Kinetic Aspects of the Blue Pigment Formation in a Maillard Reaction between D-Xylose and Glycine

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The production of blue pigments in the early stage of a Maillard reaction between D-xylose and glycine was kinetically analyzed to be associated with the mechanism for polymerization. An ion-pairing and reversed-phase HPLC method was developed for quantitatively tracing the blue pigment formation. The experiment with varied composition revealed that the reaction obeyed the following relationship:

$$v = k[\text{D-xylose}]^2 \cdot [\text{glycine}]^2$$

This equation could also be derived according to the stationary state theory. Based on Arrhenius plots, the activation energy was found to be 17.5 kcal/mol. The application of the transition state theory provided −17.4 cal/deg/mol for the activation entropy. A reaction scheme for the production of blue pigments is proposed on the basis of this kinetic analysis.

Many reports can be obtained on the mechanism for the Maillard reaction between amino acids and reducing sugars. Most of these reports have concentrated on the early stage of this reaction, including the formation of *N*-glucosides, Amadori rearrangement and the decomposition of Amadori compounds.[1] In consequence, various intermediates have been identified, whose roles in the advanced stage of this reaction have been confirmed to a great extent. Of these roles, Strecker degradation[2] and the formation of heterocyclic compounds[2] are well known to be of much significance in the development of food flavor during heat treatment.

However, investigations on the coloration which characterizes the final stage of a Maillard reaction are few, and the polymerization mechanism as well as the chemical structure of the brown pigment called melanoidin, whch accumulates in the final stage of this reaction, remain obscure. Few intermediates have been confirmed in the initial stage of polymerization, and such compounds may be very unstable under cooking conditions.

In general, however, it has been observed that strong fluorescence and a characteristic color are developed just prior to browning.[3] The detection of the intermediates associated with these phenomena may contribute to an elucidation of the polymerizing mechanism in browning by a Maillard reaction.

Some workers have reported the formation of low molecular weight pigments with a specific color tone in the reaction between amino acids and carbonyls such as reducing sugars[4] and dehydroascorbic acid.[5,6,7] Nursten and O'Reilly[8] identified a yellow intermediate produced from a D-xylose–glycine system on heating at 100 °C. Those pigments were possibly oligomers in the formation of melanoidin. On the other hand, kinetic information about the coloration process of Maillard reactions have been presented by Song and Chichester[9] and Suyama *et al.*,[10] who suggested the role of

aldimines as a monomer in polymerization.

Miura and Gomyo[3] reported that a series of blue colored substances were produced in the early stage of a Maillard reaction between D-xylose and glycine under milder conditions than those for conventional cooking, and suggested that those characteristic pigments might be involved in the initial steps of polymerization as significant oligomers.

The structures and the formation pathways of these blue pigments will be a clue to explain the entire mechanism for a Maillard reaction, especially in the production of high molecular weight compounds such as melanoidin. However, studies on the blue pigments are still difficult because of their complexity in chemical structure and their high instability.

In an attempt to understand the polymerization scheme in a Maillard reaction, this paper deals with the kinetics associated with the production of those blue pigments, and much attention will be paid to the reaction rate equation, the reaction order and the activation energy. A novel and effective HPLC method was also developed to facilitate the determination of the pigments.

Materials and Methods

Standard conditions for the reaction between D-xyolse and glycine. D-Xylose (1 M), glycine (0.2 M) and $NaHCO_3$ (0.1 M) were dissolved together in 100 ml of aqueous 60% ethanol, and the solution adjusted to pH 7.5 with HCl. The reaction solution was placed in a N_2 atmosphere and stood in a water bath maintained at the temperature of 20 ± 0.5°C, shielding the reaction vessel against any incident light.

