and methods.* Authentic specimens of glucosyl-*n*-butylamine and 1-deoxy-1-*n*-butylamino-fructose were prepared according to the literature.⁸⁾ Methylglyoxal bisphenylhydrazone was synthesized by the reaction of methylglyoxal with phenylhydrazine hydrochloride in aqueous sodium acetate, and its structure was confirmed by NMR. Other reagents were of guaranteed grade.

Analytical TLC was done on precoated Merck silica gel plates using the solvent system (EtOAc-MeOH-CHCl₃, 5:2:1). A solution of 2,4-DNP (3% in the mixture of ethanol, water, and sulfuric acid (70:20:15)) was used for visualization and detection of the spots. TLC of phenylhydrazone was developed with benzene-*n*-hexane-EtOAc (5:2:1).

NMR and mass spectra were recorded using JNM-MH-100 and JMS-D-100 spectrometers, respectively. GC analysis was done on Hitachi GC 263-30 on a 0.25 mm i.d. × 25 m fused silica capillary column coated with carbowax 20M at 170°C. Preparative and analytical HPLC were done using a JEOL TWINCLE pump with Shodex RI SE-II.

Measurement of glucosylamine and the Amadori product formed from *n*-butylamine system was done by the previously reported method using HPLC.⁴⁾ The degree of browning was measured by the absorbancy at 420 nm.

2. *Isolation of phenylhydrazone from the reaction mixture.* D-Glucose and *n*-butylamine (0.1 mol each) in 100 ml of distilled water were heated for 8 min in a boiling water bath and extracted with ether (50 ml). The extract was washed with water three times (50 ml each). An aqueous solution (16 ml) of phenylhydrazine hydrochloride (1.5 g) and sodium acetate (2.4 g) was added to the concentrated extract, and immediately extracted with ethyl acetate twice (20 ml each). The collected extract was washed with water two times (20 ml each) and then concentrated. Next, preparative TLC was done by using a silica gel plate (Wako Gel B, 0.5 mm thick, 20 × 20 cm, 8 sheets) with the same solvent as that of TLC for analysis. The topmost band among several yellow ones on TLC was scraped off and extracted with ethyl acetate. The dried matter

(66.6 mg) gave yellow plates on crystallization with ethanol. The ethyl acetate extract was chromatographed by HPLC using a Lichrosorb SI60 column (150 × 8 i.d. cm), eluted with hexane-EtOAc (90:10) as solvent (1.5 ml/min) and the fraction with retention time 11.3 min was collected.

3. *Preparation of methylglyoxal dicyclohexylimine.* A mixture prepared by the addition of methylglyoxal (10 mmol) to an aqueous solution of cyclohexylamine (22 mmol) in 10 ml of water was immediately extracted with ether (20 ml), and the extract was washed with water (20 ml) four times, dried, and concentrated to 5 ml. After addition of a small portion of water, it was stored in a freezer overnight to yield a brown precipitate, which was crystallized from ethanol; yield 0.312 g, pale brown and fine. MS *m/z* (relative intensity); 234 (M^+ , 10), 191 ($C_{12}H_{19}N_2^+$, 66), 109 (56), 96 (83), 83 ($C_6H_{11}^+$, 100), 52 (91) and 40 (70). NMR, see Fig. 3.

4. *Preparation of the ether extract of the reaction mixture of glucose and amine.* The ether extract of a reaction mixture of glucose (3.6 g) and cyclohexylamine (2.0 g) in water (20 ml), heated for 4 min in boiling water bath, was washed with water (10 ml) 5 times and dried, and then made solvent-free (0.18 g).

An extract of an *n*-butylamine system was prepared under the conditions described previously.⁴⁾

5. *Quantification of glyoxal and methylglyoxal dialkylimine.*

a) *NMR method.* A reaction mixture of glucose and *n*-butylamine was extracted with ethyl acetate. After washing with water, it was concentrated and dried, and then dissolved in CDCl₃ containing TMS and 1,2-dichloroethane as standards. The amounts of C_3 and C_2 dialkylimine were calculated from the NMR signal intensities at 7.8 and 7.9 ppm, respectively, compared to that at 6.0 ppm of the standard (1,2-dichloroethane).

b) *o*-Phenylenediamine method. The GC measurement of quinoxaline derivatives was made by a modification of the method of Moree-Testa and Saint-Jalm. A testing mixture (2 ml) was added to an aqueous solution of *o*-phenylenediamine (1%, 10 ml), and mixed well with a magnetic stirrer for 30 min. The dichloromethane (2 ml) extract of the mixture, containing naphthalene as a standard, was analyzed by a Hitachi 263-30 GC with a capillary column (Carbowax 20 M, 0.25 mm × 25 m).

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