High-performance liquid chromatography for separating the blue pigment. A packed Inertsil ODS column (25 cm × 4.6 mm ID, 5 μm particle size, Gasukuro Kogyo Inc.) was employed for separating the blue pigments, which was preceded by a precolumn (5 cm × 4.6 mm i.d.) packed with the same material. The mobile phase was a 0.005 M Tris–HCl buffer in water–methanol (60 : 40) mixed with 0.005 M tetrabutyl ammonium phosphate as an ion-pair regent, the pH being precisely adjusted to 7.50 with HCl. The flow rate was fixed at 0.5 ml/min under the pressure of 110 kg/cm^2 with a PUS-8 pump (Gasukuro Kogyo Inc., Japan) and the column temperature was maintained at 50°C. Ten microliter of the sample was loaded through a specially devised injection port (Eyla 5010, Gasukuro Kogyo Inc.). The blue pigments were detected by a Shimadzu SPD 1 spectrophotometer, and the absorbance at 630 nm was continuously recorded by a Shimadzu R-11 recorder.

Ion-pair reagent. An aqueous solution of 0.5 M tetrabutyl ammonium phosphate, which had been commercially prepared, was purchased from Gasukuro Kogyo Inc. Prior to use, the solution was diluted 100 times with the developing solvent.

Sampling and pretreatment. An appropriate volume of the reaction solution was taken out at given intervals of time. After removing the ethanol by evaporation under reduced pressure, the sample was adjusted to pH 4.5, and then applied to a short column charged with DEAE-Sephadex A-25 (3 cm × 2cm i.d., Pharmacia Fine Chemicals, Sweden, and purchased from Pharmacia Japan, Tokyo) which had been conditioned with water, and followed by washing with 25 ml of water to remove most of the D-xylose and glycine. After that, all of the colored products adsorbed on the column was washed out with a certain amount of 0.4 M NaCl. The eluate from the column was concentrated and applied to a Sephadex G-10 (Pharmacia Fine Chemicals) column (50 cm × 1.3 cm i.d.) for desalting, and followed by concentration. The desalted solution was made up to a small volume and filtrated by the use of a micro filter (Type TM-2p membrane filter, 0.45 μm, Toyo Roshi Co. Ltd., Japan) before application.

Determination of the reaction order. The reaction medium was an aqueous solution containing ethanol at 30% and was adjusted to pH 7.5 with HCL. The reaction compositions were prepared by varying D-xylose from 0.1 to 0.4 M and glycine from 0.05 to 0.4 M in $NaHCO_3$ (0.1 M). The reaction solution was kept standing in the same manner as that already described. At given intervals, a cartain volume of the reaction solution was taken out by pipetting, and followed by the determination of absorption at 630 nm, which was characteristic of the blue pigments.

Determination of the activation energy. D-Xylose (1 M), glycine (0.2 M) and $NaHCO_3$ (0.1 M) were dissolved together in 50 ml of an aqueous solution containing ethanol at 60%, and placed in a bath maintained at various temperatures of 15, 20, 25 and 30°C. The other conditions were the same as those already mentioned.

Isolation of the blue pigment. All of the separating procedures were done in a cold room maintained at 4°C. Under the standard conditions described, two liters of a strongly blue colored solution was prepared after 7 days of reaction. The ethanol was removed under reduced pressure, and the solution diluted to one liter with distilled

water. After adjusting to pH 4.5, the solution was applied to DEAE-Sephadex A-25 column (26 cm × 4 cm i.d., 40 ~ 120 μm, Pharmacia Fine Chemicals, Sweden), which had been conditioned with water alone, The coumn was then washed with one liter of water to remove most of the remaining D-xylose and glycine, and followed by elution with 0.4 M NaCl. The blue colored fraction thus obtained was concentrated to a small volume under reduced pressure, and most of the salt removed by centrifugation. The supernatant was then applied to a Sephadex G-10 column (80 cm × 2.5 cm i.d., FINE grade, Pharmacia Fine Chemicals) to complete the desalting process. The desalted sample was adjusted to pH 4.5 and adsorbed on a DEAE-cellulose column (90 cm × 2.5 cm i.d., 0.72 meq/g, Serva, Nakarai Chemicals Ltd., Japan), which had been conditioned with water alone. The development was performed with 500 ml of 0.12 M triethyl-ammonium acetate (TEA-Ac) buffer (pH 4.5), which was followed by a linear gradient system made up of 1000 ml of 0.1 M TEA-Ac buffer (pH 4.5) and 1000 ml of 0.4 M NaCl in the same buffer. Several kinds of blue pigments different in color tone flowed out successively, and were fractionated every 2 ml. The main fractions, which appeared in the greatest amount among the blue pigments produced in the reaction solution, were collected, and subjected to further purification by the use of a Bio-Gel P-2 column (55 cm × 3 cm i.d., 200 ~ 400 mesh, 1800 daltons exclusion limit, Bio-Rad Laboratories, U.S.A.) that had been equilibrated with 1 M NaCl. The main band was effectively isolated by developing with 1 M NaCl, and followed by desalting in the same manner as that already described. The final solution was lyophilized. The resulting substance, which was designated Blue 1 as described later, was found to contain a small amount of a shoulder component, which was estimated to be less than about 4% in terms of the peak area on HPLC chromatogram.

Estimation of the molecular weight of the main blue pigment. About 1 mg of the isolated blue pigment was dissolved in a mixture of 0.2 ml of 0.1 N HCl and 1 ml of methanol. To this solution, ethyl acetate was added drop by drop until some insoluble particles appeared. The suspension was centrifuged, and then the precipitate was dried under a stream of N_2 and dissolved in 0.2 ml of methanol. This sample was subjected to mass spectrometry by the use of a JMS-DX303 instrument (JEOL, Japan) with FAB (fast atom bombardment). Glycerine was employed as a matrix for FAB.

Reagents. All of the reagents used in the present experi-

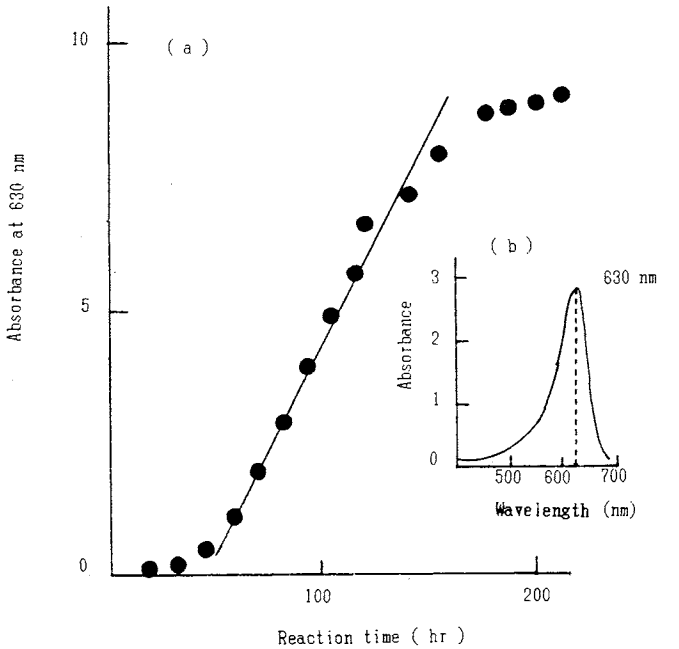

Fig. 1. (a) Change in Absorption at 630 nm during the Reaction between D-Xylose and Glycine.
D-Xylose (1 M), glycine (0.2 M) and $NaHCO_3$ (0.1 M) were dissolved in 100 ml of aqueous 60% ethanol, and the solution adjusted to pH 7.5 with HCl. The reaction solution was placed in a N_2 atmosphere and kept standing in a water bath maintained at the temperature of 20 ± 0.5°C. The reaction vessel was shielded against any incident light.
(b) Absorption Spectrum of the Solution after 64 Hours of Reaction under the Same Conditions.

ment were of commercial G. R. grade.

Results

A series of blue pigments

Figure 1(a) shows the change in absorption at 630 nm during the reaction between D-xylose and glycine under the standard conditions described in experimental section. After a certain period of induction, the reaction rate increased rapidly to attain a constant level, and finally decreased, resulting in a sigmoidal curve. The intermediary portion of the reaction curve was sufficiently linear so that the velocity of the reaction predominantly contributing to the production of blue pigments could be easily estimated in terms of the absorption at 630 nm. Figure 1(b) shows an absorption spectrum taken after 64 hr of the reaction. Figure 2 is the HPLC chromatogram obtained after 64 hr of the same reaction, revealing a series of blue pigments, although different in yield from each other. The greatest peak on the chromatogram was designated as Blue 1, and was further investigated as will be described later. As Fig. 3 shows, Blue 1 dominated the coloration in the early stage when the steady state of the reaction was realized.

Reaction orders in respect to D-*xylose and glycine*

Figure 4 shows the dependences of the reaction rate on the concentration of D-xylose in excess of glycine, and of glycine in excess of D-xylose. The reaction rate was determined on the basis of the slope of the linear portion in the reaction curve. As can be seen in Fig. 4, the reaction was found to obey good second-order kinetics with respect to both D-xylose and glycine. Accordingly, the rate equation for the production of blue compound could be expressed as follows:

$$v = k[\text{D-xylose}]^2 \cdot [\text{glycine}]^2 \qquad (1)$$

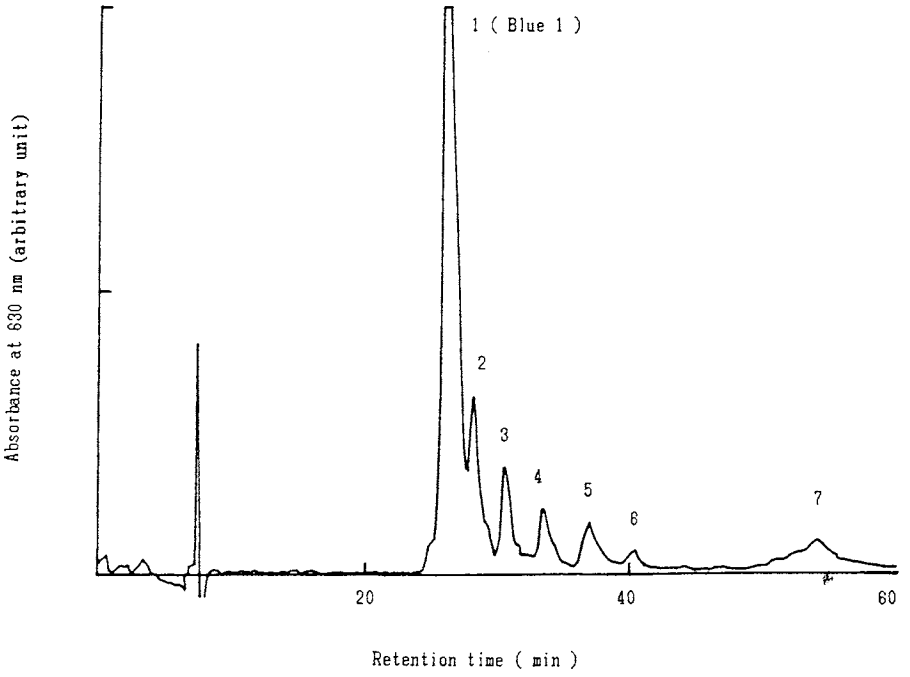

Fig. 2. HPLC Chromatogram after 64 Hours of the Reaction between D-Xylose and Glycine.

The reaction conditions were the same as those in Fig. 1(a). The chromatographic conditions were as follows: Column, Inertsil ODS (25 cm × 4.6 mm i.d., 5 μm particle size); eluent, 0.005 M Tris–HCl buffer (pH 7.50) in water–methanol (60 : 40) containing 0.005 M tetrabutylammonium phosphate as an ion pair reagent; column temp., 50°C; sample size, 10 μl; detection, absorption at 630 nm with a Shimadzu SPD 1 instrument.

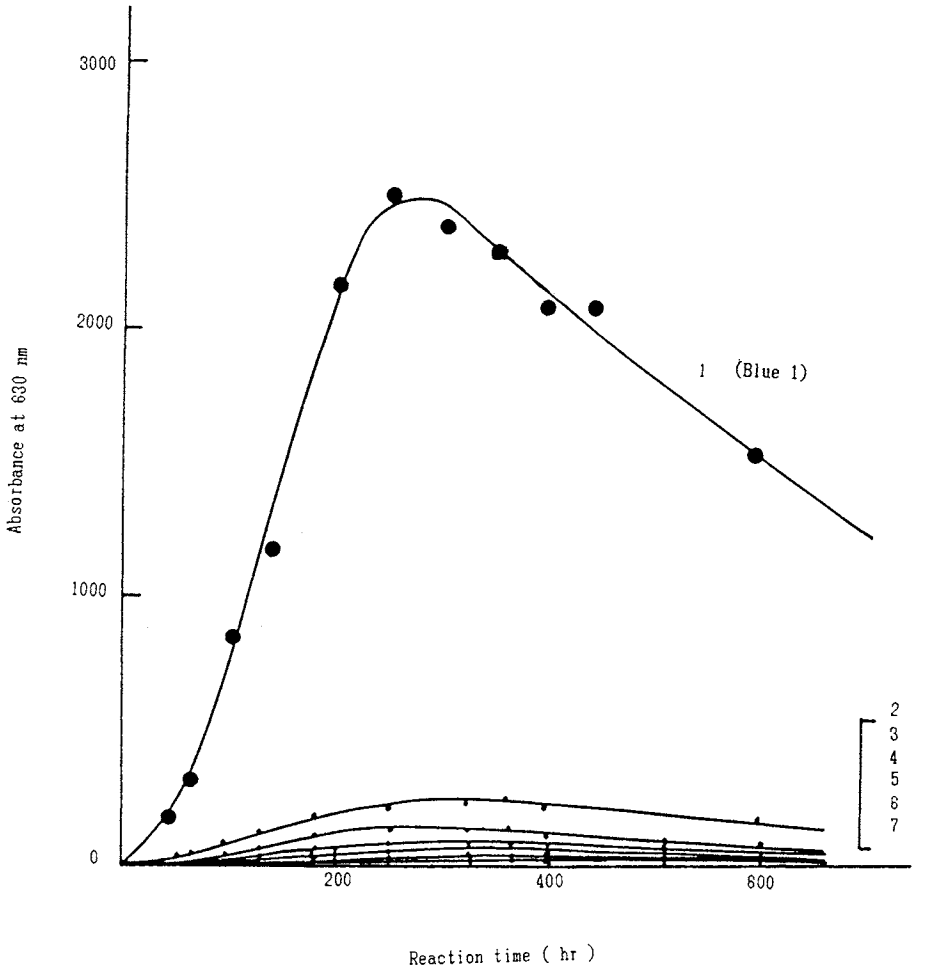

Fig. 3. Time Course for the Accumulation of Various Kinds of Pigments Produced by the Reaction between D-Xylose and Glycine.

The reaction conditions were the same as thoses in Fig. 1(a). The amount of each pigment was estimated on the basis of its peak height on HPLC chromatogram, whose conditions are explained in Fig. 2.

In this equation, v and k are the reaction rate and the rate constant, respectively. Practically, equation 1 represents the production rate of Blue 1 because of its dominance among the blue pigments formed in the reaction stage where the rate was constant.

Activation energy for the production of blue pigments

Figure 5 shows Arrhenius plots relating to the coloration rate in terms of the absorption at 630 nm, where $-E/2.303R$ was found to be 3.82×10^3. Accordingly, the activation energy (E) for the production of blue pigments could be calculated as follows:

$$E = 17.5\,\text{kcal/mol} \qquad (2)$$

Estimation of the molecular extinction coefficient (ε) of Blue 1

The mass spectrum revealed that Blue 1 had a molecular ion peak (M^+) at 797 m/z. It should be that M+2, M+4 and M+6 were detected around M^+. This observation suggested that Blue 1 contained some atoms of chlorine due to HCl in the acidic medium used

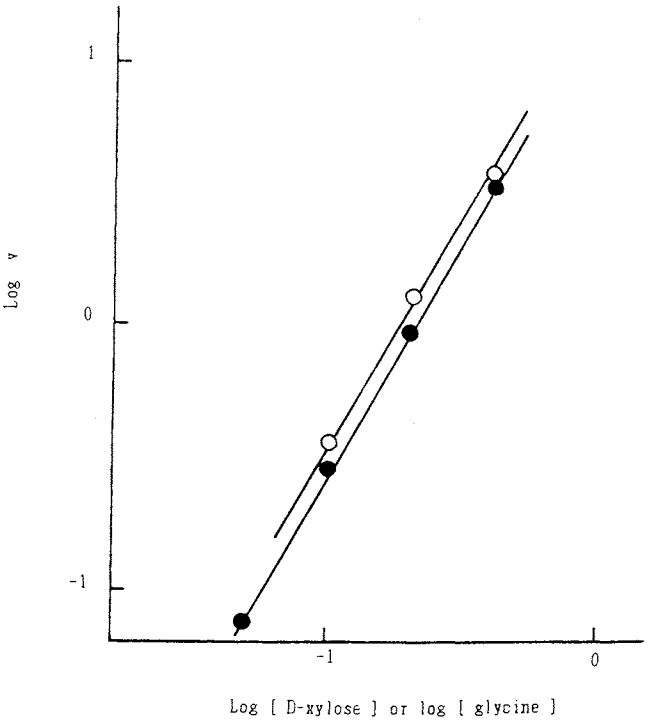

Fig. 4. Dependence of the Rate of Coloration on the Concentration of D-Xylose or Glycine.
[D-xylose] and [glycine] are molar concentrations. V indicates the rate of coloration in terms of the change in absorbance at 630 nm per min.
The concentration of D-xylose was varied from 0.1 M to 0.4 M against 2 M of glycine, and that of glycine from 0.05 M to 0.4 M against 2 M of D-xylose. The reaction medium was an aqueous solution containing ethanol at 30%. The other conditions were the same with those in Fig. 1(a). The rate of coloration was estimated in terms of the change in absorption at 630 nm in the linear stage of the sigmoidal curve shown in Fig. 1(a).
○—○, excess of glycine; ●—●, excess of D-xylose.

for preparing the sample for mass spectrometry. However, the exact number of chlorine atoms could not be determined only from this chart.

In this experiment, the molecular weight of Blue 1 was tentatively assigned as about 800 daltons. In the previous report, Gomyo and Miura[1] estimated that the molecular weight of Blue 1 was approximately 800 daltons by gel filtration chromatography on Bio Gel P-2. An aqueous solution containing Blue 1 at 0.3 mg% was 0.262 in absorbance at 630 nm. From these results, the molecular extinction coefficient (ε) could be estimated to be 7.0×10^4.

Rate equation for the production of Blue 1

In the linear portion of the reaction curve in Fig. 1, the reaction rate (v) at 20°C in terms of molar concentration could be calculated as follows:

$$v = \frac{\mathrm{d}(\mathrm{Abs}/\varepsilon)}{(\mathrm{d}\,t)} = 1.865 \times 10^{-8}\ \mathrm{mol \cdot l^{-1} \cdot min^{-1}} \quad (3)$$

At this stage, the concentration of D-xylose and glycine could be assumed to be practically unchanged, because those starting materials were probably still in great excess compared with the amount of colored products. In the equation, the respective substitution of D-xylose, glycine and v with $1\ \mathrm{mol \cdot l^{-1}}$, $0.2\ \mathrm{mol \cdot l^{-1}}$ and the value in Eq. 3 yielded the value of the rate constant (k) as follows:

$$1.865 \times 10^{-8} = k \times 1^2 \times (0.2)^2$$

$$k = 4.663 \times 10^{-7}\ \mathrm{mol^{-3} \cdot l^3 \cdot min^{-1}} \quad (4)$$

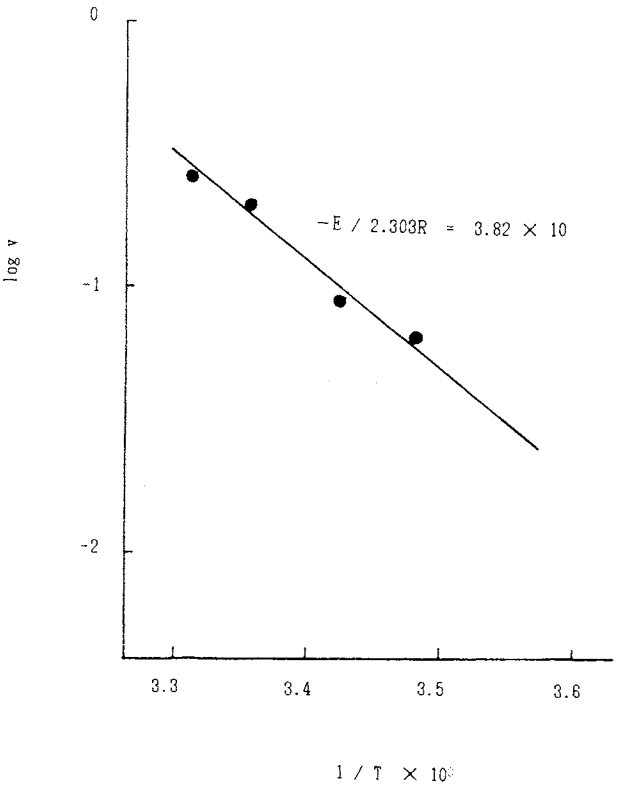

Fig. 5. Arrhenius Plots for the Rate of Coloration.

The temperature was varied at 15, 20, 25 and 30°C, and the other conditions were the same as those in Fig. 1(a). The rate of coloration was estimated in the same manner as that described in Fig. 4.

When the temperature was 20°C (293K), an Arrhenius equation substituted by the values from Eqs. (2) and (4) resulted in the following:

$$4.663 \times 10^{-7} = A \exp(-17.5 \times 10^3 / 1.987 \times 293)$$

$$A = 1.438 \times 10^7 \quad (5)$$

Here, A indicates a frequency factor.

The following result could then be obtained for Arrhenius plots representing the production of blue pigments:

$$k = 1.438 \times 10^7 \exp(-17.5 \times 10^3 / RT) \quad (6)$$

Some thermodynamic parameters associated with the production of blue pigments

According to transition state theory, the following equation holds for Arrhenius plots:

$$k' = \frac{k_B T}{h} \exp(-\Delta H^*/RT) \exp(\Delta S^*/R) \quad (7)$$

k' indicates the rate constant for the reaction from the active complex to the stable product. The other terms being as follows:

k_B, Boltzmann's constant;
h, Planck's constant;
T, absolute temperature;
R, gas constant;
ΔH^*, activation enthalpy;
ΔS^*, activation entropy.

Here, the following relations should be noted:

$$E = \Delta H^* + RT \quad (8)$$

$$\Delta F^* = \Delta H^* - T\Delta S^* \quad (9)$$

ΔF^*, ΔH^* and ΔS^* were calculated as follows:

$$\Delta F^* = 22.0 \text{ kcal/mol}$$

$$\Delta H^* = 16.9 \text{ kcal/mol}$$

$$\Delta S^* = -17.4 \text{ cal/deg/mol}$$

Discussion

The linear portion in the time course curve of Fig. 1 is the most important for analyzing the mechanism of blue pigment production. Most of the blue colored substances can be considered to be formed at this stage. On the other hand, several kinds of blue pigments were responsible for the absorption at 630 nm in the reaction solution, although Blue 1 dominated the coloration at the early stage, and the kinetic parameters obtained should be considered to represent the production of Blue 1 in a fairly good approximation. As noted from Eq. 1, the reaction proceeded as second-order for both D-xylose and glycine. This result can be explained by the following reaction scheme:

$$\text{D-xylose} + \text{glycine} \underset{}{\overset{K_1}{\rightleftharpoons}} I_1 \qquad ①$$

$$I_1 \overset{K_2}{\rightleftharpoons} I_2 \qquad ②$$

$$I_2 \overset{K_3}{\rightleftharpoons} X + \text{glycine} \qquad ③$$

$$X + \text{glycine} \overset{K_4}{\rightleftharpoons} M \qquad ④$$

$$M + M \xrightarrow[k_1]{} M_2 \qquad ⑤$$

$$M_2 + M_2 \xrightarrow[k_2]{} M_4 \qquad ⑥$$

In general, Eqs. ①, ② and ③ have been confirmed in the Maillard reaction between reducing sugar and amino acid.[9] Equations ①, ② and ③ correspond to the formation of aldosylamine (I_1), Amadori rearrangement and the decomposition of Amadori compounds (I_2) into some kinds of osones (X) accompanied by the production of glycine, respectively. In Eq. ④, an unknown intermediate (M), which was produced by the reaction between X and glycine, is postulated. The intermediate M was considered to be involved in the formation of blue colored products as those in Eqs. ⑤ and ⑥, where M_4 indicates Blue 1. On the other hand, it was assumed that an equilibrium state holds for Eqs. ①, ②, ③ and ④ with the respective constants of K_1, K_2, K_3 and K_4. In addition, k_1 and k_2 represent the rate constant for the reactions ⑤ and ⑥, respectively. Equilibrium state theory was applied for the production rate of Blue 1 as follows:

$$\mathrm{d[M_4]}/\mathrm{d}t = k_2[\mathrm{M_2}]^2 \qquad (10)$$

If $k_1 \ll k_2$ holds, the following equation can be applied concerning the production of M_2, which is postulated as a precursor to the blue pigment:

$$\mathrm{d[M_2]}/\mathrm{d}t = k_1[\mathrm{M}]^2 - k_2[\mathrm{M_2}]^2 = 0 \qquad (11)$$

This means the realization of a certain stationary state in respect of the production for M_2, which might be very slow in formation and very quick in conversion. On the other hand, from the equilibrium Eqs. ①, ②, ③ and ④, the following relationship can be obtained:

$$K = K_1K_2K_3K_4 = [\mathrm{M}]/[\text{D-xylose}]\cdot[\text{glycine}] \qquad (12)$$

So, the following equation can be derived from expressions (10), (11) and (12):

$$\mathrm{d[M_4]}/\mathrm{d}t = k_1K[\text{D-xylose}]^2\cdot[\text{glycine}]^2 \qquad (13)$$

In this way, the relationship derived theoretically agrees with that of expression (1), which was obtained empirically. This agreement suggests that Blue 1 is a tetramer composed of four units of the unknown active complex M, which possibly maintains the original skeleton of both D-xylose and glycine in an equimolar ratio. At present, the authors anticipate that this unknown intermediate M is a kind of aldimine with a conjugated double bond, which Song and Chichester had also postulated as a monomer playing a part in polymerization during a Maillard reaction.

If repeated dehydration-condensation is assumed to yield Blue 1 from the system of D-xylose and glycine, the theoretical molecular weight for the tetramer $\mathrm{M_4}$ is expected to be 774 daltons ($150\times4+75\times4-18\times7=774$). However, there is no doubt that a highly conjugated structure is responsible for the strong absorption of visible light and the development of intense fluorescence, indicating a highly unsaturated structure for Blue 1.

Therefore, the possible molecular weight for the pigment would be less than 774.

On the basis of the proposed mechanism for the production of Blue 1, the rate regulating step involves the reaction from ① to ⑤. So, the production of M_2 (⑤) could be a key step in forming the blue pigments. After the formation of M_2, polymerization might follow rapidly to form a series of blue pigments, including Blue 1. Those pigments, which were confirmed on the chromatogram from HPLC shown in Fig. 2, possibly differ in polymerization degree. Although the possible precursor M_2 attracts much interest, its accumulation during the reaction is anticipated to be too low and too unstable to be examined by such a chromatographical separation.

Furthermore, it must be paid attention to that Blue 1 became more stable on purification. This stabilization can be explained in terms of the removal of reactive intermediates such as M and M_2 by purification.

The activation energy for the production of Blue 1 pigment was 17.5 kcal/mol. This value is low when compared with other organic reactions in an aqueous medium, indicating that room temperature was enough to produce Blue 1 from the reaction system consisting of D-xylose and glycine. Based on transition state theory, some thermodynamic parameters were derived, which are directly associated with the reaction stage ⑤. As expected previously, the activation entropy ΔS^* provided a negative value (-17.4 cal/deg/mol), which indicates a decrease in the degree of freedom corresponding to the intermediate structure at the transition state. This suggests that a specific molecular orientation took place in the activation process of the reaction, where two molecules of M connected into M_2.

In the previous report, Miura and Gomyo[3] observed two blue pigments with molecular weights of 1200 and 1400, which were estimated by gel filtration chromatography on Bio-Gel P2. According to the reaction scheme just proposed, each of them can be supposed to be formed as follows:

$$M_4 + M_2 = M_6 \ (1200)$$

$$M_6 + M_2 = M_8 \ (1600)$$

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